Research Article

Application of Direct Colony-PCR for Molecular Genetic Characterization in Fission Yeast *Schizosaccharomyces Pombe*

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Abstract

In this study, an attempt was made to standardize a quick and low cost direct colony PCR method without isolating genomic DNA from fission yeast cells and then further extend its potential applications to the molecular genetic characterization studies in the *Schizosaccharomyces pombe* used as a test model organism. Standardized protocol involved the picking of a single colony or a patch of cells, suspending the cells into sterile nuclease-free water, followed by proper mixing by pipetting in and out and subsequently using this suspension for PCR based applications. Utilization of direct colony PCR in molecular genetic studies had produced better and quick results as compared to the conventional methods since it does not require any enzymatic (Zymolyase etc.), chemical (Sodium hydroxide) and bead-beater based harsh physical/mechanical treatment during isolation of genomic DNA. It was successfully demonstrated that standardized direct colony PCR had quickly detected *ura4* and *ade6* markers insertion at different heterochromatin loci, integration or tagging of HA (Influenza Virus Hemagglutinin) and c-Myc (Human c-myc) epitope at the C-terminal of specific gene of interest and *mat-1* content in newly constructed progeny strains of fission yeast which were further used in different genetic and molecular studies. Overall, it was found that, this direct colony PCR protocol had been not only very suitable for a variety of PCR based genotypic molecular analysis of all the strains of *Schizosaccharomyces pombe* but also useful in simultaneous analysis of a large number of samples (fission yeast strains) with different genotypic backgrounds.

Keywords: Genomic DNA, Kilo Base-pairs, Markers, Mating Type (*mat*) loci, PCR, *Schizosaccharomyces pombe*, Wild Type

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Introduction

The fission yeast *Schizosaccharomyces pombe* is an ideal model organism for the study of eukaryotic molecular and cellular biology [1]. Because of having easy genetic manipulations and fast growth, it is frequently used in many studies such as cell-cycle control, mitosis, meiosis, DNA replication-repair-recombination and chromosome-dynamics [2]. More recently investigators have used *S. pombe* to study a variety of other cell biology problems including signal transduction, RNA splicing and cell morphology, addressing important questions of physiology, genetics and genomics/proteomics/epigenetics [3, 4] using its published genome [5].

A PCR is most commonly used method in genotypic studies of yeast cells. Yeast mutants are often analysed by PCR-based assays for the presence of gene insertion or deletion. Any molecular and biochemical analysis needs isolation and purification of genomic DNA from yeast prior to PCR. Generally, one has to isolate and purify the genomic DNA from yeast cells for further PCR related applications. For the efficient recovery of genomic DNA from yeast cells, the cell wall is the main obstacle [6]. Therefore, the cells must be disrupted and lysed in lysis-buffer or treated with enzyme specially Zymolyase [7]. Generally, in conventional methods for genomic DNA preparation from yeast cells, one has to use either enzymatic degradation or beating with glass beads, followed by lysis of cells with detergents and extraction of genomic DNA with phenol-chloroform [8-14]. Therefore, it is documented that DNA purification from fission yeast is time-consuming and labour-intensive process and use of these harsh treatments and reagents can also damage the DNA. These reagents can inhibit subsequent PCR steps as well by affecting DNA polymerase activity. Overall, when analysing a large number of strain-samples, these methods are time consuming, human labour intensive and comparatively less cost effective.

Alternatively, colony PCR is often employed to avoid steps followed during DNA purification. Investigators used to follow colony PCR as reported in prokaryotic *E. coli* by taking single or a patch of cells in small volume of water and then boiling up for 5-7 minutes at water bath and further using this suspension for PCR amplification studies, but unfortunately most of the time it does not work or looks waste of time. There are also reports of using Zymolyase and then incubation at 37°C in water bath for 10-30 minutes [15]. Very recently, a variety of colony PCR methods have

been discussed but all of them needs a different kind of physical treatments (Boiling, Chemical and Enzymatic action) before setting up PCR reaction [16]. Overall, these protocols are not always convenient, consistent and guaranteed for routine PCR amplifications.

Here we present an easy, quick and reliable method to amplify genomic DNA from fission yeast (*S. pombe*) without prior DNA purification and also showed further its applications in the molecular and genetic characterization studies using *S. pombe* as a model organism.

Materials and Methods

Strains, Primers and Growth Media

Fission yeast strains (*S. pombe*) and primers used in this study are listed below in **Tables 1** and **2** separately with specifications. All strains were maintained at 4°C for short term storage. For long term storage, yeast cultures were maintained in 25% glycerol stock and finally stored at -80°C. The primers were mostly purchased from Biobasic Inc. (Canada) and Sigma USA. All media were prepared and dissolved in double distilled water and autoclaved for 15 minutes at 121°C under 15 pounds of pressure. Unless mentioned otherwise media Yeast Extract Agar (YEA) & sporulation PMA^+ medium were prepared according to the procedure described in [17, 13].

Table 1 List of the Fission yeast strains used in genetic characterizations

S. No.	Strains used (*Schizosaccharomyces pombe)*	Genotype	Remarks
1.	FY2002	h^+,*leu1-32, ura4-DS/E, ade6-DN/N, imr 1L-NcoI::ura4*$^+$, *otr 1R-SphI::ade6*	Allshire *et al*., (1994)
2.	SPJ541	h^{90}, *leu1-32, ura4-D18, ade6-216, his2*	Lab strains
3.	SPN3	*mat1M smt-0, his2, leu1, ura4, ura4-DS/E, ade6-DN/N,*	Lab strains
4.	ASP164	*mat1M smt-0, his2, leu1, ura4, ura4-DS/E, ade6-DN/N, cac2-3HA::Kan*^{r}	This study

Table 2 List of the Primers used in PCR amplification during the study

S. No.	Oligos Name	Oligo sequences	Length	Purification
1.	Ade6-For	*TgCgATgCA CCT gACCAggAAAgT*	24	PAGE
2.	Ade6-Rev	AgAgTTgggTgTTgA TTT CgCTgA	24	PAGE
3.	Ura-For	gAggggATg AAA AAT CCC AT	20	PAGE
4.	Ura-Rev	TTC gAC AAC Agg ATT ACg ACC	21	PAGE
5.	Kan-For	gCTAggATAgAgTTCTCACATCACATCCg	29	PAGE
6.	Cac2-rev Confirm	gACAAAAAgTACAAACgAT	19	PAGE
7.	Mat-1	AgAAgAgAgAgTAgTTgAAg	20	PAGE
8.	Mat-2P	ACggTAgTCATCggTCTTCC	20	PAGE
9.	Mat-3M	TACgTTCAgTAgACgTAgTg	20	PAGE

Method Development

Looking to the major applications of *E. coli* colony PCR, an attempt was made to standardize a direct colony PCR method in *S. pombe* aiming studies. Different and following approaches were adopted at first, (a) a patch and (b) single colony were used directly with in master mix, (c) colony picking with the help of tooth pick (d) 10 µl of cell suspension and (e) 1µl of cell suspension were used for the setting up PCR, but all gave no PCR products. Analysing all the procedures and combining all gain knowledge, finally this direct colony PCR had got success (preliminary data not shown) and summarised as below.

For quick genotyping and screening, cells were first patched on the plates and grown (further strain preservation purpose), or alternatively patch of cells or a single cell directly taken by sterile plastic tip and suspended in 10 micro-litre (µl) of sterile nuclease-free water, mixed properly by using micropipettes with little or no vortexing. Finally, 1.0 µl out of 10 µl suspension was used for setting PCR amplifications. This volume was used as a template for setting PCR reactions. Remaining suspension may be stored at 4°C for further use (1-2 days).

Genetic characterization of newly constructed strains

During strain construction, it is required to check or detect the different allele of *ade6* and *ura4* marker insertion in

each and every newly generated strain, so the above standardized protocol for colony PCR was used for this purpose. In the study, the specific primers (for *ade6* and *ura4* markers inserted at different heterochromatic locus), TsgTaq polymerase (Biobasic Inc.) in all, the PCR master mix and Thermocycler-Master cycler (Eppendorf) were used. PCR Conditions were as follows:

- 95°C for 3or 5 minutes,
- 95°C for 1 minute,
- Standard Annealing temperature for 30 seconds (such as 55°C for Ura4 and Ade6 primers)
- 72°C for 2 minutes, Repeated (step 2-4) for 25-30 cycles.
- 72°C- 10 minutes extension.
- 4°C -Hold.

Thermal cycler (PCR machine) was put on hold mode and run after keeping all PCR tubes. To check the PCR products after all cycle over, 1% agarose gel electrophoresis was carried out using 1X TAE buffer. A Standard 1 kb ladder (NEB) was also run along with other PCR products, to determine size of PCR products.

Checking of integration or epitope-tagging

For checking or detection of the integrations or tagging of HA (*Influenza* Virus Hemagglutinin), c-Myc (Human c-*myc)*, and GST (Glutathione *S*-transferase) epitope at the C-terminal of specific gene of interests in the transformants, this direct Colony PCR was used. The PCR amplified products, using specific primers as reported by [18] were transformed in the suitable background strains and these transformants were picked and patched on the new rich medium containing fresh plates (YEA). From this patch, some part of patch or cells were suspended in sterile nuclease-free water containing Eppendorf tubes and used in direct colony PCR for detection of tagging or PCR-based amplifications. The specific primers *Kan F* (*Kanamycin* forward) and gene specific reverse (*Cac2*, a gene encoding second sub unit of Chromatin assembly Factor-1 in *S. pombe*) primers and other components of PCR mix were used in this direct colony PCR. Finally, The PCR products were electrophoresed onto 1% agarose gel and photographed. Positive transformants were saved.

Detection of mat-1 locus or Mating-type-switching Assay

To extend the applications of this direct colony PCR, the direct colony PCR was employed to detect the mating-type of dark and light iodine stained homothallic (h^{90}) strains (with or without specific gene deletion).The isolated dark and light colonies were first patched and streaked onto two different plates (sporulation medium) in triplicates, grown at 30°C for 3-4 days and photographed with microscope and scanned. Some of the colonies had been taken out and used for quantitative Multiplex-PCR for detection of *mat1* content. Specific primers were used to amplify the particular region of Mat1, Mat2P and Mat3M loci (Heterochromatic locus) [19]. Each PCR products were loaded in dilutions on the 1% agarose gels and photographed. Finally, P/M ratio was calculated and got the mating-type of the homothallic strain.

Results and Discussion

It is well documented in fission yeast that genes placed within the centromeres are always transcriptionally repressed and exhibit phenotypic variegation [20]. The fission yeast centromeres consist of a central core composed of unique sequences surrounded by inner (*imr /B*) and outer (*otr /K+L*) repetitive sequences and most importantly the marker genes(*ade6* and *ura4*) inserted in centromere region were subject to reversible repression of gene expression in FY2002 strain [20]. The FY2002 strain was crossed to compatible strain and got the progeny colonies which were further genetically characterised by this direct Colony-PCR. It may be concluded and characterised from the size of obtained PCR products; which allele of *ade6* and *ura4* gene is present or absent in each lane of specific progeny strain (**Figure 1**, A and B). During the strain construction, the transfer of *ade6* (full length) and *ade6-DN/N* (truncated allele) in the obtained new strain occurred as shown in lane (1, 3, 4, 5, 12, 13) in Figure 1A. The Ura4 alleles were also transferred in the new strain obtained as shown in lane (2, 3) of Figure 1B. These screened strains with detected *ade6* and *ura4* marker were used in further experiments specifically to study whether the combined mutation or deletion of gene was involved in heterochromatin silencing in case of this study, is at the Centromere in fission yeast.

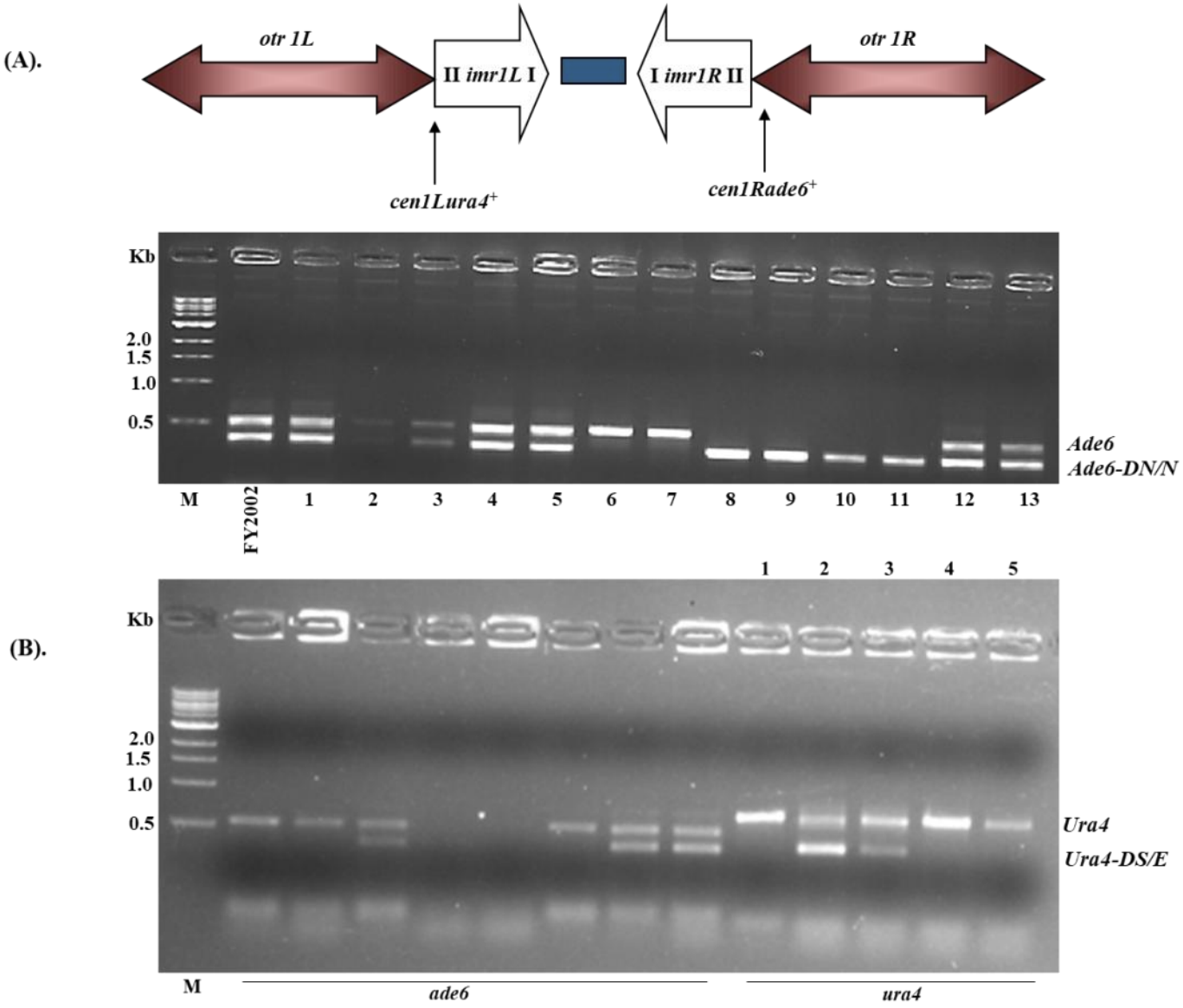

Figure 1 Detection of insertion of *ade6* and *ura4* markers in *S. pombe.*

Checking of markers or alleles in progeny strains of *S. pombe* after genetic crosses which were also having genetic manipulations (deletion background) and selected for further studies, here in case of silencing of *ura4* and *ade6* markers at the region of Centromere-1 of fission yeast (as shown above by pictorial cartoon). (A). Fourteen colony PCR reactions were set up with the use of *ade6* forward and reverse primers. An amplified PCR product of extracted genomic DNA from WT (Wild type /FY2002) strain was taken as a positive control. Both the truncated and full length *ade6* direct colony PCR products were observed in different lanes of progeny samples in the DNA gel electrophoresis. 1 kb marker/ladder was also loaded in a lane. (B). The thirteen direct colony PCR products were resolved in the 1% DNA gel electrophoresis, out of which five reactions were set up using *ura4* primers. Since the nature of PCR products were very much similar in amplification of *ade6* and *ura4* markers, so here is a choice of using or not using any positive control in DNA gel electrophoresis.

Earlier in nineties, a straight forward PCR-based approach was reported in detail for the deletion, tagging, and overexpression of genes in their normal chromosomal locations in the model organism *Schizosaccharomyces pombe* [18]. These Investigators constructed a series of plasmids containing the kanMX6 module, which certainly allowed the selection of G418-resistant cells and showed best use of this heterologous marker (kanMX6) specifically in case of epitope tagging. The modular nature of this construct allowed us to be used for variety of gene manipulations studies specifically in C-terminal protein tagging (such as with HA) in our study. The kanMX6 construct was used as template and the primers included 60 to 80 bp of flanking sequences homologous to target sequences in the genome of fission yeast, were used to amplify in the PCR amplicon. After transformation of construct in *S. pombe*, the transformants were screened for homologous integration by adopting the direct-colony PCR method. Presence of

desired band (~1 kb) was observed in transformant no.4 as shown in **Figure 2**. Here, *Spcac2* gene was found tagged with 3-HA successfully. Since commercial antibodies to the HA epitopes are available, so this screened positive strain was used as a HA-tagged strain expressing SpCac2-3HA-epitope in further molecular and biochemical studies. Similar type of HA-or other epitope tagged strain can be used in further protein–protein interactions experiments.

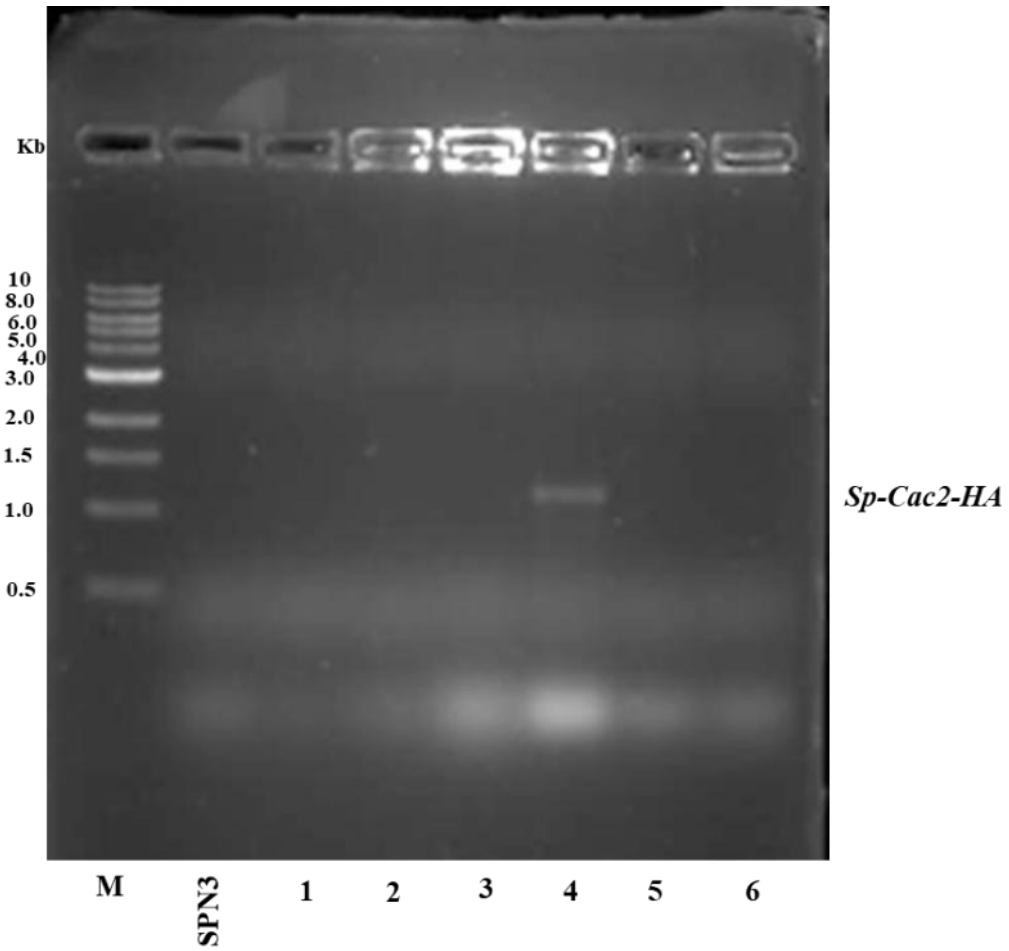

Figure 2 Detection of Hemagglutinin (HA) integration in the wild type haploid strain (SPN3 strain) by the direct colony PCR.

The direct colony PCR method was used for the checking of HA integration to the *cac2* gene in haploid clone strains or transformants. Out of thirteen, only single clone (no.4) was found positive (only six clones were shown in gel picture) and WT strain was used as a negative control.

Mating-type switching is an important phenomenon, occurs efficiently in mitotic dividing cells of *S. pombe*. The mating-type switching basically involves replacing the genetic information at the expressed *mat1* locus with sequences copied from one of two silent donor loci, *mat2-P* or *mat3-M*, located within a 20 kb heterochromatic domain. It was also reported that donor selection is dictated by the cell type of fission yeast strain i.e. the *mat2* is the preferred donor in *M* cells and while *mat3* is the preferred donor in *P* cells [19]. In wild type homothallic strains, designated h^{90}, *mat2* is separated from *mat1* by 15-kb, *L*-region, whereas *mat3* is separated from *mat2* by 11-kb, *K*-region [21]. This homothallic strain, in an assay shows a starch-like compound during sporulation which results in black staining of colonies after exposure to iodine vapours. Cells that switch efficiently to the opposite mating type form colonies with a homogenous distribution of *M* and *P* cells and thereby can readily mate and sporulate, resulting in dark iodine staining. In contrast, the cells that switch their mating type with a lower efficiency form lightly staining colonies due to poor mating and sporulation within the colony, caused by the lack of cells of opposite mating type in the vicinity. The switching efficiency of homothallic strains with gene deletion background was examined by adopting this direct colony PCR based assay. The calculated P/M ratio in light and dark colony was shown in **Figure 3**, may positively suggest the involvement of this gene in the phenomenon of mating-type switching and also preference to Minus (M) type of cells as reviewed earlier [22]. Here also, the direct colony PCR was positively worked and showed its application in the quantitative multiplex PCR technique.

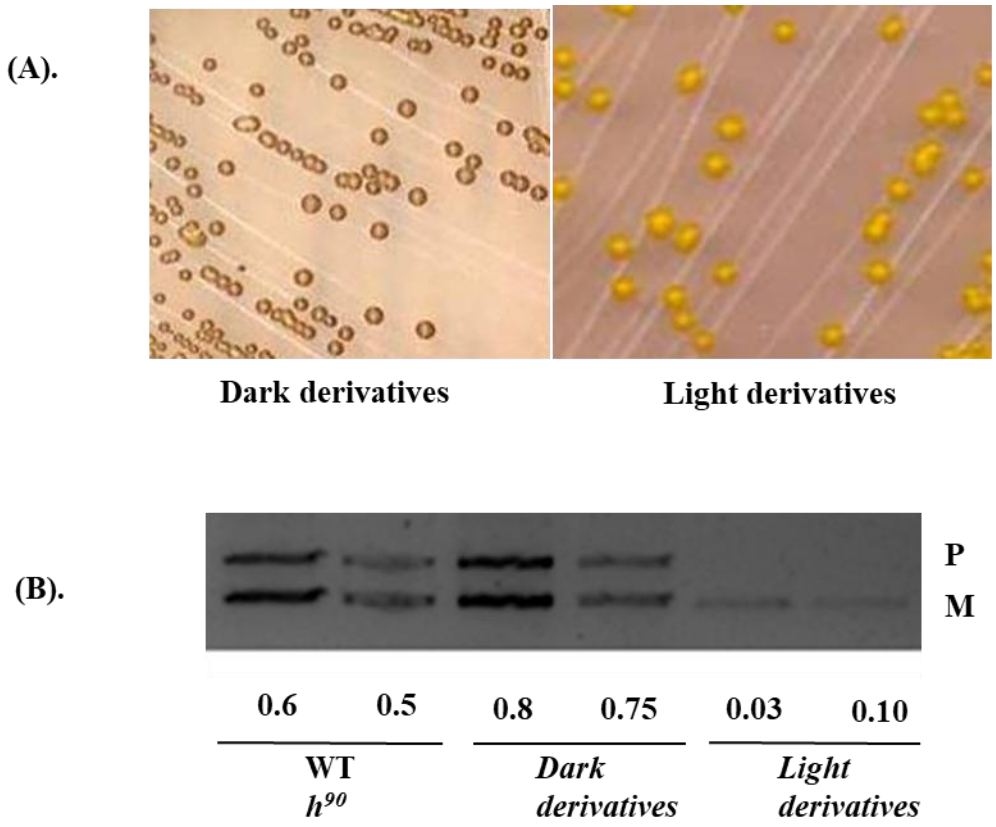

Figure 3 Application of direct colony-PCR method in Quantitative Multiplex-PCR for detection of *mat1* content.

(A). These photographs are showing the iodine staining patterns in genetic background of h^{90}, geneΔ strain where dark and light derivative colonies were streaked on the sporulation media. (B). The Colony PCR was performed in WT (h^{90}), light derivatives-(h^{90}, geneΔ), and dark derivatives-(h^{90}, geneΔ) strains and further the *mat1* content was determined with the help of multiplex-PCR using specific primers to amplify *mat1*-P (Plus) and *mat1*-M (Minus) sequences simultaneously. Each PCR product was loaded in dilutions with DNase-free sterile distilled water. Results suggest that the light derivatives with background of gene deletion have been showing preference of *mat1*-M (Minus) type. The P/M ratio is shown beneath at each lane.

The success rate of the new method was relatively high as compared to traditional ones. Using this method, the newly generated fission yeast strains of exogenous markers were screened. Compared to PCR using isolated genomic DNA, the results of colony PCR were consistent with those from isolated genomic PCR. The results indicated that this novel method was of good stability, reproducibility and suitable for the rapid screening of the fission yeast transformants in case of tagging experiments as shown in this study.

We observed this rapid and direct colony PCR technique for fission yeast specifically for *S. pombe* is very important; a challenge in respect to these methods has always been the variability in the amplification results caused by the variability in extraction of genomic DNA due to the hardy nature of yeast cell wall.

Conclusions

From this experimental study, we can conclude that the standardized direct colony PCR method is quick, easy and consistent method. The direct colony PCR, from a patch or single cell of fission yeast has been successfully standardised and described without involving any pre-treatment and showed its applications in PCR based genetic studies to fulfil the aim. We have also found that it is reliable, less-time consuming, cheap and showed good applications to the larger genetic studies. Overall, author has also successfully showed the applications of standardised colony PCR into genetic and molecular screening, genotyping, checking integration or tagging, also performed and applied into the multiplex-PCR for mating-type switching assays. In the present study, the PCR band amplicons size was observed from 400 bp to 1.6 kb which shows the direct colony-PCR as have more and wider applications in molecular and genetic studies, and much more have potential to be explored yet.

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