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SYBR safe™ efficiently replaces ethidium bromide in *Aspergillus fumigatus* gene disruption

H.M.S. Canela, L.A. Takami and M.E.S. Ferreira

Departamento de Ciências Farmacêuticas,
Faculdade de Ciências Farmacêuticas de Ribeirão Preto,
Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Corresponding author: M.E.S. Ferreira
E-mail: mesfe@fcfrp.usp.br

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ABSTRACT. Invasive aspergillosis is a disease responsible for high mortality rates, caused mainly by *Aspergillus fumigatus*. The available drugs are limited and this disease continues to occur at an unacceptable frequency. Gene disruption is essential in the search for new drug targets. An efficient protocol for *A. fumigatus* gene disruption was described but it requires ethidium bromide, a genotoxic agent, for DNA staining. Therefore, the present study tested SYBR safe™, a non-genotoxic DNA stain, in *A. fumigatus* gene disruption protocol. The chosen gene was *cipC*, which has already been disrupted successfully in our laboratory. A deletion cassette was constructed in *Saccharomyces cerevisiae* and used in *A. fumigatus* transformation. There was no statistical difference between the tested DNA stains. The success rate of *S. cerevisiae* transformation was 63.3% for ethidium bromide and 70% for SYBR safe™. For *A. fumigatus* gene disruption, the success rate for ethidium bromide was 100 and 97% for SYBR safe™. In conclusion, SYBR safe™ efficiently replaced ethidium bromide, making this dye a safe and efficient alternative for DNA staining in *A. fumigatus* gene disruption.

Key words: *Aspergillus fumigatus*; Gene disruption; DNA fragment staining

INTRODUCTION

The incidence of invasive fungal infections has increased in the last decades due to the increased number of immunocompromised patients (Brown and Goldman, 2016; Lee and Sheppard, 2016). These patients are frequently affected by invasive aspergillosis, a disease that can reach 30 to 100% of mortality and is mainly caused by *Aspergillus fumigatus* (Pfaller and Diekema, 2010; Steinbach et al., 2012; Steinmann et al., 2015; Lee and Sheppard, 2016).

The current available antifungal drugs for aspergillosis treatment are limited (Brown and Goldman, 2016). Therefore, the discovery of new antifungal targets is necessary and the study of gene function in this fungus contributes to elucidate the pathogenesis mechanisms (Malavazi and Goldman, 2012; Lamoth et al., 2016).

An efficient protocol for *A. fumigatus* gene disruption described previously requires separation and purification of amplicons (Malavazi and Goldman, 2012). DNA fragment staining is usually performed using ethidium bromide, which is known for its mutagenicity (Singer et al., 1999; Martineau et al., 2008).

The present study tested a safer DNA stain, SYBR safe™, as a replacement for ethidium bromide in *A. fumigatus* gene disruption protocol (Malavazi and Goldman, 2012).

MATERIAL AND METHODS

Aspergillus fumigatus gene disruption

The gene chosen to be disrupted was *cipC* (AFUA_5G09330), which was disrupted successfully in our laboratory (Canela et al., 2017). The used strains included *A. fumigatus* mutant D*cipC*, its wild-type, D*akuB*^{Ku80} (Ferreira et al., 2006), and *Saccharomyces cerevisiae* FGSC9721 (Winston et al., 1995).

For D*cipC* strain construction, DNA manipulations were performed as described previously (Sambrook and Russell, 2001). Polymerase chain reaction (PCR) strategy and *in vivo* recombination in *S. cerevisiae* were used to produce a *cipC* gene deletion cassette for *A. fumigatus* (Malavazi and Goldman, 2012).

Briefly, 2-kb regions on either side of the open reading frames (ORFs) were selected for primer design. The primers cipC 5'Fw and cipC 5'Rev were used to amplify the 5'-UTR flanking region of *cip*C ORF; cipC 3'Fw and cipC 3'Rev were used to amplify the 3'-UTR flanking region (Table 1). Both fragments were amplified from genomic DNA of D*aku*B^{Ku80}. The *pyr*G gene was used as an auxotrophic marker to delete the *A. fumigatus cipC* gene and was amplified from the pCDA21 plasmid (Chaveroche et al., 2000) using pyrG Fw and pyrG Rev primers (Table 1). PCRs were performed using High Fidelity PCR Enzyme mix.

The cipC 5'Fw and cipC 3'Rev primers presented cohesive ends (bold letters) with the pRS426 vector, which was double digested with *Eco*RI and *Bam*HI for linearization. PCR products and the digested vector were separated by electrophoresis, stained with ethidium bromide or SYBR safe™ (Invitrogen, Carlsbad, CA, USA) and purified using a Quiaquick PCR cleanup kit.

The purified products were transformed into *S. cerevisiae* (Malavazi and Goldman, 2012). The DNA of the yeast candidates was extracted and transformation was confirmed by PCR using pyrG Fw and pyrG Rev primers and Taq DNA Polymerase. In the *in vivo S. cerevisiae* recombination, three replicates of each DNA stain were performed and 10 candidates of each replicate were tested.

Table 1. Primers used in this study.

Primers	Sequence (5'-3')
cipC 5'Fw	**GTAACGCCAGGGTTTTCCCAGTCACGACG**GATAGCATGGCAGAGGTTCT
cipC 5'Rev	GTGCCTCCTCTCAGACAGAATTTTGAGTTGATCTGGTGAAATC
cipC 3'Fw	GAGCATTGTTTGAGGCGAATTCGACGGCTAGACAACATGTAC
cipC 3'Rev	**GCGGATAACAATTTCACACAGGAAACAGC**ATTGTGTCTGCTGAGGGCG
cipC ORF Fw	ATGGCTTGGGGCTGGG
cipC ORF Rev	TTACCAACGGTCGACAGGGC
cipC 2.2 5'Fw	ACTGTGAGGTTGGTGGGGA
pyrG Fw	ATTCTGTCTGAGAGGAGGCA
pyrG Rev	AATTCGCCTCAAACAATGCTC

Bold letters mean cohesive ends with the vector pRS426.

The deletion cassette was amplified from the DNA of the confirmed candidates using High Fidelity Takara Ex Taq™ (Takara Biotechnology, Otsu, Shiga, Japan) and cipC 5'Fw and cipC 3'Rev primers. Two mutants of each DNA stain were selected to amplify the deletion cassette.

After reaction, the PCR products were separated by electrophoresis using 1% agarose gel, stained with ethidium bromide or SYBR safe™, and purified using a Quiaquick PCR cleanup kit.

Aspergillus fumigatus transformation was performed according to that previously described and candidates were selected by their ability to grow on selective YAG medium containing 0.6 M KCl as osmotic stabilizer (Malavazi and Goldman, 2012). Confirmation of gene disruption was achieved by three different PCRs: by comparing the size of the amplicon generated by the mutant strain with that generated by the wild-type strain using cipC 5'Fw and cipC 3'Rev primers; by the absence of amplification in the mutant strain using cipC ORF Fw and cipC ORF Rev primers (Table 1), which amplify the *cipC* gene only in the wild-type strain; and using cipC 2.2 5'Fw and pyrG Rev primers (Table 1), which amplify a fragment of 4.1 kb only in the mutant strain, if the deletion was effective. The reactions for gene deletion confirmation were performed using Taq DNA Polymerase. For *A. fumigatus* transformation, three replicates of each DNA stain were performed.

Ethidium bromide DNA staining

The agarose gel was stained after the electrophoresis using a solution of 0.5 µg/mL ethidium bromide, for 30 min and visualized under UV illumination.

SYBR safe™ DNA staining

SYBR safe™ was incorporated on agarose gel at 1X concentration; the electrophoresis was performed and the gel was visualized under blue light transilluminator.

Statistical analysis

The results were evaluated by chi-square or Fisher exact tests using GraphPad Prism version 5. Values of $P \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In the *in vivo S. cerevisiae* recombination, there was no statistical difference between DNA stains. The results are shown in Table 2.

In *A. fumigatus* gene disruption, 31 candidates were obtained for SYBR safe™ and 18 for ethidium bromide (Table 2). SYBR safe proved to be as efficient as ethidium bromide; the deletion success of the SYBR safe™ DNA stain was not significantly different to the ethidium bromide ($P < 0.0001$).

Table 2. Success rate of *Saccharomyces cerevisiae* and *Aspergillus fumigatus* transformations using different DNA stains.

Transformation	DNA stain	Confirmed candidates/total	Success rate (%)
S. cerevisiae	Ethidium bromide	19/30	63.3
	SYBR safe™	21/30	70
A. fumigatus	Ethidium bromide	18/18	100
	SYBR safe™	30/31	97

DNA staining plays an essential role in molecular biology. Ethidium bromide is the most common stain used for nucleic acid detection on electrophoretic gels. This dye emits fluorescence when intercalated into DNA bases of nucleic acids and can interfere in both DNA and RNA synthesis (Singer et al., 1999; Ohta et al., 2001; Martineau et al., 2008; Haines et al., 2015). Ethidium bromide is classified as a strong mutagen and is genotoxic at the typical concentration for gel staining (Schagat and Hendricksen, 2013; Haines et al., 2015). Moreover, this stain requires special waste treatment, consequently increasing costs for laboratories (Martineau et al., 2008). Finally, ethidium bromide needs to be visualized under UV illumination, which can damage the DNA (Gründemann and Schömig, 1996; Martineau et al., 2008).

Other DNA stains were developed to be less hazardous to the handlers. SYBR safe™, a DNA stain developed by Invitrogen, is classified as non-genotoxic, non-mutagenic, and non-hazardous (Martineau et al., 2008; Evenson et al., 2012). This stain interacts with the DNA grooves instead of intercalating the DNA double stranded (Haines et al., 2015). Moreover, SYBR safe™ can be visualized under a blue light transilluminator, which does not damage the DNA (Martineau et al., 2008).

Due to mutagenicity, protocols have been tested with different and safer combinations to substitute ethidium bromide. Martineau et al. (2008) showed that SYBR safe™ can replace ethidium bromide in cesium chloride density gradients.

This study provides new support to ethidium bromide substitution, and SYBR safe™ successfully played this role. This substitution makes the *A. fumigatus* gene disruption protocol safer and less hazardous to the environment. Alternative approaches like this presented here are novel and certainly exert a high impact in the discovery of new treatment protocols for aspergillosis.

Conflicts of interest

The authors declare no conflict of interest.

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