# Benchmarks

## Zeocin for selection of bleMX6 resistance in fission yeast

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Complementation of auxotrophic nutrient deficiencies in minimal media is widely used for selection of exogenous gene introduction to fission yeast. However, only a limited number of such selection markers are available. Antibiotic resistance markers are good alternatives, but they typically work well in complete rich medium but not in minimal defined Edinburgh minimal medium (EMM). It would be ideal if both the auxotrophic and antibiotic resistance markers can be used together for molecular genetic analysis. Here we describe the use of Zeocin in Pombe minimal glutamate (PMG) media for selection and maintenance of bleMX6 resistance with a *LEU2* auxotrophic marker in fission yeast.

Expression of an exogenous gene from a plasmid, which is selected based on a selectable genetic marker in fission yeast (*Schizosaccharomyces pombe*), is an important tool for molecular genetic studies. Typically an auxotrophic marker such as *leu1*, *ura4*, or similar markers are used (1–7). Selection of these gene markers, however, requires that all strains have the corresponding auxotrophic genetic background, and the selection can only be carried out in minimal defined media such as the Edinburgh minimal medium (EMM). An alternative to auxotrophic selection is the use of antibiotic drug resistance. For example, the *kanMX6* gene cassette that confers resistance to geneticin (G418) has been commonly used in *S. pombe*. Other antibiotic-resistant genes such as *natMX6*, *hphMX6*, and *bleMX6* have also been reported in *S. pombe* that confer resistance to nourseothricin, hygromycin B, and phleomycin/Zeocin, respectively (8,9). However, many of these drugs typically work well only in rich medium but not in the commonly used EMM, which limits their combined use with the auxotrophic selection markers (Reference 8, www-bcf.usc.edu/~forsburg/drugs.html). Furthermore, the most widely used fission yeast expression vector system uses an inducible *nmt1* promoter that is only activated in minimal media (10–12). Therefore, it would be highly desirable if both the auxotrophic and antibiotic markers can be used jointly in minimal media for molecular genetic analysis.

Zeocin (Zeo) was chosen to test this possibility for several reasons. Zeocin, which is commercially available (Cat. no. R250-01; Invitrogen, Carlsbad, CA, USA), is a phleomycin derivative that has a broad range of antibiotic activity against most bacteria, yeasts, and mammalian cells. Like phleomycin, expression of *bleMX6* gene inhibits the Zeocin's antibiotic activity, thus conferring resistance to Zeocin (13), but Zeocin is considerably cheaper than phleomycin. Noticeably, phleomycin does not work well in minimal medium (8), thus limiting its use in molecular genetic studies. However, different drug sensitivity was described in various yeasts between phleomycin and Zeocin, suggesting Zeocin may have different drug uptake or mode of action. Here we describe the use of Zeocin as a drug marker for selection of *bleMX6* gene resistance in both complete and minimal media of fission yeast.

To test Zeocin resistance under the auxotrophic selective conditions, a Zeocin-resistant ($Zeo^R$) strain was generated by using an auxotrophic Zeocin-sensitive ($Zeo^S$) fission yeast strain SP223 (h-, *ade6-216 leu1-32 ura4-294*) (12). Specifically, the *bleMX6* gene was integrated at the *ura4* gene locus with an integrated vector created from the pCloneBle1 plasmid by using a high-throughput knockout method developed by Spirek et al. and Gragen et al. (13,14). The $Zeo^S$ and $Zeo^R$ phenotypes under various experimental conditions were tested as described in the following. Standard complete medium (YES) and minimal EMM or Pombe minimal glutamate (PMG) media were tested (15). Each minimal media were supplemented with 150 mg/L adenine (A), 100 mg/L uracil (U), and 20 µM thiamine.

We first measured Zeocin sensitivity on solid media in the $Zeo^S$ and $Zeo^R$ strains. Zeocin (150 mg/L) was added to the media. As shown in Figure 1A (left), 150 mg/L Zeocin completely inhibited colony formations in all three agar media. No colonies were seen on these Zeo-containing plates even after 11 days. In contrast, the *bleMX6*-carrying strain formed the same size colonies in the Zeo-containing plates as that without Zeocin, confirming the expected $Zeo^R$ phenotype (Figure 1A, right). Similar colony formations were also seen with 200 mg/L Zeocin. To further quantify the extent of $Zeo^S$ and $Zeo^R$ phenotypes, dot tests were carried out on agar plates containing 0, 200, or 300 mg/L Zeocin (Figure 1B). While expression of *bleMX6* gene in the $Zeo^R$ strain formed expected number of dots at each dilution in all three media (Figure 1B, right side of each strip), different levels of Zeocin resistance were observed in the $Zeo^S$ strain. While Zeocin completely prevented dot formations on the YES and PMG media even at a high Zeocin concentration of 300 mg/L, significantly reduced Zeo sensitivity was seen in the EMM in which complete inhibition was only observed below $5 \times 10^4$ cells/dot.

Next Zeocin sensitivity was evaluated in liquid media supplemented with 0, 150, or 200 mg/L Zeocin (Figure 2). As expected, the $Zeo^S$ strain grew normally

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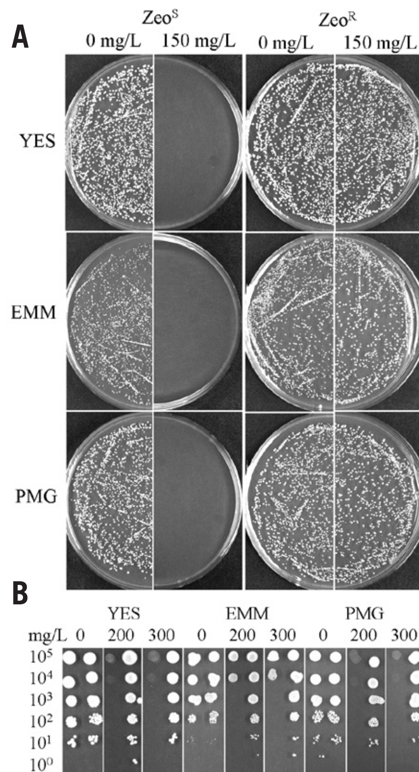


**Figure 1. Sensitivity of fission yeast cells to Zeocin on complete (YES) or minimal (EMM or PMG) agar plates.** (A) Log-phase cultures (100 μL) of Zeo$^S$ or Zeo$^R$ yeast culture with the concentration of $5 \times 10^4$ cells/mL were plated onto three different agar media supplemented with 0 or 150 mg/L Zeocin. Pictures were taken 4 days after incubation at 30°C. (B) The same log-phase cultures (2 μL) were spotted onto each media with 10-fold dilutions from $5 \times 10^7$ to $5 \times 10^2$ cells/mL. Left colonies in each strip are Zeo$^S$, and right colonies are the Zeo$^R$ strain.

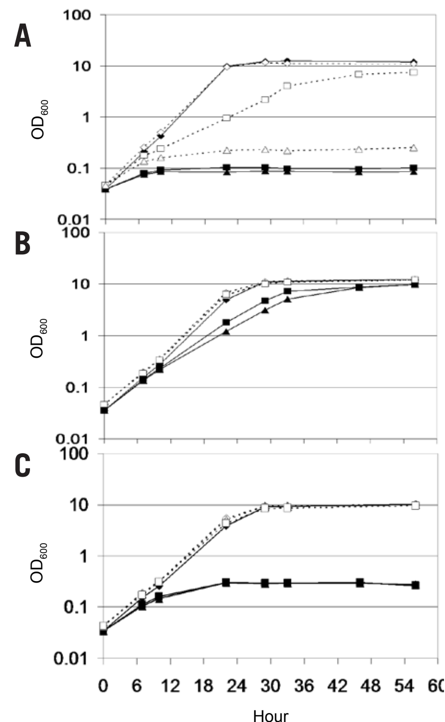


**Figure 2. Sensitivity of fission yeast cells to Zeocin in YES (A), EMM (B), or PMG (C) liquid medium.** Log-phase cultures (5 mL) of Zeo$^S$ (solid symbol) or Zeo$^R$ (open symbol) yeast culture with the initial concentration of $4 \times 10^5$ cells/mL were inoculated in three different liquid media supplemented with Zeocin of various concentration, i.e., 0 (◆ or ◊), 150 (■ or □), and 200 (▲ or △) mg/L. The cultures were shaken at 300 rpm at 30°C. Zeocin was purchased from Invitrogen and dissolved in deionized and autoclaved water. Optical density was measured at different time points as indicated. Please note that in panel C, the closed triangle curve completely overlaps the closed square curve, while the other four curves are mostly overlapping.

in every medium without Zeocin (◆). Zeocin (150–200 mg/L) completely stopped Zeo$^S$ cell growth in the YES and PMG media (Figure 2, A and C, ■ and ▲). This growth inhibition was very stable, because no growth was seen even after 5 days. Similarly in solid EMM, 150–200 mg/L Zeocin were unable to completely prevent growth of Zeo$^S$ cells in liquid EMM (Figure 2B). A similar pattern was also observed at 600 mg/L Zeocin or lower cell concentrations, suggesting the observed growth was not due to low drug concentrations or high cell concentrations. Since the only difference between EMM and PMG is the nitrogen source (i.e., EMM uses ammonium chloride but PMG contains L-glutamate), it is possible that either ammonium chloride reduces uptake of Zeocin or attenuates its activity in EMM.

Resistance of Zeo$^R$ cells was also evaluated in liquid. The Zeo$^R$ strain grew normally in both EMM and PMG minimal media regardless of drug treatment, suggesting that the *bleMX6* gene was fully expressed, thus leading to Zeocin resistance. Interestingly, significant growth delay was found in the YES medium with 150 or 200 mg/L Zeocin (Figure 2A, □ and △). It is unclear what causes this growth delay. Potential explanations include suboptimal activation of the foreign *TEF* promoter, which drives the *bleMX6* gene expression (16). Low level or delayed expression of the *bleMX6* gene expression could thus lead to the observed partial resistance to Zeocin.

Next, we tested whether Zeocin resistance could be maintained through an

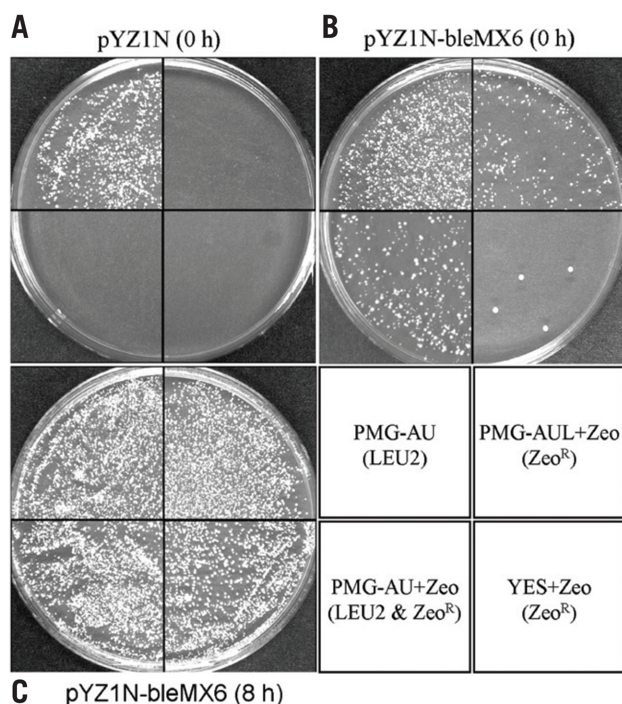


**Figure 3. Transformation and co-selection of Zeocin resistance and Leu2 auxotrophic marker with an autonomously replicating pYZ1N-bleMX6 plasmid in PMG minimal medium.** Zeo$^S$ SP223 cells ($1.3 \times 10^7$) were transformed with 700 ng pYZ1N (A) or pYZ1N-bleMX6 (B) plasmid DNA (18) and plated onto PMG minimal agar plates containing 150 mg/L adenine (A), 100 mg/L uracil (U), and 200 mg/L leucine (L) for counting of viable cells (not shown), AU + 150 mg/L Zeocin (AU+Zeo) for selection of *LEU2* marker, AUL+Zeo for selection of Zeo$^R$, and the AU+Zeo plates for double selection of *LEU2* and Zeo$^R$. The YES+Zeo plates were also used here for quantitative comparison of Zeo$^R$ with that of PMG plates. Equal amount of cells were plated in all plates. Plates shown in panels A and B were plated immediately after cell transformation (0 h). Plates shown in panel C were cells plated after 8 h of incubation in nonselective PMG+AUL medium at 30°C. After 8 h, cells were washed carefully and resuspended in distilled water for plating. Plates were incubated at 30°C for 4 days before photography.

autonomous plasmid and/or in conjunction with selection of an auxotrophic nutrient marker such as *LEU2* in the minimal PMG medium. A new pYZ1N-bleMX6 plasmid, which contains both the *LEU2* gene of *Saccharomyces cerevisiae* that complements *S. pombe leu1* gene and the *bleMX6* cassette on the same plasmid, was created by inserting the *bleMX6* gene cassette at the *Bam*HI-*Not*I sites of the pYZ1N vector (12). The pYZ1N or pYZ1N-bleMX6 plasmid was transformed into the Zeo$^S$ SP223 cells using the Suga method (17). Transformed cells were plated directly onto different PMG selective media to select for *LEU2* only (AU), for Zeocin resistance only (AUL+Zeo), or for both LEU2 and Zeocin (AU+Zeo), respectively. In addition, complete YES medium with Zeocin (YES+Zeo) was also used as a comparison for Zeocin resistance in PMG medium; pYZ1N plasmid was used as a positive control for *LEU2* selection but a negative control for Zeocin drug resistance. Cell viability on each agar plate was verified by plating the same amount of cells on a nonselective PMG+AUL plate (data not shown). The DNA transformation efficiency under each experimental condition was calculated based on the number of colonies formed on the testing plates divided by the number of colonies formed on the nonselective plates and calibrated with the amount of DNA used in the transformation. Results of these experiments are summarized in Figure 3. As shown in Figure 3A, colony formation was only observed in the PMG agar plate supplemented with adenine and uracil, confirming complementation of the *leu1* deficiency by the *LEU2*-containing pYZ1N plasmid. However, the same pYZ1N-carrying cells were unable to form colonies on Zeo-containing plates. In contrast, colonies were seen in all four testing plates when the pYZ1N-bleMX6 plasmid was used (Figure 3B). DNA transformation efficiency of ~$1.2 \times 10^5$ transformants/µg DNA was seen in both pYZ1N and pYZ1N-bleMX6 plasmid DNA, indicating that addition of the bleMX6 gene cassette to the pYZ1N vector did not affect the efficiency of *LEU2* selection (Figure 3, A and B, left top). Threefold reduction in transformation efficiency was observed both in the Zeo-selecting AUL+Zeo plate and double selection AU+Zeo plate, suggesting the observed decrease was likely due to the efficiency of Zeocin resistance. Surprisingly, the transformation efficiency was much lower (~$2.4 \times 10^2$ transformants/µg DNA) in the YES+Zeo medium than in the AUL+Zeo PMG medium. One possibility for the observed discrepancy is that the bleMX6 protein level produced from the plasmid might be temporarily lower in rich medium than in that of minimal medium, which is somewhat reflected by the delayed growth of Zeo$^R$ cells in YES (Figure 2A). Conceivably, cells with low or no bleMX6 protein would have died due to treatment of Zeocin. To test this possibility, we have further incubated the transformed cells in nonselective PMG liquid medium up to 8 h before onset of cell divisions, which was constantly monitored under the microscope. Indeed, the same level and higher transformation efficiency

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were seen in all four plates. It should be mentioned that even though we were unable to observe obvious cell division during those 8 h, the fact that significantly higher transformation efficiency was seen than that of 0 h indicates that possible cell doubling might contribute to the observed additional increase of colony formations. Nevertheless, the notion here is that, after brief incubation, similar level of transformation efficiency could be achieved in all four experimental conditions.

In summary, we demonstrated here that Zeocin, a phleomycin analog, can be used for selection of bleMX6 resistance in fission yeast either from an integrated gene in the chromosome or from an autonomously replicating plasmid. To maximize transformation efficiency, 2–3 h of cell incubation in nonselective medium is recommended before plating on Zeocin-containing plates. Unlike phleomycin and similar to G418, Zeocin works well both in YES and PMG media (Reference 8, www-bcf.usc.edu/~forsburg/drugs.html). Based on the described results, we recommend using 150–200 mg/L Zeocin for proper inhibition of fission yeast cell growth or for selection of bleMX6 resistance. The minimal PMG medium appears to be the medium of choice, because it works well in both solid and liquid media. Most importantly, it allows combined use and co-selection of Zeocin with other auxotrophic markers such as *LEU2* from an autonomously replicating plasmid as shown in this study. Finally, Zeocin should be used with caution, because Zeocin, like bleomycin and phleomycin, is a radiomimetic agent that can cause DNA double-strand breaks (DSBs). It is highly possible that Zeo$^R$ cells experience DSBs in the presence of Zeocin, resulting in activation of cell cycle checkpoint. This suggests that Zeocin (also phleomycin) may not be an appropriate selection drug for cells that harbor mutations in checkpoint/DNA damage response/DNA repair genes.

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## Competing interests

The authors declare no competing interests.

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