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Reagent/Equipment used in your research:

Name of the reagent	Company	Catalogue number	Comments (optional)
2-deoxy-galactose (2-DOG)	Sigma Aldrich	D4407	Also purchase Sucrose, Dextrose, and glycerol from Sigma Aldrich
Middlebrook 7H10 Agar	BD Biosciences	262710	
32P dCTP	Perkin Elmer	BLU013H250UC	
Gene Pulser Xcell Microbial System	Bio-Rad	165-2662	
Hybridization Oven	VWR	47746-130	

Please include short abstract below. The abstract can already be published, as this will be sent the company on your behalf, and won't be reproduced by JoVE in any fashion. The company who sponsors your article will NOT have any input into your article, as you and your group will be responsible for the science.

Counterselectable markers are powerful tools in genetics because they allow selection for loss of a genetic marker rather than its presence. In mycobacteria, a widely used counterselectable marker is the gene encoding levan sucrase (*sacB*), which confers sensitivity to sucrose, but frequent spontaneous inactivation complicates its use. We have shown that the *Escherichia coli*

galactokinase gene (*galK*) can be used as a counterselectable marker in both *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. Expression of *E. coli galK*, but not the putative *M. tuberculosis galK*, conferred sensitivity to 2-deoxy-galactose (2-DOG) in both *M. smegmatis* and *M. tuberculosis*. Here, we demonstrate a method using *E. coli galK* in combination with *sacB* as counterselectable markers in a two-step allelic exchange method to make unmarked gene deletions in mycobacteria. We show that when galK and sacB are used as dual counterselectable markers and marker loss is selected for on 0.2% 2-DOG/5% sucrose, 98.6–100% of sucrose/2-DOG resistant clones had undergone recombination, indicating that the frequency of mutational inactivation of both markers was lower than the recombination frequency. This method establishes a new counterselectable marker system for use in mycobacteria that shortens the time to generate unmarked mutations in *M. smegmatis* and *M. tuberculosis*. This system further expands the available methods for genetic manipulation of mycobacteria and provides tools for counterselection that can be adapted to other bacteria.