

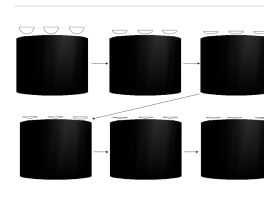
May 10, 2020

Version 1

Modified protocol for *URA3* counter-selection at highly expressed regions of *Saccharomyces cerevisiae* V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.bf7njrme>



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Protocol Citation: Lenny Teytelman, Jasper Rine 2020. Modified protocol for *URA3* counter-selection at highly expressed regions of *Saccharomyces cerevisiae*. protocols.io <https://dx.doi.org/10.17504/protocols.io.bf7njrme>



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Protocol status: Working

This protocol was suggested by Gille Fischer. In the Rine lab, it worked for Lenny Teytelman and Erin Osborne.

Created: May 10, 2020

Last Modified: May 10, 2020

Protocol Integer ID: 36814

Keywords: ura3 protein, saccharomyces cerevisiae, appropriate for genomic loci, regions of saccharomyces cerevisiae, ura3, genomic loci, modified protocol, similar yeast, replica, many plates for the replica

Abstract

This is a modified protocol for *URA3* counter-selection in *S. cerevisiae* and similar yeasts. It is identical to the standard counter-selection with two transformations, with the exception of the replica-plating step.

This protocol is appropriate for genomic loci where *URA3* is over-expressed and the Ura3 protein is likely to be present in the colonies and cells at higher toxic levels. Because this method required five times as many plates for the replica-plating, it is more wasteful and should only be used when necessary.

Guidelines

[The following is from Appendix A of my 2009 doctoral dissertation at UC Berkeley.]

INTRODUCTION

To compare the efficiency of the establishment of silencing of the Rap1 binding site variants at *HMR-E*, I needed to substitute the original Rap1 binding site at *HMR-E* with the genomic consensus for Rap1. Bilge "Ozaydin constructed the fragment with the desired changes, and I just needed to perform two transformations to replace the native *HMR-E* sequence. The first transformation would delete the *HMR-E* sequence, replacing it with a *URA3* cassette, and the subsequent transformation would replace the *URA3* with the new construct from Bilge.

This type of counter-selection is a standard procedure and is usually done in a span of two-three weeks in budding yeasts. The first transformation to delete *HMR-E* was immediately successful, with high efficiency. Exactly as expected, it required only a single week. I confirmed the presence of the *URA3* cassette at the right position within *HMR-E* by sequencing. The next step was like a battle between the single-cell eukaryote and the graduate student. Based on suggestions from countless lab meetings and personal troubleshooting sessions with members of the Eisen and Rine labs, over the course of three years, I repeated the second transformation numerous times, with some modifications at each step.

While I have efficiently performed numerous transformations in *S. cerevisiae* and in *S. bayanus*, at different positions within each of their genomes, the specific Rap1 binding site counter-selection at *HMR-E* took three years of trial and error. The final, successful transformation required a modification of the standard protocol that warrants a description in this appendix. This is especially note-worthy because several member of the Rine lab have had persistent difficulties with this type of counterselection in the silenced regions of *S. cerevisiae*.

RESULTS

1. Logical changes in attempted transformations led to consistent failure

The first and successful transformation replaced a 450 base-pair sequence with a longer 1,600 base-pair cassette. The next transformation was to perform the reverse, replacing a long region with a shorter one. The efficiency of transforming with a short DNA sequence is lower than with long sequence (Annie Tsong, personal communication), so I scaled up the second transformation, performing it three times in parallel on five cultures, instead of just one. A separate attempt at increasing the efficiency was to transform with a much higher concentration of the target construct, amplified by PCR and concentrated after gel-extraction. Yet another approach was to perform a co-transformation, with a plasmid and the genomic-Rap1-HMR-E. The plasmid included the *LEU2* marker, allowing for initial selection for plasmid presence, followed by selection for the loss of *URA3*. This ensured that the cells had taken up DNA, at least as a plasmid, then asking if homologous recombination had occurred.

In addition to the above efforts to increase efficiency of the transformation, I attempted to control for silencing-specific interference. Theoretically, if silencing were to interfere with the homologous recombination of a transformation, this impact would be strongest in the initial transformation to replace *HMR-E* with *URA3*. However, this step worked well, despite the silencing, and with *HMR-E* deleted, the locus would be derepressed. Nevertheless, as some Sir proteins may be recruited through the neighboring *HMR-I*, I performed the second transformation in the presence of nicotinamide. This chemical inhibits the catalytic activity of Sir2, leading to loss of silencing [91], and I grew the pre-transformation cultures with nicotinamide in the media. Erin Osbourne had similar difficulties with *URA3* deletion at *HMR*. She also tried to perform counter-selection transformation in derepressed cells, using a strain without the *SIR3* gene. Neither one of us got the desired transformants with these methods.

Another potential problem could be the *URA3* cassette. I initially used a *Candida albicans* *URA3* cassette, driven by the *TEF* promoter [68]. The *TEF* promoter consists of multiple Rap1 binding sites and is a very strong activator [123]. To avoid overexpression of *URA3* or any other *TEF*-related impact on homologous recombination, I constructed new strains, replacing the *HMR-E* with a *Kluyveromyces lactis* *URA3*, driven by its endogenous promoter [69]. Alas, as with the *C. albicans* cassette, I failed to counter-select against the *K. lactis* *URA3*.

Because colony-PCR may some times fail to detect successful transformations, for each of the above trials, I single-colony streaked up to 40 colonies, and performed regular PCR on DNA genomic preparations.

2. Crazy but successful modification

The counter-selection against *URA3* relies on the toxicity of the Ura3 protein in the presence of 5-fluoroorotic-acid (5-FOA) [124, 125]. Because of a generation lag, where the Ura⁺ phenotype persists for several generations in a ura genotype, the standard protocol uses replica-plating from a YPD to a 5-FOA plate after transformation.

This allows the Ura3 protein to be diluted out of cells through division. An increase in *URA3* expression or increased activity of the Ura3 protein could lead to a more persistent toxicity under 5-FOA. I allowed extra cell divisions by replica-plating from YPD to YPD, multiple times, before transferring cells to the 5-FOA plates.

While this procedure did not solve the problem, a variation on this dilution theme, suggested by Gille Fischer, finally worked. The modified step is the replica-plating from YPD to 5-FOA: after transferring the colonies onto the velvet, five YPD plates are successively pressed to the velvet and discarded before the main transfer to the 5-FOA plate. The modified protocol is described in detail in the Materials and Methods section below. The resulting transformation efficiency is very high, with eight out of ten screened colonies having the correct integration.

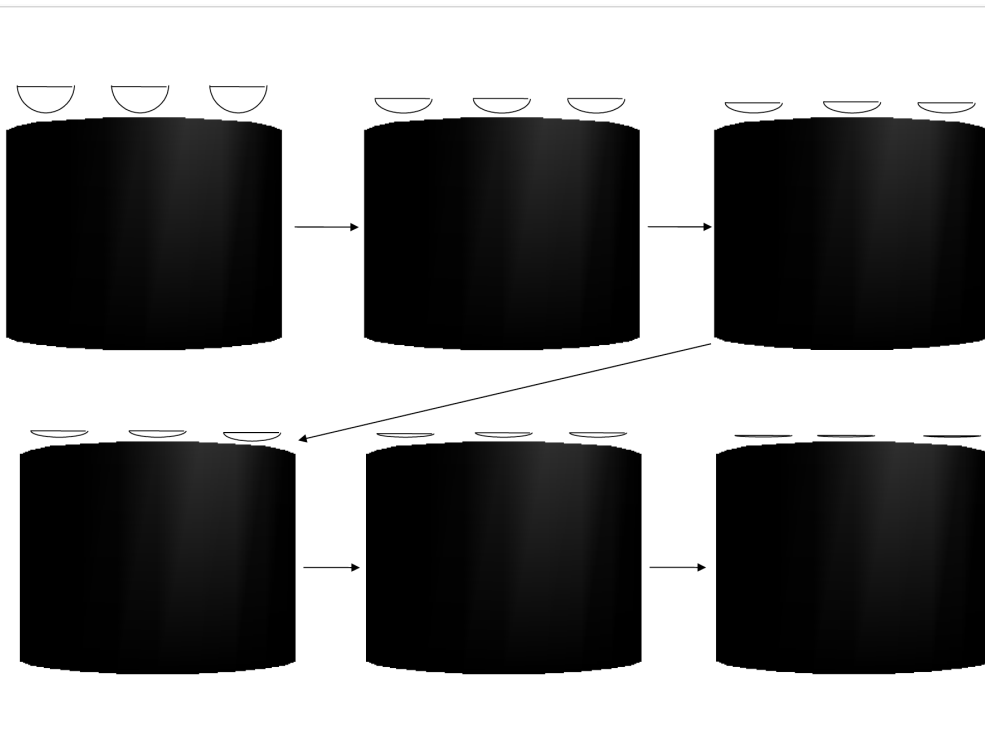
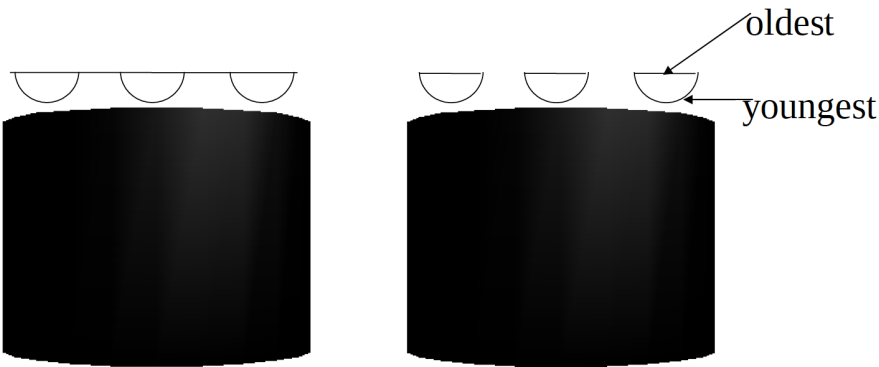
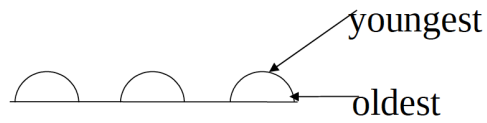
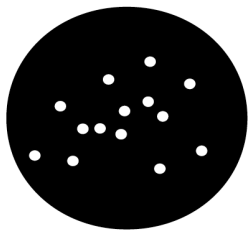
DISCUSSION

The final success of the *URA3* counter-selection at *HMR-E* of *S. cerevisiae* is a small personal triumph over a technical difficulty, but the procedural change is certain to be of use for other researchers. Erin Osborne, a lab mate in the Rine lab, had similar difficulties with a counter-selection against *URA3* at *HMR*, and the modified protocol has also worked for her with a high efficiency.

The particular difficulty at *HMR* may be due to subtelomere-related overexpression of *URA3* from the TEF promoter. There is preliminary evidence that TEF-driven expression increases dramatically at positions closer to the telomeres (Menzies Chen, personal communication). One hypothesis is that the Rap1 presence at telomeres creates a locally high pool of Rap1 in the vicinity of subtelomeric regions, resulting in overexpression from the TEF promoter, only at loci that are telomere-proximal.

In turn, this increase in expression may result in increased generational lag in the toxicity of Ura3 on 5-FOA. This may explain the difficulty of replacing the *C. albicans URA3* with the TEF promoter, but I also failed to replace the *K. lactis URA3* under the control of its endogenous promoter. However, I performed the *K. lactis* transformation only once, and these comparisons would need to be extended for a definitive test. It is also possible that the *K. lactis* promoter also has one or more Rap1 binding sites.

The most likely explanation for the success of the modified protocol is dilution of Ura3. After the transformation heat-shock, cells are spread on YPD plates for over-night growth. As a colony forms on the plate, starting from a single cell, it spreads out in three dimensions, in a cone-like shape. The most recently-divided cells will be at the top of the cone. However, for replica-plating to the 5-FOA plate, the YPD plate is pressed onto the velvet, inverting the cone. In contrast to the order on the YPD plate, the newest cells will be directly on the velvet, and the surface layer will include the oldest cells. The mock-replica-plating onto five YPD plates before the real transfer to the 5-FOA plate, may be sectioning through the colony layers in reverse-chronological order, enriching for the youngest cells for the 5-FOA plate. This sectioning may dilute the Ura3 protein concentration in the cells that end up on the 5-FOA plate, reducing the toxicity. A careful controlled test of this model has not been performed. The experiment suggested by this model is a transformation and replica-plating with successive 5-FOA plates instead of the mock-replicas with YPD plates. If the dilution hypothesis is correct, the number of successful transformed colonies should increase on successive 5-FOA plates.



MATERIALS AND METHODS

1. Transformation to replace HMR-E with URA3

The HMR-E sequence was replaced in *S. cerevisiae* JRY3009 with the *K. lactis* URA3 construct (EUROSCARF plasmid pUG72 [69]), using primers TTTCAATTTTTTATTAAACAATGTTTGATTTTTTAAATCGcagctgaagcttcgtacgc and AGATTAAGCTCATAACTTGGACGGGGATCGTTCGTATTTTgcataggccactagtggatctg to amplify the *K. lactis* URA3 cassette. Transformation was performed using the LiAc method [126]. The parental cells were heat-shocked in the transformation mix for 40 minutes at 42°C, and then resuspended in 250 µl of YPD and plated directly onto a CSM-Ura plate. The resulting *hmr-eΔ::KL* URA3 strains (JRY8991, JRY8992) were two distinct colonies from the CSM-Ura3 plate. The correct integration was confirmed by sequencing. A similar replacement was performed with a *Candida albicans* URA3 cassette (EUROSCARF plasmid pAG60 [68]), resulting in the *hmre Δ::CA* URA3 strain (JRY8993). Primers with 40 base-pairs of HMR-E homology for amplification of the URA3 cassette were TTTCAATTTTTTATTAAACAATGTTTGATTTTTTAAATCGcggatccccgggtaattaa and AGATTAAGCTCATAACTTGGACGGGGATCGTTCGTATTTTcgatgaattcgagctcgttt.

2. Modified protocol to counter-select against URA3

The transformation to replace the URA3 cassette with the genomic-Rap1-HMR-E sequence was performed largely as above. The two strains with *hmr-eΔ::KL* URA3 (JRY8991, JRY8992) were grown in CSM-Ura cultures. After the heat-shock, the cells were plated onto YPD-plates, for overnight growth. In the subsequent replica-plating, the YPD plate is pressed onto the velvet on the replica-plating block. Then, five distinct YPD plates are pressed against the velvet, with each plate discarded. After the five mock-YPD replicas, a single 5-FOA plate is pressed against the same velvet. To screen for the correct integration, I picked ten colonies on each of the two 5-FOA plates, single-colony streaked each one, and then isolated genomic DNA with a Qiagen genomic prep. PCR fragments from the colonies with the expected PCR products were sequenced with primers TCCTTCACATCATGAAATATAA and ACCAGGAGTACCTGCGCTTATTCT, confirming the correct integration of the genomic-Rap1- HMR-E sequence. The resulting strains (JRY8994, JRY8995) were used in the experiments described in Chapter 3 of the thesis.

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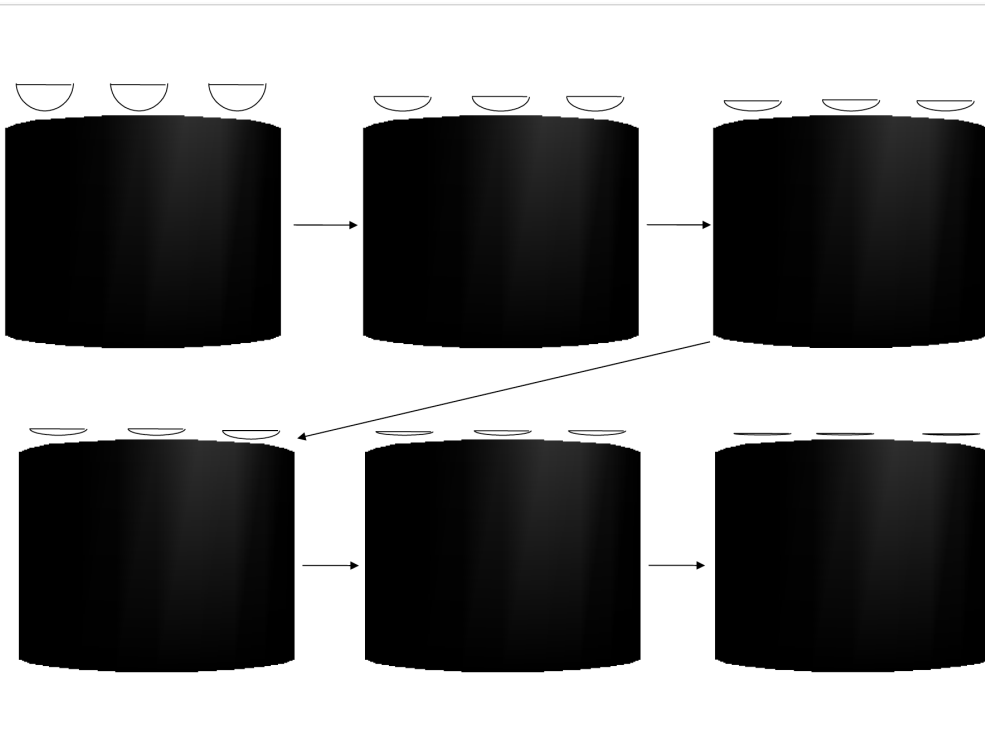
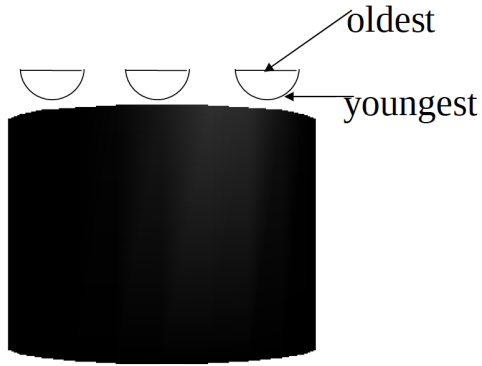
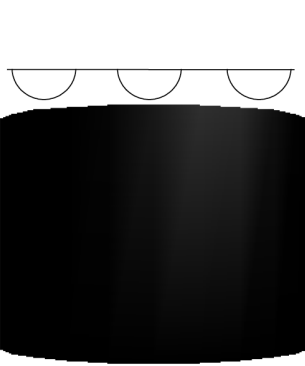
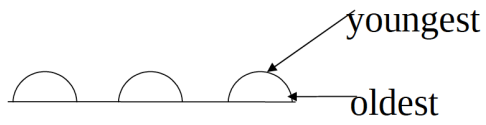
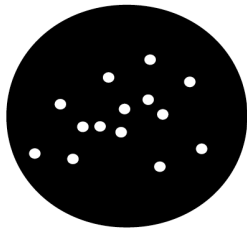


Transformation and selection

- 1 Perform the standard LiAc transformation to replace the locus of interest with the *URA3* cassette.
- 2 Grow on CSM-Ura3 plates and confirm that the integration is correct by sequencing.

Counter-selection against *URA3*

- 3 Perform the standard LiAc transformation to replace the *URA3* cassette with the target sequence.
- 4 After the heat-shock, plate the cells onto YPD-plates, for  Overnight growth.
- 5 Press the YPD plate onto the velvet on the replica-plating block.
- 6 [Mock replica-plating] Press a new YPD plate against the velvet and discard the plate.
- 7 [Mock replica-plating] Repeat Step6 another 4 times to slice through the layers with excess Ura3.
- 8 [Replica-plating] After the five mock-YPD replicas, press a single **5-FOA plate** against the same velvet.





- 9 Grow the colonies and screen for the correct integration using sequencing.