**ABSTRACT** The budding yeast Saccharomyces cerevisiae has many traits that make it useful for studies of quantitative inheritance. Genome-wide association studies and bulk segregant analyses often serve as first steps toward the identification of quantitative trait loci. These approaches benefit from having large numbers of ascospores pooled by mating type without contamination by vegetative cells. To this end, we inserted a gene encoding red fluorescent protein into the MATa locus. Red fluorescent protein expression caused MATa and α diploid vegetative cells and MATa ascospores to fluoresce; MATα cells without the gene did not fluoresce. Heterozygous diploids segregated fluorescent and nonfluorescent ascospores 2:2 in tetrads and bulk populations. The two populations of spores were separable by fluorescence-activated cell sorting with little cross contamination or contamination with diploid vegetative cells. This approach, which we call Fluorescent Ascospore Technique for Efficient Recovery of Mating Type (FASTER MT), should be applicable to laboratory, industrial, and undomesticated, strains.

In yeast, meiotic segregants can be isolated by micromanipulation of individual tetrads to separate the four ascospores or as random spores, where ascus walls are enzymatically removed and the population of released spores is plated. Because tetrad analysis is time consuming and not automated it is ill suited to produce sufficient numbers of recombinant progeny for QTL studies. Isolation of large numbers of random spores without micromanipulation is straightforward but has at least two technical shortcomings. First, a diploid culture subjected to meiosis-inducing conditions contains contaminating diploids that failed to undergo meiosis in addition to the desired haploid meiotic spores. Second, the population of haploid meiotic cells consists of equal numbers of the two mating types, which when plated could mate to form diploids. Without a method for removing diploids and separating haploids into α and α mating types, the random spore population is not useful for QTL mapping. Thus, simple, rapid, and efficient methods for bulk isolation of pure ascospores sorted by mating type are needed.

Rapid separation of haploids and diploids has been accomplished by incorporation of genetic markers that allow for selection by (1) insertion of a gene-promoter construct expressed only in haploids of one mating type and (2) the use of a recessive resistance marker [e.g., canavanine resistance (Whelan et al. 1979)] to select against diploids (Tong and Boone 2007; Ehrenreich et al. 2010). Although effective, these approaches require the introduction of engineered cassettes via multiple manipulations and entail selections that could bias some analyses. Further, they may not be applicable to wild strains, which are rich sources of quantitative variation but are diploid, often homothallic, and lack genetic markers needed for introduction of some engineered cassettes (Timberlake et al. 2011).
Thacker et al. 2011 demonstrated the feasibility of obtaining ascospore-autonomous expression of fluorescent protein constructs and used these to visualize meiotic events. Fluorescently tagged ascospores would be well suited for preparation of QTL mapping populations if expression of the tag could be limited to one mating type. The approach we describe here is based on the integration of a red fluorescent protein (RFP) gene at the MATα locus, with selection provided by a hygromycin-resistance gene so that the cassette can be introduced into any transformable, haploid or diploid, hygromycin-sensitive strain. MATα vegetative cells and ascospores thus tagged contain a visible marker useful for separation of cells by hand or fluorescence-activated cell sorting (FACS).

**MATERIALS AND METHODS**

We used standard yeast molecular genetic techniques (Guthrie and Fink 2004; Amberg et al. 2005) to obtain the *S. cerevisiae* Σ1278b (http://wiki.yeastgenome.org/index.php/History_of_Sigma) strains given in Table 1.

Plasmid pBC58 (Figure 1A) was constructed as follows: A BamHI fragment from plasmid yEpGAP-Cherry (Keppler-Ross et al. 2008) containing a yeast-optimized red fluorescent protein gene and promoter (TDH3pyEmRFP) was cloned into pAG35 (Goldstein and McCusker 1999). A polymerase chain reaction (PCR) product (BCP538-539; Table 2) encompassing the RFP-hygMX genes and adding approximately 50 bp of homology at the 5′ end of MATα2 was used to direct integration at MATα. A second PCR product (BCP569-571; Table 2) spanning MATα and adding terminal StuI sites was then made from genomic DNA and cloned into pCR TOPO2.1 (Invitrogen) to produce pBC58, which is available upon request.

Cells were examined with a 40×/0.75 M/N2 dry objective or 100×/1.30 H/N2 oil immersion objective at room temperature. Fluorescence was monitored at 590 nm with a G-2E/C blocking filter (Nikon). FACS was performed with either a BD Biosciences FACS AriaIIU or a BD Biosciences FACS AriaIII laser with a 561 nm laser and 610/20 filter. Growth curves were performed in microtiter plates with 150 μL of medium/well. Wells were inoculated with 10 μL of 1 OD600/mL aqueous suspensions of cells. Plates were incubated at 30° and OD600 measurements were taken at 30-min intervals after shaking for 15 sec.

Ascospores were isolated by scraping well-sporulated colonies from SM plates, suspending them in 1 mL of phosphate-buffered saline, and adding 1000 units of lyticase (Sigma-Aldrich). After incubation at 30° for 8 hr, sodium dodecyl sulfate was added to 1%. The ascospores were washed twice with 0.1% Tween-20, 5 mM EDTA, and suspended at approximately 10^9/mL.

**Table 1. *S. cerevisiae* strains used in the study**

| Strain | Genotype |
|--------|----------|
| ML1    | ura3-52/ura3-52 his3::hisG/HIS3  
         | leu2::hisG/LEU2 trp1::hisG/TRP1  
         | tec1::KANMX/TEC1 MATα  
         | (matα2::yEmRFP-HYGMX)/MATα |
| ML2    | ura3-52/URA3 his3::hisG/his3::hisG  
         | leu2::hisG/LEU2 trp1::hisG/trp1::hisG  
         | tec1::KANMX/TEC1 MATα/MATα |
| ML3    | ura3-52 leu2::hisG MATα  
         | (matα2::yEmRFP-HYGMX) |
| ML4    | ura3-52 leu2::hisG MATα |
| ML5    | his3::hisG trp1::hisG tec1::KANMX MATα |
| ML6    | his3::hisG trp1::hisG tec1::KANMX MATα |

**RESULTS AND DISCUSSION**

Transformation of haploid MATα strains with the Stul fragment of pBC58 (Figure 1A) resulted in the formation of hygromycin-resistant (hyg^R), pink colonies. The intensity of the color increased upon incubation at 4°. We crossed one transformant to produce heterozygous diploid ML1 (Table 1), whose color was approximately one-half as intense as that of the haploid. Figure 1, B–D shows that segregation of the marker in ML1 tetrads was 2 RFP⁺:2 rfp⁻. PCR analysis of transformants indicated that a single copy of RFP-hygMX had integrated at MATα. Moreover, mating type was completely linked to RFP in 20 tetrads. These results indicate that transformation was due to integration by homology at MATα.

Transformation of a diploid strain with the Stul fragment also resulted in formation of hyg^R, pink colonies. Of these, approximately 10% were converted from a/α to a/α diploids, as evidenced by acquisition of mating competence with a MATα tester strain. This is predicted by transplacement of the MATα locus by the pBC58 Stul fragment, which contains homologous sequences flanking MAT (BUD5/TAF2; Figure 1A). The ability to make RFP⁺/rfp⁻ diploids by transformation speeds up analysis because strains can be sporulated

![Figure 1](transformation_with_RFP_cassette.png)
without intermediate steps to obtain segregants. Further, Klar (1980) showed that a/a diploids could be induced to sporulate after transient mating with a MATa haploid containing a kar1 mutation that interfered with karyogamy (Conde and Fink 1976). This approach, which is expected to produce equal numbers of MATa spores containing and lacking the RASTER insert, could be used to obtain untagged MATa populations.

We subjected vegetative cells and ascospores to FACS to assess the feasibility of separating them by mating type. Figure 2A shows that control haploid cells (nontransformed or MATa derivatives of transformed diploids) and transformed haploids are separated by approximately 3 logs of intensity, whereas heterozygous diploids are intermediate. Gateway permitted separation of the three classes: diploids and MATa and MATa haploids. Separation of ascospores is more relevant to most studies. Figure 2B shows that forward and side scatter analysis separated a crude ascospore preparation into four populations, one of which contained equal numbers of individual fluorescent and nonfluorescent cells. Microscopic examination of these cells showed that they were unaggregated ascospores. Figure 1C shows that this population could be sorted into nonoverlapping, nonfluorescent and fluorescent subpopulations, present in equal proportions.

We tested each population for viability and cross-contamination (MATa → MATa and converse). Table 3 shows that spor viability was high (60%–70%) even after the rigorous enzymatic and detergent treatments used to eliminate ascus walls and vegetative cells, and FACS. For the MATa (nonfluorescent) population, the contamination with hygR cells was <0.2%, which should be acceptable for most purposes. Moreover, as the contaminating cells, which we presume are the result of aggregation, are RFP+, they can be removed without much effort after plating because the colonies are red. The MATa (fluorescent) population was contaminated with approximately 0.2% of fluorescent diploid cells (Table 3). These could be removed by further enzymatic and detergent treatments.

These results lead to the following conclusions:

1. Large, pure populations of MATa and MATa spores can be obtained by FACS. These have high viability making them suitable for GWAS and BSA.

2. RFP is expressed at high enough levels to be detected visually in colonies. Therefore, because RFP and hygR are completely linked to MATa, haploid colonies can be separated into mating types by fluorescence or drug resistance.

3. The ability to use both MATa and MATa populations lacking the introduced marker provides a way to get around potential distortions arising from linkage of genes of interest to MATa.

Although the deletion of MATa2 has been reported to have no effect on growth, mating, or sporulation (Dranginis 1989), we assessed the growth characteristics and mating competence of some of our MATa2 transplacement strains. Figure 3 shows growth curves of strains ML1-4 (Table 1). ML1, a diploid containing the RFP cassette, and ML2, a related diploid lacking the cassette, had similar growth profiles on either YPD or supplemented SD, although ML1 reproducibly grew a little slower. By contrast, ML3, a haploid containing the cassette, grew much more slowly and to a lower final OD in YPD than isogenic ML4 lacking the cassette. However, this difference was moderated and reversed in SD. These differences could be a consequence

| Table 2 Primers used in the study |
|-----------------------------------|
| Primer   | Sequence                                    |
|----------|---------------------------------------------|
| BCP538   | 5′-TGCAAAACACATCTCAACTCTACTACCTATTACTGTATT ACTCAAGAAGAAGCTTCTGACGCTGCA |
| BCP539   | 5′-TTTTCTCTGTGTAAGTTAATTACTCTATCGTTTTCT ATGCTGCGCATATCGATGAATTCGAGCTCG |
| BCP569   | 5′-AGGCCGTGTTAGAAAAAGTGGAATACAAAT    |
| BCP571   | 5′-AGGCCCTTTACGTTAGACCAATGTAATGAA      |

Figure 2 FACS. (A) Vegetative cells. Top: nontransformed MATa haploids; middle: transformed MATa haploids; bottom: heterozygous diploid. (B) Separation of ascospores. An ascospore suspension was subjected to FACS. For the treatments used to eliminate ascus walls and vegetative cells, and FACS. For the MATa (nonfluorescent) population, the contamination with hygR cells was <0.2%, which should be acceptable for most purposes. Moreover, as the contaminating cells, which we presume are the result of aggregation, are RFP+, they can be removed without much effort after plating because the colonies are red. The MATa (fluorescent) population was contaminated with approximately 0.2% of fluorescent diploid cells (Table 3). These could be removed by further enzymatic and detergent treatments.

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| Table 3 Characteristics of sorted ascospores |
|--------------------------------------------|
| Parameter Tested | Sorted Ascospores |                  |
|                  | RFP− | RFP+ |
| Physical counta | 6.7 × 10⁶ | 6.3 × 10⁶ |
| Viable countb   | 4.2 × 10⁶ | 4.5 × 10⁶ |
| Viability, %    | 69    | 71    |
| Contamination with hygR cells, %c | 0.16 | N/A |
| Contamination with diploid cells, %d | N/A  | 0.21 |

a Counted in a hemocytometer.
b Serial dilutions were spread onto YPD plates and colonies were counted after 2 days at 30⁰C.
c RFP-negative cells (2 × 10⁶ CFU/plate) were spread onto YPD plates containing 200 µg/mL of hygromycin-B. Colonies were counted after 3 days at 30⁰C.
d RFP-positive cells (20–50 CFU/plate) were grown on YPD for 2 days at 30⁰C and replica-plated onto lawns of a MATa tester strain. After 2 days at 30⁰C, the colonies were scored for halo formation. Diploids were implicated by lack of halo formation.
of the insertion of two strong promoters at \textit{MATa2} and suggest that controlled measurements of growth rates (or other traits of interest) are required for strains containing the RFP cassette. Of course this caution applies to any strains carrying residual markers, selection cassettes, chromosome abnormalities, etc., introduced to facilitate QTL studies, because they might modify or bias traits of interest directly or indirectly.

We found that RFP strains mated as well as non-RFP strains in routine strain constructions. However, in a mating assay where congenic RFP\(^+\) and \textit{rfp}\(^-\) strains were in competition for a common mating partner the RFP\(^+\) strain mated somewhat less efficiently than the \textit{rfp}\(^-\) strain. This disadvantage decreased with increased mating time. Dranginis, 1989, reported that strains containing a complete deletion of \textit{MATa2} had normal mating characteristics, but this conclusion was not based on the sensitive competitive assays employed here. Whatever the function of \textit{MATa2} and the effect of the insertion, RFP strains in which it is disrupted mate well under the standard, noncompetitive conditions used for strain construction.

These results lead to the following conclusions:

1. Integration of the RFP cassette at \textit{MATa} does not influence growth rate on one medium but does on another. Growth rates of selective markers should be assessed in QTL studies.
2. The RFP cassette does not interfere with standard genetic manipulations, but may reduce mating efficiency in more sensitive assays.

Summary
Integration of a cassette containing RFP and \textit{hpy}\(^{6}\) into the \textit{MATa} locus provides a simple, robust means for marking mating type so that \textit{a} and \textit{\(\alpha\)} ascospores can be separated and purified by FACS. The fact that the cassette can be transformed into most haploid or diploid \textit{S. cerevisiae} strains without introduction of other mutations means that it should be useful for studies of quantitative inheritance in laboratory, industrial, and wild strains. Moreover, it can serve as a mating type indicator without compromising other genotypic or phenotypic features.

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