points are distributed along lines that represent the expected differences in signal intensity from each channel. Without a systematic dye effect or after proper normalization, we would see three sets of data points along three equally spaced lines parallel with slope one. On the other hand, the direct comparison of measurements for each dye on different slides can be interpreted with no normalization because our experimental setting does not require across-slide normalization. Therefore, the most direct way to assess the efficiency of RNA amplification and Cy3 and Cy5 dye incorporation during RNA labeling is to directly compare measured quantities for each channel on pairs of slides. Figure 2C illustrates the results of these comparisons. For each channel, we show the estimated slope for the corresponding intensity comparison, which corresponds precisely to the expected values.

This procedure can be used to monitor the quality of targets after the very last step of the labeling protocol, without compromising the amount of labeled target available for hybridization. Importantly, other commercially available products can efficiently control for amplification but cannot determine the efficiency of the labeling reaction because this would require laser excitation or equivalent wavelengths. Furthermore, one can determine label efficiency using data (Figure 1C) to correct for the specific activity of labeled targets to facilitate data normalization. In the case of greater differences in labeling efficiency, one can repeat the labeling step prior to hybridization, thus avoiding data of lower quality that could impose potential distortions during normalization and eliminating the costs of hybridization.

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Counter-selection facilitated plasmid construction by homologous recombination in *Saccharomyces cerevisiae*

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The ability to shuffle and combine separate DNA fragments is one of the most fundamental activities in molecular biology. For this reason, *in vitro* manipulation of bacterial plasmid DNA using restriction enzymes is a staple technique of virtually every molecular biologist. Unfortunately, the position and availability of unique or rare restriction enzyme recognition sequences typically dictate the entire direction of subcloning strategies.

Recombinational cloning techniques based on homologous recombination in *Saccharomyces cerevisiae* can be used to circumvent tedious multiple-step plasmid construction strategies without the need for specific restriction sites (1-4). Unfortunately, the time investment and additional workload associated with the isolation and characterization of the desired recombinant plasmid from heterogeneous populations of *S. cerevisiae* transformants is a limitation of these otherwise valuable techniques.

The screening workload associated with *in vivo* plasmid construction techniques in *S. cerevisiae* has been partially abrogated by the use of various counter-selection techniques to isolate yeast transformants containing only

recombinant plasmid (1,3). However, these useful systems have, to this point, been limited exclusively to the construction of specific yeast expression vectors or required time-consuming replica plating to distinguish yeast transformants carrying recombinant plasmid. Here we describe a general method for *URA3*-based counter-selection during recombinational cloning that effectively eliminates the growth of background yeast transformants carrying regenerated acceptor plasmid by selecting for the growth of yeast containing only recombinant plasmid. Positive yeast transformants generated using this technique harbor exclusively the recombinant plasmid of interest without the need for replica plating (Figure 1).

The utility of the *URA3*-based counter-selection technique was demonstrated during *in vivo* construction of adenovirus E1-deletion rescue plasmids (used for the production of recombinant adenovirus vectors). The *in vitro* manipulation of such viral genomes as bacterial plasmids is typically difficult due to their large size and general lack of unique or rare restriction sites within the virus coding regions being manipulated. An adenovirus rescue vector capable of undergoing recombinational

cloning in *S. cerevisiae* (YRpXC38) was generated by inserting the 1456-bp *TRP1*/*ARS1* (chromosomal origin of replication) *EcoRI* fragment from YRp7 (5) into the unique *EcoRI* site of pXC38 (6). In addition to the appropriate yeast and bacterial replication origins and selectable markers, this vector contains nucleotides 22 to 5788 from the left end of the human adenovirus type 5 (Ad5) (GenBank® accession no. X02996). *URA3*-based counter-selection of this plasmid during recombinational cloning was then made possible by embedding the *S. cerevisiae URA3* gene within the region of the acceptor molecule that would be deleted during a successful recombinational cloning reaction. Briefly, PCR amplification of the *URA3* gene from the plasmid pYES2 (Invitrogen, Carlsbad, CA, USA) was carried out with primers containing 5' overhangs encoding *KpnI* recognition sequences

(forward primer 5'-GGGGGTACC-GCTTTTCAATTCAATTCATC-3'; reverse primer 5'-GGGGGTACCGGGTA-ATAACTGATATAATTA-3', *KpnI* sites indicated by underline). This PCR fragment was subsequently purified from agarose gels using the GENECLAN® DNA isolation kit (Qbiogene, Carlsbad, CA, USA), digested with *KpnI*, and inserted into the adenovirus sequences within YRpXC38 at a unique *KpnI* site, producing the plasmid YRpETE. Electroporation of electrocompetent *S. cerevisiae* with YRpETE generated yeast colonies capable of growth on *trp*⁻/*ura*⁻ synthetic complete medium, but incapable of growth on *trp*⁻ synthetic complete medium fortified with 0.1% 5-fluoroorotic acid (5-FOA) (7).

Recombinant adenovirus vectors are often used as mammalian expression vectors. Consequently, PCR-

derived mutations to any gene being inserted into the adenovirus backbone are undesirable. Linker-assisted homologous recombination (1,4) was utilized to eliminate the need to PCR amplify the donor fragment. The relative positions of the regions that underwent homologous recombination and the reaction conditions and sequences of the oligonucleotides used to generate the linkers are depicted in Figure 2.

The donor fragment [a 1697-bp *AfIII*/*AfIII* fragment of pEGFP-N1 containing the enhanced green fluorescent protein (EGFP) reporter gene] was purified from agarose gels and resuspended at a final concentration of 1 µg/µL. Typically, several isolations had to be pooled and concentrated by ethanol precipitation in order to obtain a donor fragment solution of sufficient concentration.

Figure 1. *URA3*-based counter-selection facilitated recombinational cloning strategy. A *Saccharomyces cerevisiae URA3* selectable marker is inserted into the acceptor plasmid within a region that will be deleted during a successful recombinational cloning reaction. Subsequent deletion of the *URA3* selectable marker resulting from homologous recombination in *S. cerevisiae* allows transformants harboring recombinant plasmid to survive *URA3*/5-fluoroorotic acid (5-FOA) counter-selection, while cells harboring the parental acceptor plasmid containing the *URA3* marker (generated by resealing of cohesive ends within the acceptor plasmid) do not survive.

Figure 2. Production of double-stranded linkers and positions of linearizing restriction sites in the YRpETE acceptor. PCR amplification of the linkers was carried out using strategies described previously (1). Oligonucleotides Lt1 and Lt2 were used to prime PCR amplification of bases 152 to 384 of the Ad5 genome from YRpETE. Oligonucleotides Rt3 and Rt4 were used to prime PCR amplification of bases 3514 to 3757 of the Ad5 genome from YRpETE. Lt2 and Rt3 each contain 40-base 5' overhangs (nucleotide sequences in boldface) possessing homology to the first 40 bp of the cytomegalovirus (CMV) promoter and the 3' end of the green fluorescent protein (GFP) mRNA in pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA, USA), respectively. PCRs were carried out in 50-µL reaction volumes containing 50 pmol of both forward and reverse primers, 2.5 U of *Taq* DNA polymerase (New England Biolabs, Mississauga, ON, Canada), and the appropriate concentration of the accompanying buffers for 35 cycles of denaturation at 95°C for 1 min, primer annealing at 47°C for 1 min, and extension at 72°C for 1 min. Reaction products were examined by agarose gel electrophoresis followed by ethanol precipitation and resuspension in Tris-EDTA (TE) buffer at a final concentration of 1 µg/µL.

Recombinational cloning reactions were carried out by introducing 1 µg of donor fragment, 1 µg of each linker, and 100 ng of *Afl*II linearized YRpETE acceptor (linearizing restriction site locations depicted in Figure 2) into *S. cerevisiae* strain JY128 (*ura3-52*, *trp1-Δ63*) by electroporation (8). The reaction mixture was subsequently plated on *trp*⁻ synthetic complete medium fortified with 5-FOA. Recombinational cloning reactions were performed in triplicate, yielding an average of 200 ± 11 yeast transformants per reaction. Yeast transformant plasmid DNA isolation (Reference 9, modified by the addition of chloroform extraction prior to ethanol precipitation) was carried out 3–5 days later and used to transform competent *Escherichia coli* (note that one half of the precipitated DNA was used per *E. coli* transformation). Restriction fragment banding patterns of plasmid DNA isolated from 50 *E. coli* transformant lines revealed that all harbored recombinant plasmids possessing the predicted banding patterns. Dideoxy sequencing of three recombinant plasmids within the regions of PCR-generated homology was performed (sequencing reactions performed at and results obtained from Norgen Biotek, St. Catharines, ON, Canada). A single thymine base deletion was detected within the PCR-generated regions of homology in one of the three plasmids analyzed. In addition, the integrity of the EGFP reporter gene was maintained throughout recombinational cloning, as evidenced by GFP fluorescence (visualized under 488 nm wavelengths) of HEK 293 cells (CRL-1573; ATCC, Manassas, VA, USA) (10) transfected with CsCl density gradient centrifugation purified recombinant plasmid (using ExGen™ 500 In Vitro Transfection Reagent; MBI Fermentas, Burlington, ON, Canada).

In order to further demonstrate the utility of *URA3* counter-selection during in vivo plasmid construction, partially digested YRpETE acceptor was utilized as substrate during in vivo plasmid construction. It has been demonstrated that partially digested acceptor can be utilized as substrate for in vivo plasmid construction, provided a suitable selection system is available to decrease screening workload (3). The YRpETE acceptor contains three

*Sac*II recognition sites, only one of which [labeled *Sac*II(358) in Figure 2] is in a suitable location to facilitate recombinational cloning with the linkers described in Figure 2. *Sac*II partial digests of YRpETE were carried out as described previously (11). YRpETE acceptor plasmid, cut only once by *Sac*II, was purified from agarose gels for use in recombinational cloning reactions. When purified single-cut *Sac*II partial digests of YRpETE were substituted for *Afl*II-digested YRpETE during recombinational cloning reactions, an average of 50 ± 9 yeast transformants growing on *trp*⁻ synthetic complete medium fortified with 5-FOA was observed. All plasmids analyzed from these transformants possessed the predicted restriction fragment banding patterns (20/20, as determined by isolation of yeast plasmid DNA, *E. coli* transformation, and restriction enzyme fragment banding pattern analysis).

Very high proportions of background yeast transformants accompanied similar recombinational cloning reactions without *URA3* counter-selection. When *Afl*II-digested YRpXC38 acceptor was utilized under identical recombinational cloning reaction conditions without *URA3*/5-FOA counter-selection, restriction fragment banding patterns of plasmid DNA isolated from 70 ampicillin-resistant bacterial colonies revealed that only three harbored the recombinant plasmid (the remainder contained regenerated acceptor plasmid). Similar recombinational cloning experiments using single-cut *Sac*II partial digests of YRpXC38 yielded only regenerated acceptor plasmid in 100 ampicillin-resistant *E. coli* transformants screened by restriction fragment banding pattern analysis.

The restriction sites necessary for incorporation of both the yeast selectable markers and ARS were present in positions convenient for the construction of the acceptor plasmids described herein. This may not be the case for other cloning vector systems. The *TRP1*/*ARS1* and *URA3* fragments utilized in this report are sufficiently short that they can be amplified by PCR and subsequently utilized for two-fragment recombinational cloning reactions (4). Such a strategy has been undertaken, and we have yet to observe any recombinants

other than those of the expected conformation (as examined by restriction fragment banding pattern analysis, unpublished data). Any integration events or PCR/recombination-induced mutations disrupting the proper function of the markers or replication sequences appear to be effectively filtered out by the selective pressure of plating on the appropriate yeast selective medium and recovery of the recombinant plasmid by transformation into *E. coli*.

Counter-selection facilitated recombinational cloning is capable of generating multiple plasmid constructs with similar backbone DNA sequences in a short time frame. The convenient zero background selection system allows the isolation of recombinant plasmid from a recombinational cloning reaction without the need for time-consuming replica plating. More important than its ability to merely decrease screening workload, this counter-selection technique eliminates problematic background yeast transformants harboring regenerated acceptor plasmid. The elimination of background yeast transformants allows the use of partially digested acceptor as substrate for recombinational cloning, thus increasing the number of useful restriction sites available to the researcher (a common dilemma when manipulating large plasmid systems). Counter-selection facilitated recombinational cloning, therefore, provides an alternative to the generation of novel restriction sites by site-directed mutagenesis or the insertion of multiple cloning sites and should facilitate research involving plasmid systems that are typically refractory to manipulation by traditional in vitro techniques.

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A/T-rich inverted DNA repeats are destabilized by chaotrope-containing buffer during purification using silica gel membrane technology

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The recovery of DNA from agarose gel is a frequently used step in nearly all gene engineering procedures. Various methods have been developed for this purpose, and the use of silica gel or glass matrices seem to be the most efficient ones. Here we describe the difficulties encountered when purifying DNA fragments consisting of inverted repeat sequences.

In our study, we wanted to determine the knock-down phenotype of several novel *Dictyostelium discoideum* genes by using RNA interference (RNAi) (1), which is the method of choice when assaying gene function both in genetically well established and in genetically less well-characterized organisms (2). The RNAi method that we employed consisted of the introduction of double-stranded RNA (dsRNA) molecules (usually several hundred base pairs

long) that are processed into the effector short interfering RNAs (siRNAs; Reference 3), resulting in sequence-specific mRNA degradation (4,5).

Using the sequences of our candidate genes, we constructed an inverted repeat targeting cassette (RNAi cassette) for each gene, which should produce a hairpin RNA upon transcription. Figure 1 depicts the RNAi cassette construction. The identity of the constructed RNAi cassettes was verified by restriction analysis; Table 1 summarizes their properties.

To reclone the prepared RNAi cassettes from the cloning vector into the expression vector, we cut the RNAi cassettes using *Bam*HI, separated them from the vector DNA by electrophoresis on 1% agarose gel, and purified them by gel purification using the QIAquick™ Gel Extraction Kit (Qia-

Figure 1. The RNA interference (RNAi) cassette construction procedure and transcribed RNA structure.