Chapter 17

DNA Double-Strand Break-Induced Gene Amplification in Yeast

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Abstract

Precise control of the gene copy number in the model yeast *Saccharomyces cerevisiae* may facilitate elucidation of enzyme functions or, in cell factory design, can be used to optimize production of proteins and metabolites. Currently, available methods can provide high gene-expression levels but fail to achieve accurate gene dosage. Moreover, strains generated using these methods often suffer from genetic instability resulting in loss of gene copies during prolonged cultivation. Here we present a method, CASCADE, which enables construction of strains with defined gene copy number. With our present system, gene(s) of interest can be amplified up to nine copies, but the upper copy limit of the system can be expanded. Importantly, the resulting strains can be stably propagated in selection-free media.

Key words CEN.PK, DNA double-strand break, Gene amplification, Gene targeting, Homology-directed recombination, I-SceI nuclease, Metabolic engineering, *Saccharomyces cerevisiae*

1 Introduction

S. cerevisiae is a widely used eukaryotic model organism and an increasingly popular host in cell factory designs for bio-production of valuable compounds [1–7]. In both basic and applied research experiments it is often necessary to increase the copy number of relevant genes in order to understand the gene function or to improve protein yields or to fine tune metabolic pathways for optimal production. In *S. cerevisiae*, high expression levels are most commonly achieved using self-replicating, 2 μ-based plasmids [8]. Unfortunately, the copy number of these plasmids may fluctuate by several orders of magnitude, which usually results in significant productivity loss in long-term cultivations [9–11]. Genomic integrations are more stable. However, multiple copies need to be integrated to reach high expression levels. This can be accomplished by repetitive cycles of transformation into unique genomic loci [12–14] or by multiple insertions of the gene-targeting substrate into naturally occurring repeated genome sequences, i.e.,

Andrés Aguilera and Aura Carreira (eds.), *Homologous Recombination: Methods and Protocols*, Methods in Molecular Biology, vol. 2153, https://doi.org/10.1007/978-1-0716-0644-5_17,

rDNA, Ty, and δ-sequences [15–22]. The advent of CRISPR/Cas tools has further improved construction of strains with multiple copies of integrated genes [23–26]. However, strains possessing multiple gene copies (integrated either in tandem arrangements or dispersed throughout the genome) may be unstable as amplified genes are prone to loss via direct-repeat recombination (DRR).

Here we describe a method, CASCADE, which enables construction of stable strains with defined gene copy numbers integrated in the *S. cerevisiae* genome. The basis of the system is the gene-*a*cceptor-*s*tarter (GAS) strain, which contains *g*ene-*a*cceptor (GA) cassettes in different numbers and in different positions in the genome; and the number of GA cassettes in a strain defines the level of gene amplification. The GA cassette contains an *URA3* counter-selectable marker and an I-SceI recognition sequence. Importantly, all sites containing a GA have been tested for the ability to support high gene-expression levels [12]. In a gene amplification experiment, a strain with a given number of GA sites is targeted by a donor amplicon (DA) sequence, which may contain one or more genes of interest [27] (Fig. 1). In such experiments, typically only one DA sequence integrates into one of the several GAs in the strain. Next, amplification is achieved via I-SceI-induced double-strand breaks that trigger gene conversion [28] between empty GA cassettes and the DA sequence that serve as a repair template. When gene amplification is complete, no *URA3* markers are left in the genome as all GAs have been replaced with the DAs. These strains can be selected on solid 5-FOA medium. Hence, precise control of the DA copy number is achieved by choosing the GAS strain with the desired number of GA cassettes. In the CASCADE setup, two sets of starter strains (mating type *MAT***a** or *MAT*α) containing 1–4, 7, and 9 GA cassettes are available [27] (Table 1).

A gene-targeting construct containing DA is assembled by USER cloning (or a similar method for seamless fusion of DNA fragments) in a designated vector denoted as pCSN [27], (Fig. 2). After insertion into pCSN, the gene(s) of interest is (are) positioned next to a selectable and recyclable *TRP1* marker. Moreover, the selectable DA cassette is flanked by the 'A' and 'B' regions, which will target the construct into the GA cassettes in the genome via homology-directed recombination (HDR). After transforming the DA gene-targeting substrate into a GAS strain, the *TRP1* marker can be eliminated from the DA cassette by selection on solid 5-FAA medium as the *TRP1* marker is positioned between a direct repeat. A strain containing a DA is then transformed with a 2 μ-*TRP1*-based plasmid containing an I-SceI coding gene under the control of the *GAL1* promoter, and subsequent plating on galactose medium induces gene amplification. Finally, strains where all GA cassettes have been replaced by DA are selected on solid medium containing 5-FOA. During this step the I-SceI plasmid is often

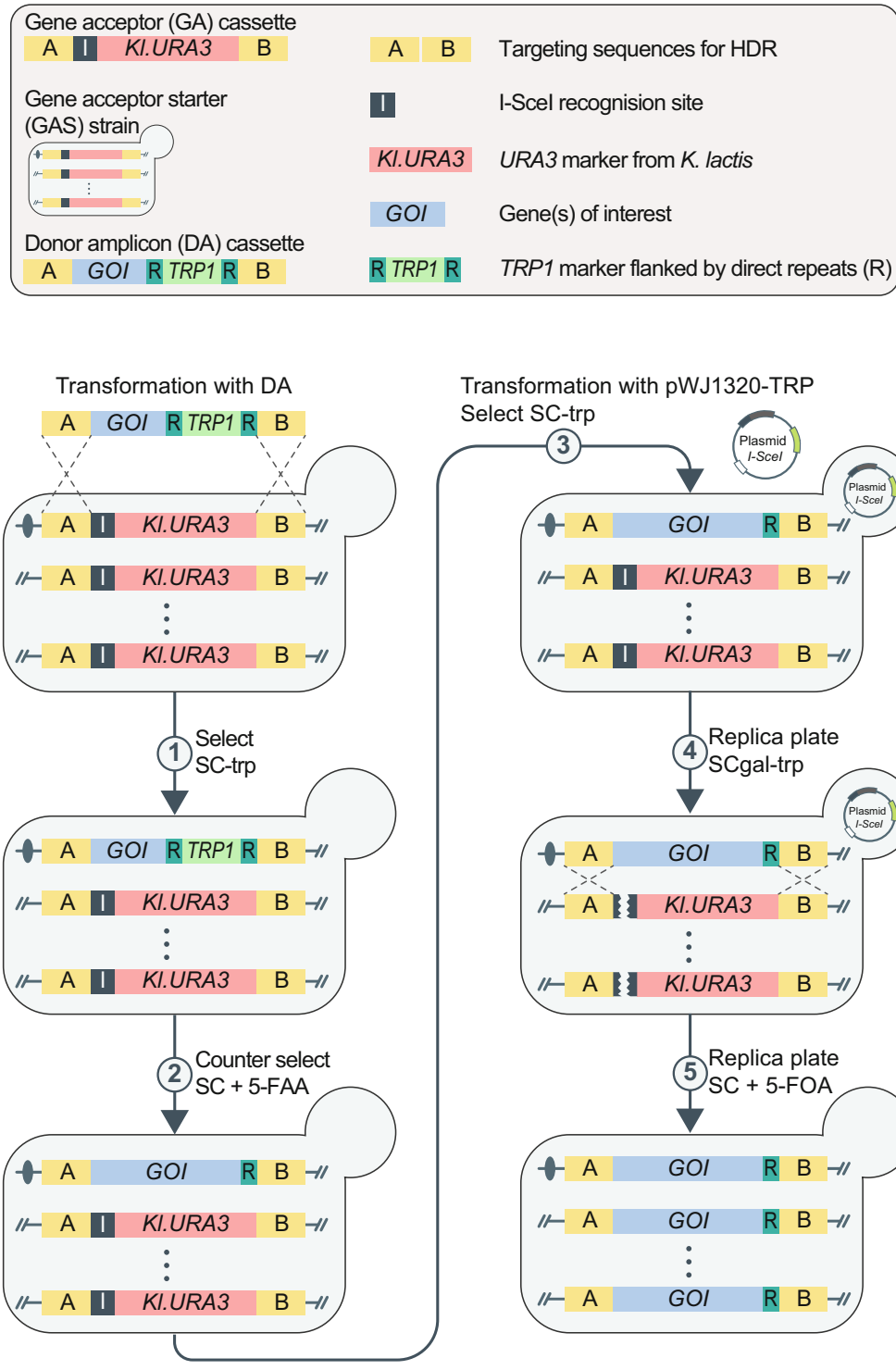

Fig. 1 Overview of the CASCADE amplification system. The five major steps are annotated by gray circles. In step 1, the DA fragment is transformed into one or more GAS-X ("X" is the number of GA cassettes) strains. In

spontaneously lost as there is no selection pressure to retain it. If not, plasmid loss can be selected on 5-FAA medium. Using CASCADE for gene amplification, we have demonstrated that DAs containing one or more genes can be efficiently amplified to produce strains with up to nine integrated copies [27]. More specifically, genes coding for proteins of different sizes and functions i.e., red and cyan fluorescent proteins, 6-MSA polyketide synthase, and different enzymes of de novo vanillin-glucoside biosynthetic pathway were successfully amplified. Lastly, the CASCADE method was proven to be a powerful tool for optimization of biosynthetic pathways as it can be used to identify the rate-limiting step [27].

2 Materials

2.1 PCR

1. 2× Phusion U Hot Start PCR Master Mix (ThermoFisher Scientific) (*see* **Note 1**).
2. DNA template.
3. 10 μM primer solutions.
4. FastDigest DpnI enzyme (ThermoFisher Scientific).
5. Gel band purification kit e.g., illustra GFX™ PCR DNA and gel band purification kit (GE Life Sciences).

2.2 Assembly of DNA Donor Construct

1. Vector pCSN [27].
2. AsiSI (10,000 units/mL) enzyme (New England Biolabs).
3. Nb.bsmI (10,000 units/mL) enzyme (New England Biolabs).
4. USER™ (1000 units/mL) enzyme mix (New England Biolabs).
5. NotI-HF® (20,000 units/mL) or SwaI (10,000 units/mL) enzyme (New England Biolabs).
6. Buffers CutSmart® and NEBuffer™ 3.1 (New England Biolabs).
7. Plasmid isolation kit e.g., GenElute Plasmid Miniprep Kit (Sigma-Aldrich).

Fig. 1 (continued) step 2, the *TRP1* marker gene is recycled by selecting strains on medium containing 5-FAA. In step 3, a 2 μ-based plasmid harboring a galactose-inducible I-SceI gene is transformed into strains from step 2 using *TRP1* for selection. In step 4, the gene amplification is activated by induced I-SceI, which cuts all remaining GA cassettes. As a result, the A and B sequences of the broken GA cassettes mediate gene amplification using DA sequences as a template for repair. In step 5, strains in which all GA cassettes have been replaced by the DA constructs are selected on medium containing 5-FOA; hence, exploiting that *KI.URA3* genes embedded in GA cassettes are eliminated. In this step, most of the strains will also be cured for the plasmid expressing I-SceI as the 5-FOA medium contains tryptophan. Additionally, plasmid-free strains can easily be selected on medium containing 5-FAA

Table 1
List of available GAS strains

Name	*MAT*a	*MAT*α	*his3*	*leu2*	*trp1*	Copy No.	Chromosome number and integration site of the GA cassette
GAS-1A		x	x		x	1	X-3
GAS-1B		x	x		x	1	XI-1
GAS-1C	x			x	x	1	XII-1
GAS-1D	x		x		x	1	XII-5
GAS-2A		x	x		x	2	X-3; X-4
GAS-2B	x			x	x	2	XII-1; XII-2
GAS-2C	x			x	x	2	XII-1; XII-4
GAS-2D	x		x		x	2	X-3; XII-5
GAS-2E		x		x	x	2	VII-1; XI-2
GAS-2F		x	x		x	2	XI-1; XI-4A
GAS-3A		x	x		x	3	X-2; X-3; X-4
GAS-3B	x			x	x	3	XII-1; XII-2; XII-4
GAS-4A	x			x	x	4	XII-1; XII-2; XII-3; XII-4
GAS-4B,C		x		x	x	4	XII-1; XII-2; XII-3; XII-4
GAS-4D	x		x		x	4	XII-1; XII-2; XII-3; XII-4
GAS-4E		x			x	4	XII-1; XII-2; XII-3; XII-4
GAS-7A, B, C		x		x	x	7	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4
GAS-7D		x	x		x	7	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4
GAS-7E,F	x		x		x	7	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4
GAS-7G	x		x	x	x	7	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4
GAS-9A, B,C	x		x		x	9	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4; XI-1; XI-4A
GAS-9D, E,F		x		x	x	9	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4; XI-1; XI-4A
GAS-9G		x	x		x	9	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4; XI-1; XI-4A
GAS-9H	x		x	x	x	9	X-2; X-3; X-4; XII-1; XII-2; XII-3; XII-4; XI-1; XI-4A

Chromosome number and integration site of the GA cassette are annotated as in Mikkelsen et al. [12]

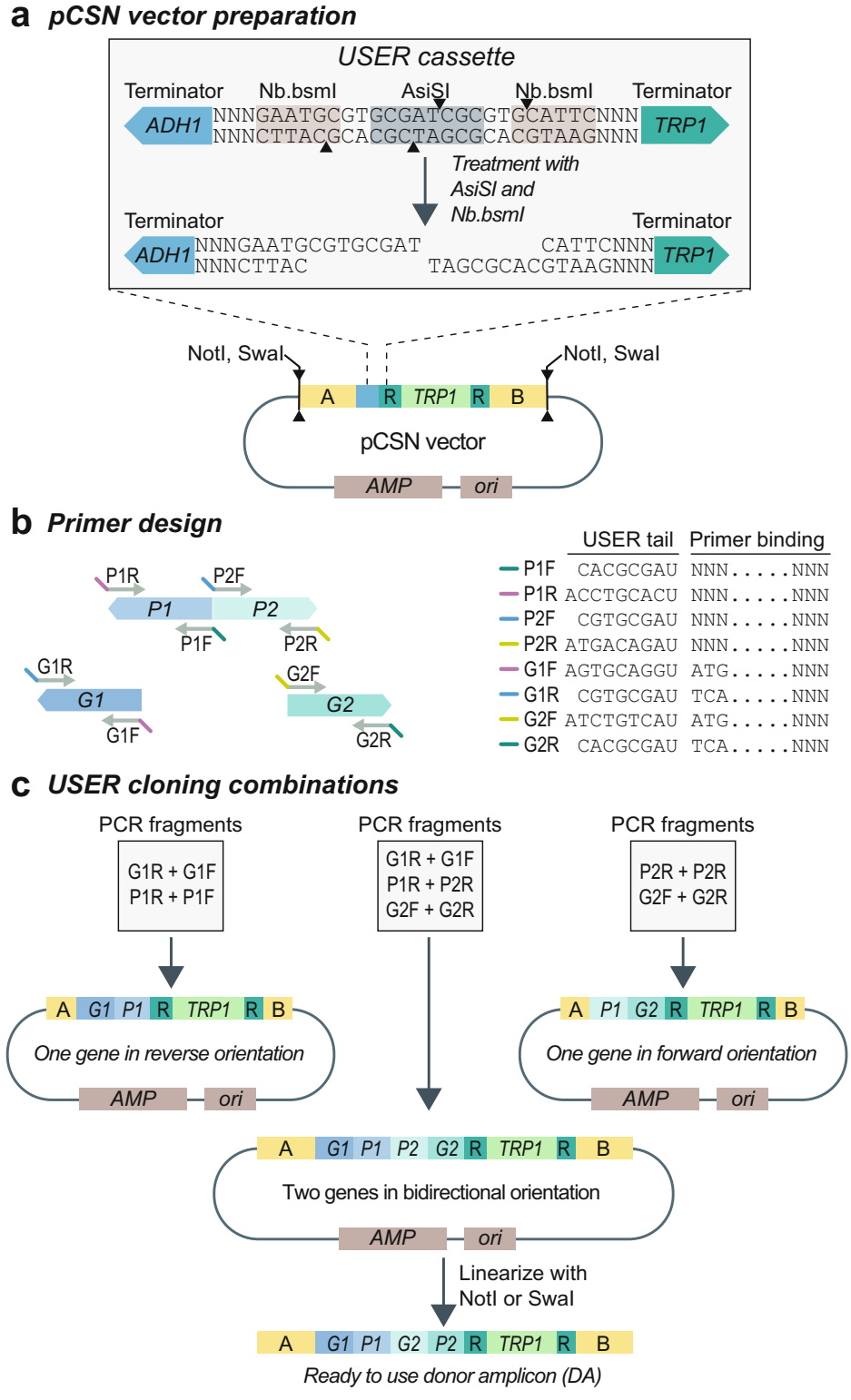

Fig. 2 Overview of the procedure to construct the donor amplicon (DA). (**a**) Map of the pCSN vector. The vector contains AsiSI/Nb.bsmI USER cassette that after treatment with the corresponding enzymes results in specific 8-nt overhangs. The USER cassette is flanked by two terminators (*ADH1* and *TRP1*) in divergent orientation.

2.3 Bacteria and Yeast Strains

1. Competent *Escherichia coli*cells, e.g., DH5α, TOP10, or similar.
2. *S. cerevisiae* CEN.PK-based GAS strains (*see* Table 1).

2.4 Cultivation Media

All media are prepared as described by Sherman et al. [29] with a minor modification as the concentration of leucine is increased to 60 mg/L. Alternatively, commercially available, (e.g., Sigma-Aldrich or others) ready-to-use powder mix of synthetic complete (drop-out) media lacking one or more components can be used instead.

1. Liquid/solid yeast extract peptone dextrose (YPD) medium. For 1 L mix 10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of glucose. If the medium has to be solidified, add 20 g/L of agar prior to autoclave.
2. Solid synthetic complete (SC) medium lacking an appropriate nucleobase or amino acid (SC-ura without uracil and SC-trp without tryptophan).
3. Solid SC + 5-FAA supplemented with 500 mg/L of 5-fluoroanthranilic acid or SC + 5-FOA supplemented with 30 mg/L uracil and 740 mg/L 5-fluoroorotic acid.
4. Solid galactose-based SC medium lacking tryptophan (SCgal-trp) is prepared in the same way as SC medium, but 2% of galactose is used instead of glucose.

2.5 Yeast Transformation

All materials and procedures for yeast transformation are adapted from Gietz et al. [30].

1. Yeast cells (GAS strains).
2. 50% PEG3350 solution. Dissolve 50 g of PEG3350 in approximately 30 mL of ddH_2O in a glass beaker. Gently warm the solution and stir until it dissolves. Adjust the volume to 100 mL in a measuring cylinder and mix thoroughly. Transfer the solution to a glass storage bottle and autoclave.
3. 1 M lithium acetate solution. Dissolve 10.2 g of lithium acetate dihydrate in 100 mL of ddH_2O, autoclave for 15 min, and store at room temperature.

Fig. 2 (continued) The pCSN also features NotI and SwaI restriction sites, thus allowing liberation of the DA construct. (**b**) Primer design strategy to amplify PCR fragments compatible with the pCSN vector (*P1*, *P2*—promoters of your choice; *G1*, *G2*—open reading frames of your choice). All primer sequences depicted in 5′–3′ orientation. (**c**) Possible outcomes of the USER cloning by assembling different PCR fragments (represented by primer pairs in gray boxes)

4. Denatured salmon sperm ssDNA (10 mg/mL). Heat the tube containing ssDNA in a boiling water for 5 min, cool down on ice, and store at −20 °C.
5. Sterile ddH_2O.
6. Linear DA fragments or plasmid pWJ1320-TRP [27].
7. Selection media (Subheading 2.4).

3 Methods

3.1 Preparation of USER Vector

This subsection describes linearization of the pCSN vector at the AsiSI/Nb.bsmI USER cassette locus (*see* Fig. 2a). Once the vector is linearized, one or more PCR fragments (*see* Fig. 2b, c) can be cloned into the USER cassette (*see* **Note 2**).

1. Digest overnight approx. 3 μg of pCSN plasmid with 20 units of the AsiSI restriction enzyme to a final volume of 30 μL, following the manufacturer's recommendations.
2. Next day, add 20 units of Nb.bsmI nicking enzyme and increase the reaction volume to 100 μL by adding extra restriction buffer and ddH_2O. Incubate for 2 h according to the manufacturer's instructions.
3. Column-purify the reaction mix using GFX™ PCR DNA and gel band purification kit (GE Life Sciences) according to the manufacturer's instructions, and elute in 60 μL.
4. Store the linearized pCSN plasmid at −20 °C.

3.2 Preparation of PCR Fragments

This subsection describes preparation of PCR fragments containing overhangs only compatible with USER cloning technique (*see* **Note 3**).

1. Run PCR reactions using primers containing overhangs with uracil base (for example *see* Fig. 2b). For each PCR, mix all components and run the reactions as described in Tables 2 and 3 (*see* **Note 4**).
2. Add 1 μL of FastDigest DpnI enzyme to each of the PCRs and incubate for 1 h at 37 °C. Heat inactivate by incubating at 80 °C for 20 min (*see* **Note 5**).
3. Check a small fraction (approx. 2 μL) of DpnI-treated PCR reaction by 1% agarose gel electrophoresis.
4. Gel-purify each PCR fragment using GFX™ Gel Band Purification Kit (GE Life Sciences) following the manufacturer's instructions, and elute in 40 μL.

Table 2
PCR mix in a total volume of 50 μL per reaction

Component	Volume, μL
2× Phusion U hot start PCR master mix	25
Primer 1 (10 μM)	2
Primer 2 (10 μM)	2
DNA template (1–10 ng)	1
DNA template (1–10 ng)	20

Table 3
PCR conditions

Temperature	Time	
98 °C	2 min	
98 °C 58 °C 72 °C	10 s 20 s 1 min (1 kb/30s)	35 cycles
72 °C	5 min	
10 °C	∞	

Table 4
USER cloning reaction final volume 10 μL

Component	Volume, μL
pCSN linear vector (Subheading 3.1)	1
PCR fragment mix (Subheading 3.2) + ddH_2O	7
CutSmart® buffer	1
USER™ enzyme	1

3.3 Construction of Donor Amplicon by USER Cloning

This subsection explains how to assemble your genes of interest into linearized pCSN vector (Subheading 3.1). The assembled construct is subsequently linearized by NotI or SwaI restriction enzymes to liberate the final DA fragment (*see* Fig. 2).

1. Mix all components for the USER cloning reaction in a 1.5 mL Eppendorf™ tube as described in Table 4. PCR fragments should be in equimolar (50–100 ng/kb per PCR product) mixture, and adjust with ddH_2O to a final volume of 7 μL. For negative controls use water instead of PCR fragments.

2. Incubate the USER reaction mix for 30 min at 37 °C and then for 30 min at 25 °C.
3. Transform chemically competent *E. coli* (Subheading 2.3) cells (*see* **Note 6**). Transfer the entire mix from **step 2** into 50 μL of competent *E. coli* on ice and follow manufacturer's instructions.
4. Plate the entire transformation mix onto LB media with ampicillin (Subheading 2.4) and incubate overnight at 37 °C.
5. Next day, prepare several cultures by inoculating 3 mL of liquid LB containing ampicillin with colonies from the plate in **step 4**. Incubate culture tubes overnight at 37 °C with 200 rpm shaking.
6. Extract plasmids from the cultures using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Verify correct assembly of the PCR product by restriction analysis. Eventually, validate the construct by sequencing.
7. Perform restriction digest of the correct plasmids with NotI or SwaI enzymes following manufacturer's recommendation.
8. Gel-purify the final DA linear fragment using GFX™ Gel Band Purification Kit (GE Life Sciences), following the manufacturer's instructions. Store the purified fragment at −20 °C until further use.

3.4 Yeast Transformation Procedure

Transform yeast cells using a standard transformation protocol. In this method all yeast transformations are done using LiAc/PEG protocol from Gietz et al. [30].

3.5 Gene Amplification Procedure

This subsection describes the step-by-step amplification procedure using the CASCADE method (*see* Fig. 3).

1. Transform a GAS-X strain with the linear DA fragment (0.2–0.5 μg per transformation) containing your GOIs (Subheading 3.3). Plate the cells on SC-trp medium and incubate at 30 °C for 2–3 days.
2. Streak-purify 2–3 single colonies from transformation plate on SC-trp medium and incubate at 30 °C for 2 days.
3. Assemble a master plate by patching single colonies from **step 2** onto YPD medium. Incubate at 30 °C for 1 day.
4. Take a scoop of cells (1–2 mm in size) from the master plate and re-streak onto SC + 5-FAA plates. Incubate plates at 30 °C for 3 days. Subsequently, plate several single colonies from SC + 5-FAA on YPD and SC-trp plates. Colonies that grow on YPD and do not grow on SC-trp are considered for the next step.

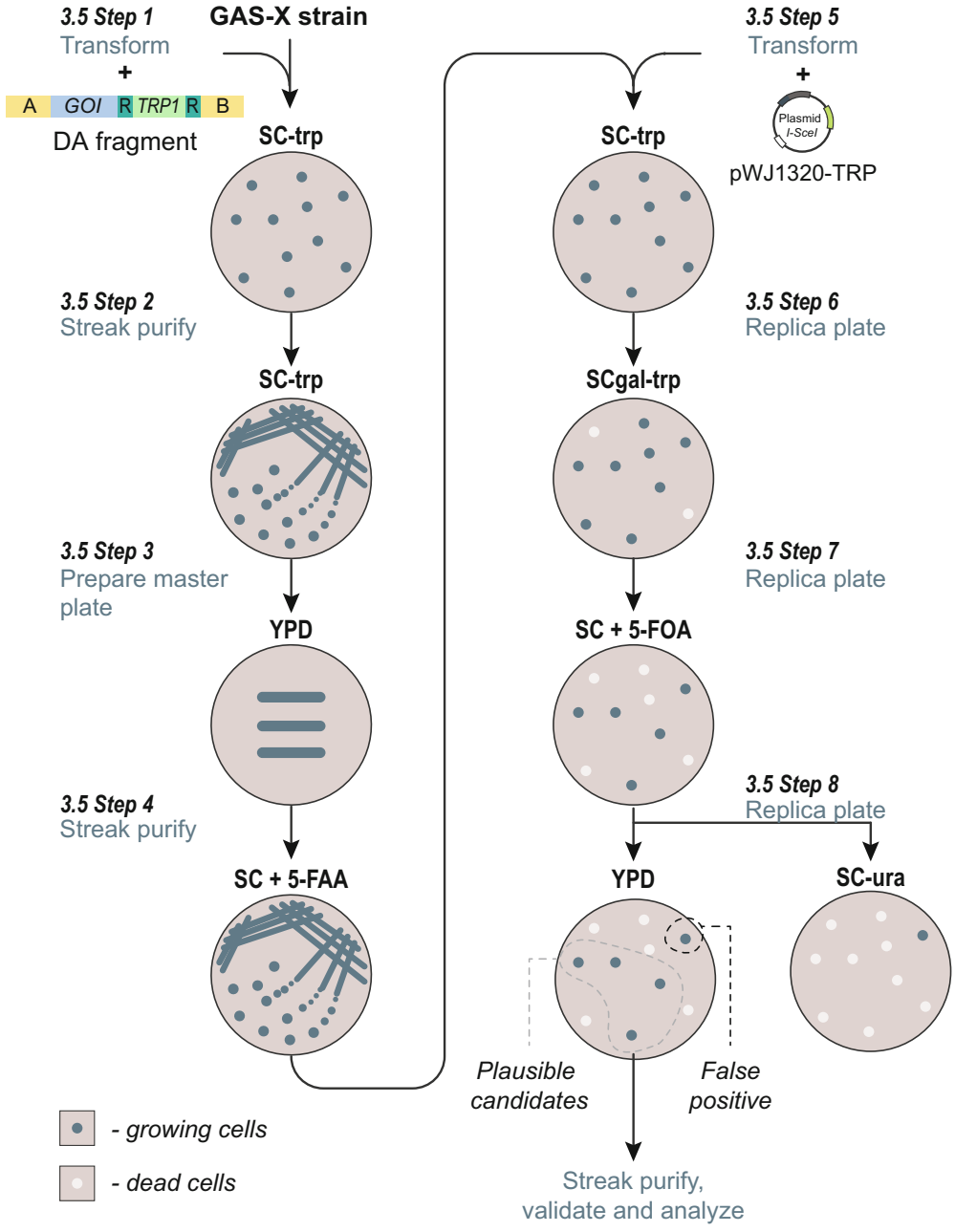

Fig. 3 Step-by-step procedure for DA amplification. Each step is described in detail in Subheading 3.5. The GAS-X represents a strain with a given number (X) of GA cassettes

5. Make competent cells from a single colony from **step 4** (YPD plate) and transform with 2 μ-based plasmid pWJ1320-TRP (up to 0.5 μg per transformation). Plate the cells on SC-trp medium and incubate at 30 °C for 2–3 days (*see* **Note 7**).

6. Induce the gene amplification by replica plating the transformation plate from **step 5** onto SCgal-trp selection medium and incubate at 30 °C for 3 days or until yeast colonies appear.
7. Replica plate the SCgal-trp plate from **step 6** onto SC-5-FOA medium and incubate for 2–3 days at 30 °C.
8. Replica plate the SC + 5-FOA plate onto YPD and SC-ura plates and incubate overnight at 30 °C. Colonies growing on YPD plates but not on SC-ura represent plausible candidates where all GA cassettes were replaced by the DA fragment.

4 Notes

1. Other proof-reading polymerases compatible with uracil in the primers can be used.
2. Assembly of the donor amplicon construct is not limited to USER cloning. Other techniques can be applied, e.g., Gibson assembly [31] or similar.
3. Please follow the manufacturer's instruction or specific protocols on how to create PCR fragments if different than USER cloning technique is used.
4. If USER cloning is not used as the method for donor amplicon construction; then follow manufacturer's instruction or specific protocols on how to create PCR fragments compatible with a selected cloning strategy.
5. The FastDigest DpnI (ThermoFisher) is functional in the reaction buffer of Phusion U polymerase (ThermoFisher).
6. Do not use electroporation to transform the *E. coli*, because electric current will dissociate the hybridized DNA overhangs.
7. Plate several dilution series to achieve approx. 50–200 single colonies per selection plate.

Acknowledgments

This work was supported by the Villum Foundation to ML, grant DNRF99 from the Danish National Research Foundation, and by grant 0603-00323B from the Danish Council for Strategic Research to UHM.

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