A novel and effective Cre/*lox*-based genetic tool for repeated, targeted and markerless integration in *Yarrowia lipolytica*

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Abstract

The unconventional yeast *Yarrowia lipolytica* is extensively applied in bioproduction fields owing to its excellent metabolite and protein production ability. Nonetheless, utilization of this promising host is still restricted by limited availability of precise and effective gene integration tools. In this study, a novel and efficient genetic tool was developed for targeted, repeated, and markerless gene integration based on Cre/*lox* site-specific recombination system. The developed tool required only a single selection marker and could completely excise all of the unnecessary sequences. A total of three plasmids were created and seven rounds of marker-free gene integration were examined in *Y. lipolytica*. All the integration efficiencies remained above 90%, and analysis of protein production and growth characteristics of the engineered strains confirmed that genome modification via the novel genetic tool was feasible. Further work also confirmed the genetic tool was effective for integration of other genes, loci, and strains. Thus, this study significantly promotes the application of Cre/*lox* system and presents a powerful tool for genome engineering in *Y. lipolytica*.

36 **Introduction**

37 With the development of synthetic biology, various molecular genetic tools are being
38 increasingly designed for precise genome modification in yeasts. As a promising host, the
39 generally regarded as safe (GRAS) yeast, *Yarrowia lipolytica,* has been employed in a
40 wide range of fields such as protein production, metabolic engineering, degradation of *n*-
41 alkanes, and dimorphism (Blazeck et al. 2014; Madzak 2015; Carly et al. 2017; Abdel-
42 Mawgoud et al. 2018; Czajka et al. 2018; Madzak 2018). Nevertheless, only few
43 effective genetic tools have been developed for repeatedly targeted gene integration in
44 this host, which still restricts further exploitation and application of *Y. lipolytica*.

45 In traditional methods, heterologous and endogenous genes are transformed into *Y.*
46 *lipolytica* genome via insertion of the whole plasmid at target loci or integration of
47 tandem fragment of target gene and selection marker at zeta loci (Barth and Gaillardin
48 1996; Pignede et al. 2000; Larroude et al. 2018). However, introduction of non-target
49 DNA fragments, especially antibiotics resistance genes, may harm the GRAS status of *Y.*
50 *lipolytica* and hinder its application in food and medicine industries. Besides, the
51 bioproduction of *Y. lipolytica* usually requires multi-round integrations of target gene or
52 co-expression of various genes (Ledesma-Amaro and Nicaud 2016; Carly et al. 2017;
53 Larroude et al. 2018; Markham et al. 2018; Yang et al. 2019), which can be hardly
54 realized via these conventional methods owing to the limited availability of selection
55 markers. Consequently, there is an urgent need to develop efficient genetic tool(s) for
56 targeted, repeatable, and markerless gene integration in *Y. lipolytica*.

57 To date, several site-specific nuclease technologies have been established for
58 genome modification in various species (Cong et al. 2013; Gaj et al. 2013; Hsu et al.

59 2014; Wu et al. 2014). Among them, CRISPR-Cas9 system can accomplish relatively
60 high-efficiency gene disruption and marker-free gene integration in *Y. lipolytica*
61 (Schwartz et al. 2017a; Holkenbrink et al. 2018; Bae et al. 2020). A previous study
62 examined 17 loci for targeted and markerless gene integration in *Y. lipolytica,* among
63 which only five loci exhibited gene integration, with highest integration efficiency of
64 68.9%±25.5% (Schwartz et al. 2017b). Another study accomplished gene integration into
65 *PEX*10 locus via homologous recombination (HR) and homology-mediated end-joining,
66 with integration efficiencies of 6.7%±3.6% and 37.5%±8.8%, respectively (Gao et al.
67 2018). These studies indicate the need for further improvement in achieving more
68 desirable gene integration efficiency (similar to gene disruption efficiency of about 90%)
69 via CRISPR-Cas9-based tools. Besides, gene insertion via HR-mediated double-strand
70 break (DSB) repair usually demands synchronous introduction of two plasmids harboring
71 different selection markers (Schwartz et al. 2017b; Gao et al. 2018), resulting in reduction
72 of transformation efficiency and additional procedure for plasmids recovery.

73 The Cre/*lox*-site-specific recombination system derived from bacteriophage P1 is
74 known to be appropriate for genome editing (Kuhn and Torres 2002; Kawano et al. 2016;
75 Richardson et al. 2017; Wu et al. 2018), and its action mechanism has been clearly
76 described (Albert et al. 1995; Leibig et al. 2008; Yan et al. 2008). Cre recombinase can
77 recognize and bind to *lox*P site, and can catalyze molecular recombination reactions
78 including insertion, translocation, inversion and deletion (Albert et al. 1995). However,
79 these reactions can retain redundant *lox*P sites in the genome, which may disturb further
80 recombination (Albert et al. 1995). To address this issue, some mutational *lox* sites have
81 been successfully designed (Leibig et al. 2008; Yan et al. 2008). For example, the

82 recombination between a left element-mutant *lox* site (LE) *lox*71 and a right element-
83 mutant *lox* site (RE) *lox*66 can generate a double element-mutant *lox* site (LE+RE) *lox*72
84 and a *lox*P site (Leibig et al. 2008; Yan et al. 2008). Meanwhile, *lox*P site can be deleted
85 and *lox*72 site can be retained in the genome through rational design. In particular, the
86 *lox*72 site, which can weakly bind to Cre recombinase, is less likely to participate in
87 subsequent recombination (Leibig et al. 2008; Yan et al. 2008). As a result, *lox* mutants
88 can exhibit enhanced controllability of recombination and accelerate the utilization of
89 Cre/*lox* system in yeasts (Gueldener et al. 2002; Fickers et al. 2003; Hentges et al. 2005;
90 Hochrein et al. 2018). Although this method can employ marker-free genome
91 modification, it often presents low efficiency or is time-consuming. In addition, a few
92 studies have attempted markerless gene integration via Cre/*lox* system in *Y. lipolytica*.
93 Among them, an inspired method combining Cre/*lox* system and 26S rDNA achieved
94 79.2% integration efficiency in *Y. lipolytica* (Lv et al. 2019); however, the integration
95 loci were random and the copy numbers of target genes were uncontrollable, requiring lot
96 of time for selection and evaluation of transformants. Moreover, to rescue selection
97 marker in the genome, an additional plasmid harboring *cre* gene must be introduced into
98 the recombinants by another transformation, and this plasmid should subsequently be
99 recovered. These tedious procedures require two different selection markers and further
100 extend the experimental period.

101 Therefore, in the present study, a novel and effective genetic tool was designed
102 based on Cre/*lox* system. Only a single selection marker was adequate to enable repeated,
103 targeted, and markerless gene integration, and all unnecessary fragments could be totally
104 excised. To demonstrate the efficacy of the developed tool, an important lipase gene from

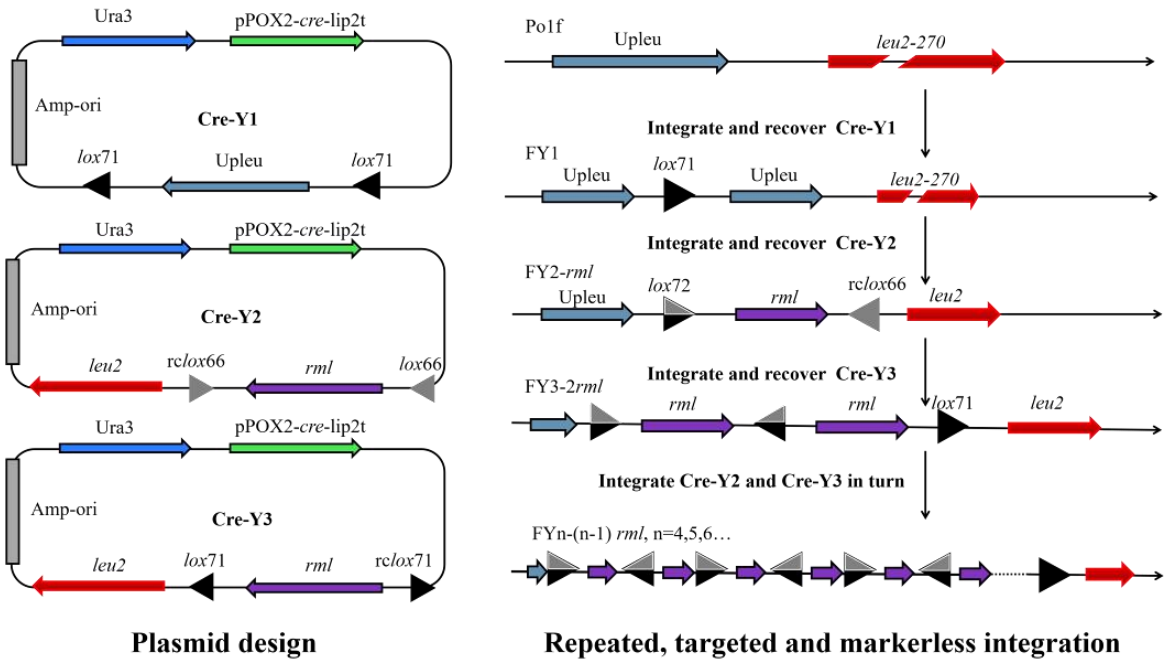
105 *Rhizomucor miehei* (*rml*) was chosen as a qualitative reporter, and three new plasmids
106 were constructed. A total of seven rounds of marker-recyclable integration were
107 implemented, and the positive colony proportions reached 90.63%–100%. The
108 experiment conditions, feasibility, and controllability of the genetic tool were evaluated
109 and optimized. Moreover, to confirm the universality of the developed tool, other six
110 different genes were further integrated into *leu2* locus, and a derived tool was
111 successfully employed to integrate another target gene into the *axp1-2* locus. The results
112 obtained revealed that Cre/*lox*-based genetic tool could resolve the limitations of the
113 existing methods and bring significant advancement in gene editing in yeast species.
114

115 **Results**

116 **Genetic tool design and integration process analysis**

117 The novel Cre/*lox*-based genetic tool comprised three plasmids, namely, Cre-Y1, Cre-Y2,
118 and Cre-Y3 (Figure 1). Plasmid Cre-Y1 was applied for generating the initial strain, and
119 plasmids Cre-Y2 and Cre-Y3 were employed to iteratively introduce target genes into the
120 host strain. Cre-Y1 consisted of a yeast selection marker, *cre* gene expression cassette,
121 bacterial element (antibiotic resistance gene and origin of replication), homologous
122 fragment of target locus in the genome (HF-A), and two convergently oriented *lox*
123 mutants. The *cre* expression cassette should adopt an inducible promoter to control the
124 production of Cre recombinase. Cre-Y2 was derived from Cre-Y1 by replacing HF-A and
125 two convergently oriented *lox* mutants with another homologous fragment of target locus
126 (HF-B), a *lox* mutant and its reverse complement (rc*lox*), and a target gene expression
127 cassette. To avoid excising original segments from the genome, HF-B should be close to

128 HF-A. Cre-Y3 was similar to Cre-Y2, but with the corresponding *lox* mutants of Cre-Y2
129 (e.g., LE for Cre-Y2 and RE for Cre-Y3). All of the tool elements were flexible and
130 nonspecific; thus, the Cre/*lox*-based genetic tool was appropriate for different species.



131

132 **Figure 1.** Plasmid design and integration process analysis.

133 In the present study, the plasmid elements used for *Y. lipolytica* were as follows
134 (Figure 1): the auxotroph Ura3 (UniProtKB, Q12724) was adopted as selection marker,
135 *cre* expression cassette was assembled as pPOX2-*cre*-lip2t (pPOX2 promoter, *cre* gene,
136 and *lip2* terminator, GenBank Accession Nos. AJ001300.1, AB449974.1, and
137 AJ012632.1), bacterial element (Amp-ori) and target gene expression cassette (*rml*) were
138 subcloned from plasmid hp12d-*rml*, and HF-B and HF-A were respectively designed as
139 *leu2* gene (GenBank Accession No. AF260230.1) and its upstream segment (Upleu) for
140 the convenience of plasmid construction and linearization. In addition, two *lox*71 sites, a
141 *lox*66 site and a reverse complement of *lox*66 site (rc*lox*66) and a reverse complement of

142 *lox*71 site (rc*lox*71) and a *lox*71 site were used in plasmids Cre-Y1, Cre-Y2, and Cre-Y3,
143 respectively.

144 To demonstrate repeated and markerless gene integration in *Y. lipolytica* via Cre/*lox*-
145 based genetic tool, the experimental process was as follows (Figure 1). First, Cre-Y1
146 containing two *lox*71 sites was introduced into the Upleu locus of Po1f to construct an
147 initial strain harboring a single *lox*71 site. Under the condition of suitable screening and
148 induction, recombination between two *lox*71 sites could remove unnecessary plasmid
149 elements and produce the initial strain. Then, Cre-Y2 was integrated into *leu2* locus of
150 the initial strain, forming a transitional strain harboring three *lox* sites (*lox*71, *lox*66, and
151 rc*lox*66) in the genome. Various recombination reactions among these three *lox* sites
152 could excise unnecessary DNA fragments and retain a *lox*72 site, an rc*lox*66 site, and a
153 target gene expression cassette in the genome. Meanwhile, two types of stable strains
154 were eventually generated. Similarly, integration of Cre-Y3 produced strains harboring
155 two target gene expression cassettes. Consequently, repeated, targeted, and markerless
156 gene integration could be efficiently realized following iterative integration of Cre-Y2
157 and Cre-Y3.

158

159 **First-round integration to construct initial strain FY1**

160 To construct strain harboring a single *lox*71 fragment, the plasmid Cre-Y1 was inserted
161 into auxotrophic host Po1f (Figure 2). The transformed cell pellet was screened twice in 5
162 mL of MD-Leu medium to enrich the Ura^+ recombinant strain Po1f/Cre-Y1 (Ura^+ and
163 Leu^-) and avoid interference from untransformed Po1f cells (Supplemental Fig. S10).
164 Subsequently, the culture solution containing Po1f/Cre-Y1 was transferred into MO-Leu-

165 Ura medium for 12 h with oleic acid as the sole carbon source, which could activate
166 pPOX2 to induce the expression of *cre* gene. In this case, the produced Cre recombinase
167 could mediate deletion reaction between two repeat *lox*71 sites in the Po1f/Cre-Y1
168 genome, causing excision of the bacterial element (Amp-ori), selection marker (Ura3),
169 and *cre* expression cassette, further generating Ura^- recombinants. Meanwhile, the extra
170 added uracil allowed Ura^- recombinants to grow normally. The induced cell suspension
171 was streaked on YPD plate for isolating single colony with Ura^- genotype, and the
172 phenotype of the isolated colonies were determined on YPD and MD-Leu media. As
173 shown in Supplemental Fig. S1A, a total of 32 recombinants were randomly chosen and
174 all of them could not grow on MD-Leu plate, proving that the selection marker Ura3 was
175 recycled successfully. Moreover, 10 positive colonies were picked up randomly and
176 identified by genome PCR (Supplemental Fig. S2A), and the PCR product obtained via
177 primers Upleu-F3/Upleu-R3 was further sequenced. The results shown in Supplemental
178 Fig. S3A indicated that only a single *lox*71 site was retained in Upleu locus of the
179 eventually obtained strain (named FY1). This finding also revealed that all recombination
180 and integration events occurred as expected (Figure 2), and the adopted plasmid elements
181 of the novel genetic tool were well suitable for *Y. lipolytica*.

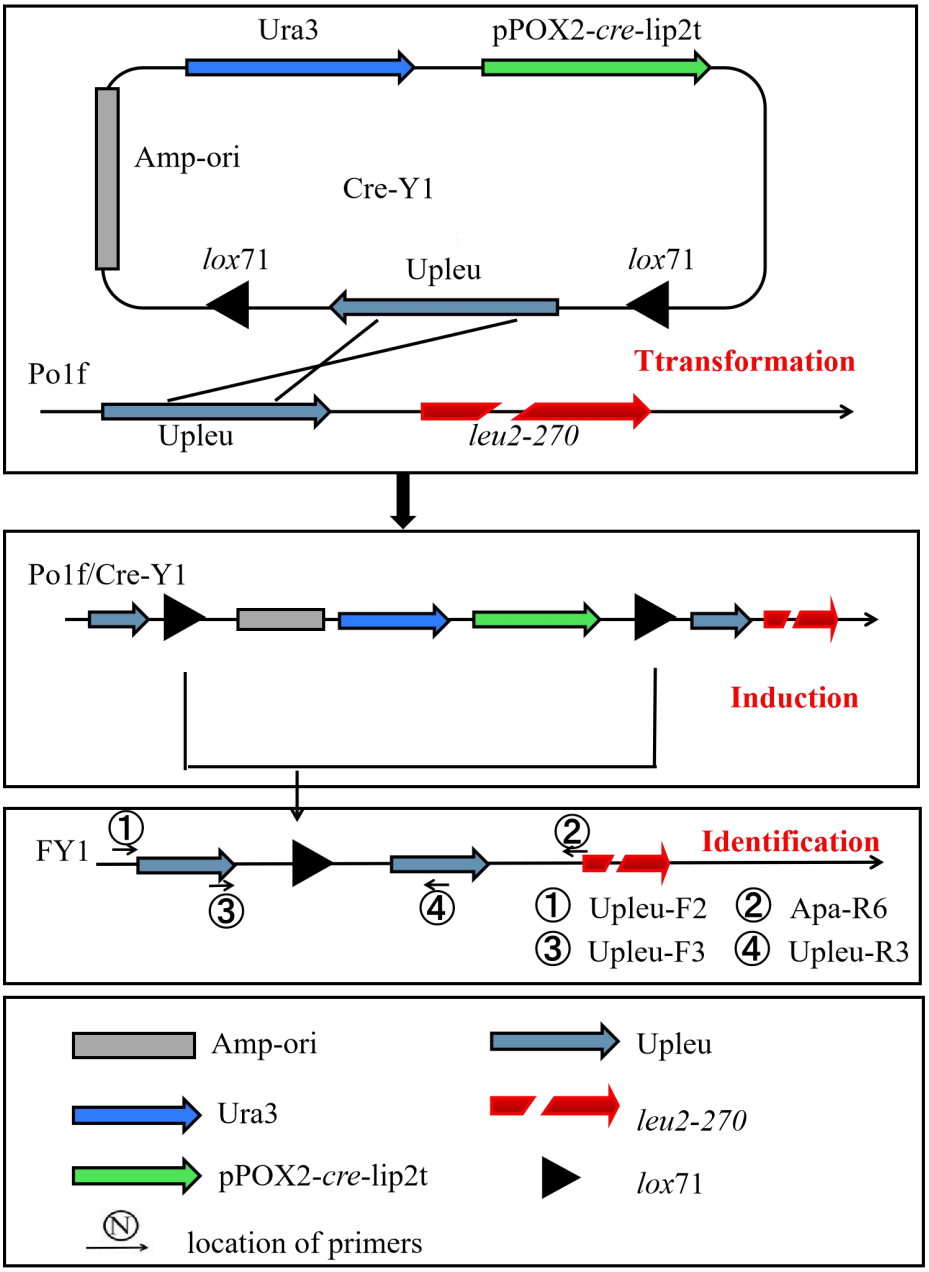
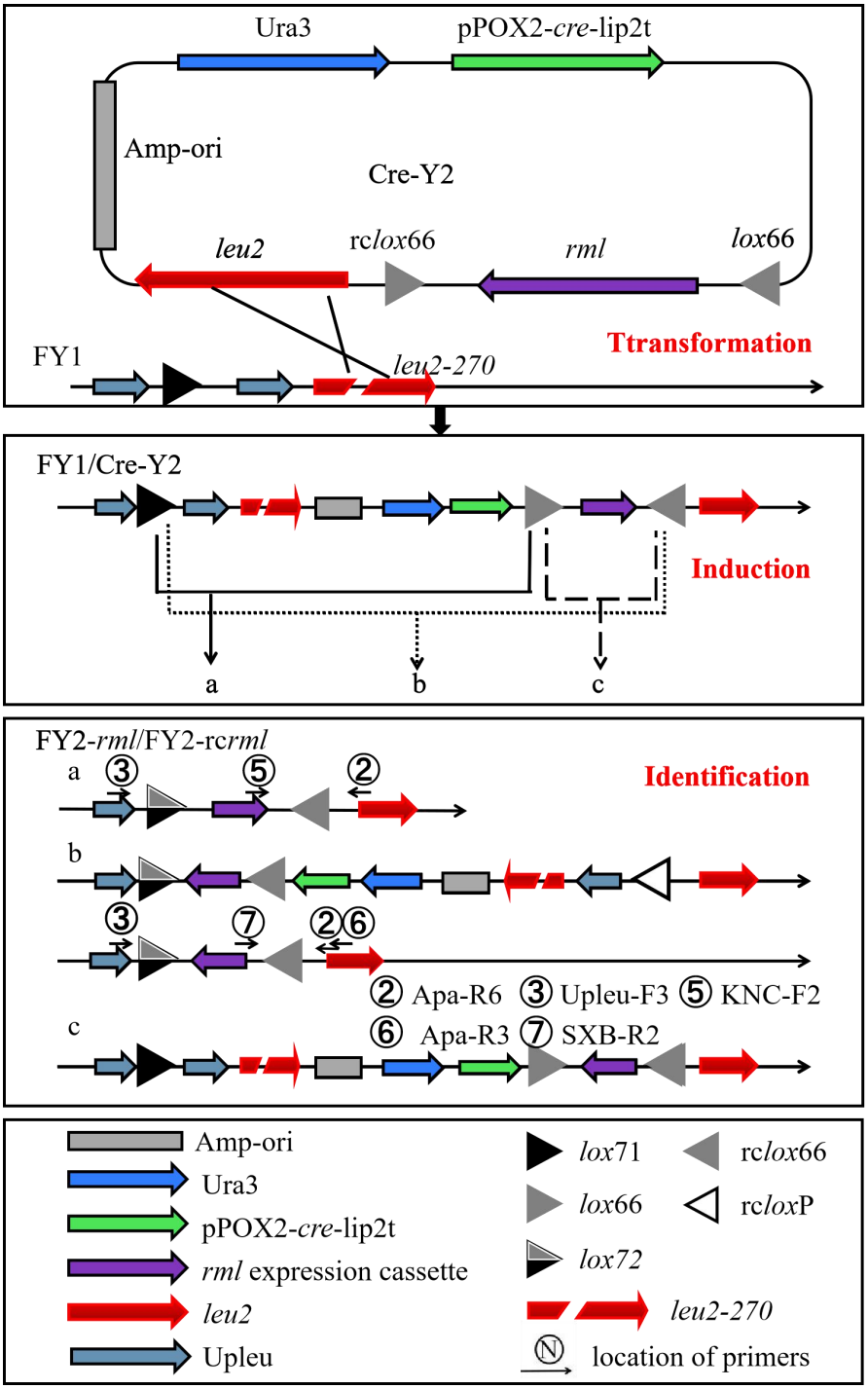


Figure 2. Schematic diagram of first-round integration to obtain initial strain FY1. The Cre-Y1 was linearized at homologous fragment Upleu and introduced into Po1f. The Po1f/Cre-Y1 was screened twice in MD-Leu medium and then inoculated into MO-Leu-Ura medium for inducible expression of *cre* gene. The bacterial element (Amp-ori), selection marker (Ura3) and *cre* expression cassette (pPOX2-*cre*-lip2t) were removed by deletion reaction of two *lox*71 sites, retaining a single *lox*71 site

188 in the FY1 genome. Primers used for PCR amplification are labeled in the figure and described in
189 Supplemental Table S3.
190
191 **Second-round integration and analysis**
192 To evaluate whether the designed plasmids could realize markerless gene integration,
193 Cre-Y2 was linearized within *leu2* and introduced into FY1, to obtain the strain FY1/Cre-
194 Y2. As shown in Figure 3, three *lox* sites (*lox*71, *lox*66, and rc*lox*66) were noted in the
195 FY1/Cre-Y2 genome, and three kinds of recombination reactions could occur when Cre
196 recombinase was expressed. In the first-type recombination (a), deletion reaction
197 occurred between the *lox*71 and *lox*66 sites in the FY1/Cre-Y2 genome, and the strain
198 FY2-*rml* carrying *rml* expression cassette was obtained. In the second-type
199 recombination (b), the *lox*71 and rc*lox*66 sites were combined with Cre proteins,
200 producing a *lox*72 site and reverse complement of *lox*P site (rc*lox*P). Here, the *lox*72 site
201 was hardly bound to Cre recombinase, and the rc*lox*P site could not stably coexist with
202 the unreacted rc*lox*66 site in the genome, which could recombine with Cre recombinase
203 again, generating FY2-rc*rml*, a derived strain carrying the reverse complement of *rml*
204 gene expression cassette (rc*rml*). In the third-type recombination (c), the *lox*66 and
205 rc*lox*66 sites were recombined for inversion reaction, forming a transitional strain that
206 also contained three *lox* sites similar to those of FY1/Cre-Y2. The recombination reaction
207 among the three *lox* sites continued until the first or second type of recombination
208 occurred, and the strain FY2-*rml* or FY2-rc*rml* was finally generated. Consequently, two
209 kinds of stable strains were obtained from three types of recombination reactions in
210 second-round integration. Here, all of the unnecessary and redundant fragments,
211 including Upleu, *leu2-270*, bacterial element, selection marker, and *cre* expression

212 cassette, were completely deleted, and only gene expression cassette and rc*lox*66 site

213 remained in the genome.



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215 **Figure 3.** Schematic diagram of second-round integration to generate FY2-*rml*/FY2-rc*rml*. The Cre-

216 Y2 was linearized on *leu2* and integrated into the FY1 genome to obtain recombinant strain FY1/Cre-

217 Y2. The FY1/Cre-Y2 was screened twice in MD medium and then induced in MO-Ura medium for
218 the production of Cre protein. Therein, three kinds of different recombination reactions for *lox* sites
219 were noted in the genome: deletion reaction between *lox*71 and *lox*66 sites (a), inversion reaction
220 between *lox*71 and rc*lox*66 sites (b), and inversion reaction between *lox*66 and rc*lox*66 sites (c).
221 Eventually, two forms of stable strains FY2-*rml* and FY2-rc*rml* were produced. Meanwhile, all of the
222 unnecessary and redundant sequences were excised, the *rml* expression cassette was integrated into
223 the genome, and the remaining rc*lox*66 site locus was employed in third-round integration. Primers
224 used for PCR amplification are labeled in the figure and described in Supplemental Table S3.

225

226 **Induction time optimization in second-round integration**

227 In the present study, oleic acid induced pPOX2 promoter controlled the production of Cre
228 recombinase in MO liquid medium. Therein, induction time affected Cre protein
229 concentration in the cell, and further influenced the recombination efficiency in the
230 engineered strains. In the first-round gene integration, there were two *lox* sites in the
231 yeast genome. The recombinants were induced for sufficiently long time to obtain high
232 proportion of positive colonies. However, in subsequent gene integrations, there were
233 three or more *lox* sites in the genome and the recombination reaction was complex and
234 diverse. As reported in previous studies (Albert et al. 1995; Suzuki et al. 2005), high
235 concentration Cre protein is more likely to mediate recombination in *lox*72 site, resulting
236 in the removal of the target gene expression cassette from the engineering strain genome.
237 Therefore, induction of *cre* gene expression should be controlled in an appropriate time
238 range. In the present study, to determine the optimal induction time, the recombinant
239 strain FY1/Cre-Y2 was incubated in MO-Ura liquid medium for 4–16 h and streaked
240 onto YPD medium every 2 h (approximately one cell generation cycle). Furthermore, 32

241 recombinants were randomly selected from each YPD medium and then transferred onto
242 MD and BMSY media. The proportions of positive colonies under different induction
243 times were examined, and the results are shown in Table 1 and Supplemental Fig. S1B.
244 All the selected recombinants could complete recombination reaction after 12 h of
245 induction. For more accurate confirmation, 64 colonies induced for 12 h were chosen and
246 none of them could normally grow on MD medium. Consequently, the induction time
247 was set as 12 h, which could be further increased if the proportion of positive colonies
248 decreased in multi-round integrations.

249 **Table 1. Positive colony proportion, genotype rate, and *rml* copy number**
250 **determined in this study.**

Integration round	Induction time (h)	Positive colonies/ all colonies	Proportion (%)	Selected colonies		
				Genotype	Rate	*rml* copy number
1	12	32/32	100	FY1	-	0
2	4	2/32	6.25	FY2-*rml*, FY2-rc*rml*	5/5	0.93
	6	2/32	6.25			
	8	14/32	43.75			
	10	24/32	75			
	12	32/32	100			
	14	32/32	100			
3	12	32/32	100	FY3-2*rml*, FY3-*rml*-rc*rml*	17/13	1.99
4	12	31/32	96.88	FY4-3*rml*, FY4-2*rml*-rc*rml*	4/2	3.10
5	12	29/32	90.63	FY5-4*rml*, FY5-3*rml*-rc*rml*	3/3	4.08
6	12	17/32	53.12	FY6-5*rml*, FY6-4*rml*-rc*rml*	4/2	5.12
	14	28/32	87.5			
	16	32/32	100			
7	16	32/32	100	FY7-6*rml*, FY7-5*rml*-rc*rml*	3/3	5.94

251

252 A total of 10 positive colonies were randomly chosen for identification via PCR
253 sequencing. The *rml*/rc*rml* expression cassette and rc*lox*66 site in all the selected colonies

254 matched with the genome of FY2-*rml*/FY2-rc*rml* (Supplemental Figs. S3B, C),
255 demonstrating that the second-round gene integration was performed according to the
256 predictive analysis (Figure 3). In addition, for the convenience of subsequent protein
257 production evaluation, strains that do not carry rc*rml* expression cassette were employed
258 for further gene integration, because the existing plasmids and method (Zhou et al. 2019)
259 are more applicable to construct strains only harboring *rml* expression cassettes.
260

261 **Iterative insertion of Cre-Y3 and Cre-Y2**

262 To achieve third-round integration, Cre-Y3 was utilized to transform FY2, and the
263 schematic diagram is presented in Figure 4. Similar to the second-round integration, there
264 were three *lox* mutants (rc*lox*66, rc*lox*71, and *lox*71) and a *lox*72 site in FY2-*rml*/Cre-Y3
265 genome, resulting in three different recombination reactions and eventually generating
266 two genotypes of the recombinant strains (FY3-2*rml* and FY3-*rml*-rc*rml*). Subsequently,
267 FY3-2*rml* and FY3-*rml*-rc*rml* were distinguished by PCR amplification with primer pairs
268 KNC-F2/Apa-R6 and SXB-R2/Apa-R3 (Supplemental Fig. S2C). The obtained products
269 were further sequenced and the results (Supplemental Fig. S3D, E) were consistent with
270 the prediction presented in Fig. 4. Thus, another copy of *rml* gene was targeted on *leu2*
271 locus and all unnecessary DNA fragments were excised in the third-round integration.

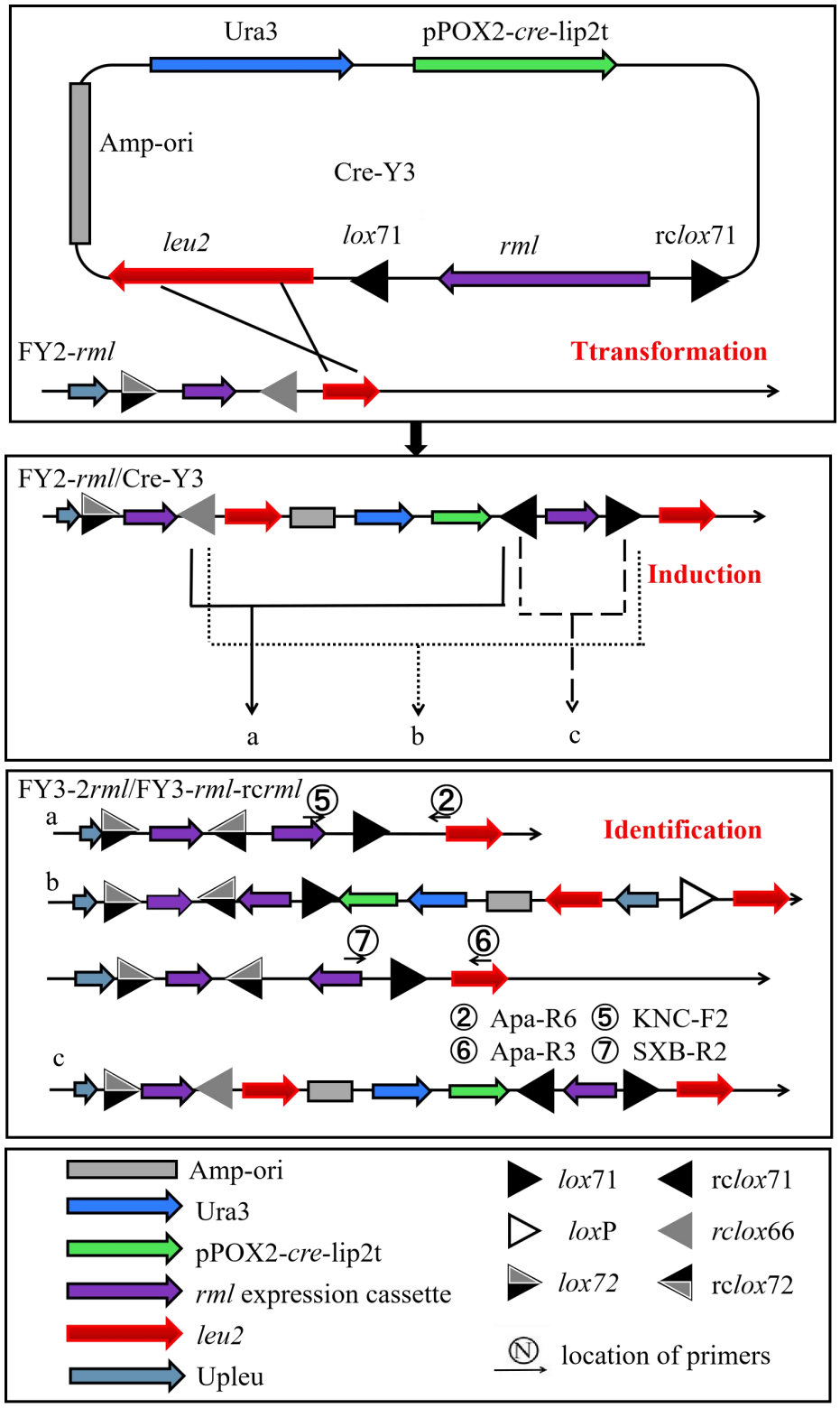


Figure 4. Schematic diagram of third-round integration to construct FY3-2*rml*/FY3-*rml*-rc*rml*. The *Apa*I-linearized Cre-Y3 was used to transform FY2-*rml* 3#, generating the strain FY2-*rml*/Cre-Y3.

275 There were three kinds of different recombination reactions for *lox* sites in FY2-*rml*/Cre-Y3 genome.
276 Herein, the recombination reactions excised unnecessary DNA fragments (a *leu2*, Amp-ori, Ura3, and
277 pPOX2-*cre*-lip2t) and eventually generated two kinds of stable strains FY3-2*rml* and FY3-*rml*-rc*rml.*
278 The remaining *lox*71 site could be used for subsequent integration process. Primers used for PCR
279 amplification are labeled in the figure and described in Supplemental Table S3.

280

281 Similarly, Cre-Y2 was introduced into FY3-2*rml* to generate FY4-3*rml*/FY4-2*rml*-
282 rc*rml*, and Cre-Y3 was used to transform FY4-2*rml* for constructing FY5-4*rml*/FY5-
283 3*rml*-rc*rml* (Supplemental Fig. S4A). Subsequently, Cre-Y2 and Cre-Y3 were iteratively
284 integrated into the corresponding recombinant strains to obtain FY6-5*rml*/FY6-4*rml*-
285 rc*rml* and FY7-6*rml*/FY7-5*rml*-rc*rml.* The genome PCR identification results
286 (Supplemental Fig. S4B) proved that repeated, targeted, and markerless gene integration
287 was successfully implemented by the novel genetic tool.

288

289 **Increasing induction time in sixth and seventh rounds of integration**

290 As shown in Table 1, the positive colony proportions slightly decreased in multi-round
291 integration processes, which might be owing to the following possibility: an additional
292 *lox*72 site could have been inserted into the genome in each round of gene integration,
293 except in the first-round integration, and all of the *lox* sites can bind to Cre recombinase
294 (Albert et al. 1995; Leibig et al. 2008; Yan et al. 2008). Thus, *lox*72 site might disturb the
295 combination between LE/RE *lox* sites and Cre recombinase, resulting in increasingly less
296 recombination reactions during the induction process and a decrease in efficiency in
297 multi-round integrations. Nevertheless, an increase in induction time caused higher

298 accumulation of Cre protein in the cell, and more Cre recombinase could recognize
299 LE/RE mutants and mediate effective recombination.

300 Accordingly, the transformants were induced for longer time to obtain higher
301 recombination efficiency in the sixth-round integration. As a result, the proportion of
302 positive colonies was enhanced, and 16 h was adequate to complete recombination in the
303 engineered strains (Table 1 and Supplemental Fig. S1C). In the seventh-round integration,
304 16 h was employed as the induction time and all the chosen colonies could not grow on
305 MD medium (Supplemental Fig. S1C). Therefore, recombination efficiency might be
306 roughly inversely proportional to the integration round; however, this problem could be
307 alleviated by increasing the induction time in MO-Ura liquid medium. Finally, seven
308 rounds of gene integration were implemented in this study, and the positive colony
309 proportions reached 90.63%–100% with an induction time of 12–16 h (Table 1). In fact,
310 the realized integration rounds were adequate to meet the demands of engineering strains
311 construction, and the potential of the developed genetic tool could also be explored in
312 future research.

313

314 **Protein expression level and growth characteristics evaluation**

315 The recombinants harboring different *rml* gene copies were cultured in BMSY liquid
316 medium for protein production, and the results are presented in Figure 5A. The
317 recombinant strains FY5-4*rml* 31# showed the highest RML expression level, while the
318 lipase activity of FY6-5*rml*/FY6-4*rml*-rc*rml* and FY7-6*rml*/FY7-5*rml*-rc*rml* was slightly
319 lower. Thus, gene integration was terminated in the seventh round. Moreover, several
320 colonies were chosen as representatives for each type of the recombinant strains, and

321 their *rml* gene copies were determined via qPCR. As shown in Table 1, all the
322 transformants carried the same gene copy number as previously predicted, indicating that
323 no *rml* expression cassette was excised from the genome and also confirming the
324 excellent controllability and stability of the genetic tool.

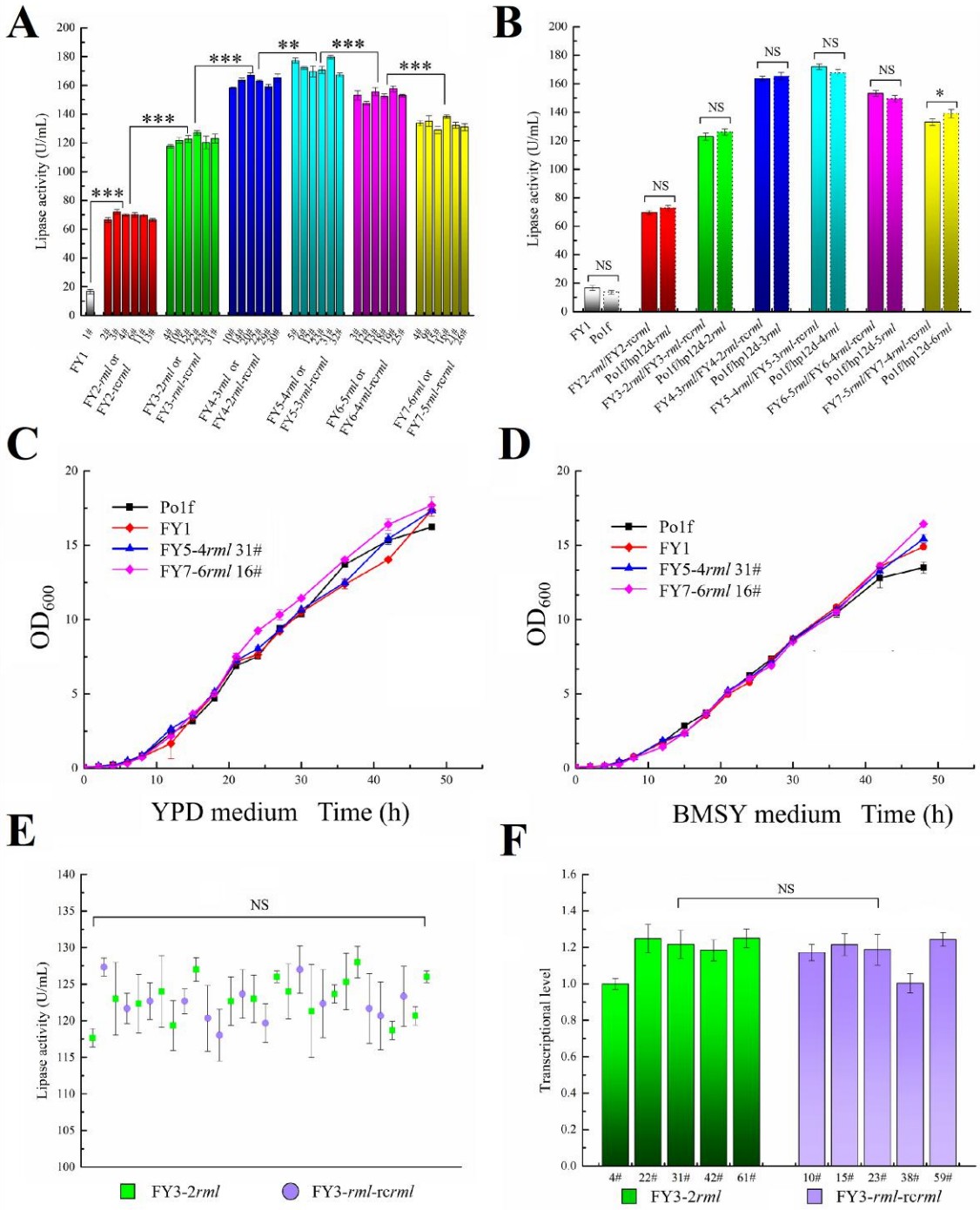
325 To evaluate the protein expression level of the recombinant strains generated via the
326 developed genetic tool, the plasmids hp12d-5*rml* and hp12d-6*rml* were constructed
327 according to the previous method (Zhou et al. 2019). Subsequently, the plasmid hp12d-
328 n*rml* (n=1–6) were linearized and introduced into Po1f to form the recombinant strains
329 Po1f/hp12d-n*rml* (carrying n copies of *rml* expression cassette, n=1–6). Then,
330 Po1f/hp12d-n*rml* (n=1–6) and engineered strains from seven rounds of gene integration
331 were respectively inoculated into BMSY medium under the same culture conditions, and
332 their lipase activity was measured (Figure 5B), which revealed that the two types of
333 recombinant strains showed similar protein expression level. Therefore, it can be
334 concluded that genome editing via the developed genetic tool did not affect target protein
335 production of the engineering strains. When compared with the previous method (Zhou et
336 al. 2019), the developed genetic tool has many advantages such as utilization of reusable
337 marker, only requiring three plasmids, no gene redundancy, and flexible direction of gene
338 expression cassette. Moreover, the novel genetic tool could assemble engineering strains
339 harboring any copies of the target gene, only requiring increase/decrease in integration
340 round.

341 Furthermore, the strains Po1f, FY1, FY5-4*rml* 31#, and FY7-6*rml* 13# were
342 cultivated in YPD and BMSY media, respectively, and their growth characteristics were
343 tested by measuring the optical density at 600 nm (OD_{600}) at different incubation times

344 (Fig. 5C, D). The results revealed no observable differences in the growth curves of the

345 four types of strains, indicating that the genome modification in *leu2* locus via Cre/*lox*-

346 based genetic tool did not influence cell growth.



347

348 **Figure 5.** *(A)* Lipase activity of recombinant strains harboring different copies of *rml* expression
349 cassettes. *(B)* Comparison of lipase activity of recombinant strains obtained via different methods.
350 Data are the mean values of each kind of recombinant strain. The strains Po1f and FY1 showed low
351 lipase activity owing to expression of autologous lipase genes. Weakly significant difference was
352 observed between the strains FY7-6*rml*/FY7-5*rml*-rc*rml* and Po1f/hp12d-6*rml* (P=0.044). *(C)* Lipase
353 activity of transformants from third-round integration. *(D)* Transcriptional level analysis of *rml*
354 mRNA from FY3-2*rml* and FY3-*rml*-*rcrml*. The endogenous *act1* was adopted as reference gene, and
355 the *rml* transcriptional level of FY3-2*rml* 4# was defined as 1. *(E)* Growth curves of Po1f, FY1, FY5-
356 4*rml* 31#, and FY7-6*rml* 13# in YPD medium. *(F)* Growth curves of Po1f, FY1, FY5-4*rml* 31#, and
357 FY7-6*rml* 13# in BMSY medium. NS, *, **, and ***indicate non-significant, $P < 0.05$, $P < 0.01$, and P
358 < 0.001, respectively.

359

360 **Comparison of two-genotype recombinant strains**

361 Two genotypes of the recombinant strains were generated in each round of integration
362 (except for the first round), and their numbers were presented in Table 1. The rates of two
363 forms of strains were approximately equal, suggesting that there was nearly equal
364 possibility to choose a strain with certain genotype. The colonies with high lipase
365 activities were selected for protein production evaluation. The results shown in Table 1
366 also implied that the protein expression levels of the recombinant strains in each round of
367 integration were similar, which was confirmed in Figure 5A. In contrast, some previous
368 studies have reported that the relative arrangement of multiple genes might affect
369 corresponding transcription and protein production in host cells (Eszterhas et al. 2002;
370 Lee et al. 2014). Thus, a total of 30 positive colonies from the third-round integration
371 were identified via the primer pairs KNC-F2/Apa-R6 and SXB-R2/Apa-R3 (data not

372 shown), and their lipase activities were measured. The results presented in Figure 5E
373 showed that the RML activity of each recombinant randomly changed within a proper
374 range. Moreover, transcriptional level analysis further indicated that there was no
375 significant difference in the transcriptional quantity of *rml* mRNA isolated from FY3-
376 2*rml* and FY3-*rml*-rc*rml* cells (Figure 5F). Thus, the relative direction of *rml* expression
377 cassettes had no observable effect on protein production in this study, which might be
378 owing to the difference in the host type. Accordingly, both the genotypes of the strains
379 were concluded to be suitable for RML production in *Y. lipolytica*.
380
381 **Successfully expanding to six different genes and *axp1-2* locus**
382 To evaluate the universality of the novel genetic tool, six genes with varied lengths and
383 resources were additionally integrated into the engineered strain FY5-4*rml*. The genes
384 *ire1*, *kar2*, *pdi*, *sls1,* and *hac1* are the homologous genes of *Y. lipolytica*, and *vgb* is the
385 coding gene of *Vitreoscilla* hemoglobin. These genes may differentially affect RML
386 expression level, which determines their integration times. The corresponding plasmids
387 were derived from Cre-Y2 and Cre-Y3 by replacing the *rml* gene with different target
388 genes (Supplemental Fig. S5A), and then used to transform FY5-4*rml*. The subsequent
389 integration processes were similar to those of Cre-Y2 and Cre-Y3, and the proportions of
390 positive colonies were all above 90% (Table 2), confirming the availability of the
391 developed genetic tool for repeated, targeted, and markerless integration of different
392 genes.
393 Furthermore, a derived tool was established to verify whether the developed genetic
394 tool could be applied for other target genes, integration loci, and strains. For this purpose,

395 three plasmids containing *Rhizopus oryzae* lipase (*rol*) gene were respectively
396 constructed based on Cre-Y1, Cre-Y2, and Cre-Y3 (Supplemental Fig. S5B), and
397 integrated into defective *axp* gene locus (*axp1-2*) of *Y. lipolytica* Po1h. The
398 transformation, screening and induction, isolation of positive colonies, and identification
399 processes were similar to those employed for *rml* gene integration into *leu2* locus of Po1f.
400 First, the Cre-axp1 plasmid was linearized by *Nsi*I and inserted into the Upaxp locus to
401 generate an initial strain. Then, the Cre-axp2 and Cre-axp3 plasmids were digested with
402 *Cla*I and iteratively introduced into the *axp1-2* locus for repeated, targeted, and
403 markerless integration of *rol* in Po1h. As a result, three rounds of integration were
404 conducted and all of the integration rates were above 96%. These findings revealed that
405 such extension of the novel genetic tool is feasible and convenient.

406 **Table 2. Integration rates of various genes, loci and strains in this study.**

Gene	GenBank	Lenth (bp)	Integration round	Positive colonies/ all colonies	Proportion (%)
Integration locus: *leu2* gene of FY5-4*rml*, induction time: 16 h					
ire1	XM_002142952.1	3663	1	32/32	100
kar2	U63136.1	2013	1	32/32	100
			2	32/32	100
			3	32/32	100
pdi	XM_503481.1	1515	1	32/32	100
			2	32/32	100
			3	31/32	96.88
			4	31/32	96.88
sls1	Z50154.1	1281	1	32/32	100
			2	32/32	100
hac1	XM_500811.1	918	1	32/32	100
vgb	AF292694.1	441	1	32/32	100
			2	32/32	100
Integration locus: *axp1-2* of Po1h, induction time: 12 h					
rol	AF229435.1	1101	1	31/32	96.88
			2	32/32	100
			3	32/32	100

407

408 **Discussion**

409 In the present study, a Cre/*lox*-based genetic tool was precisely and skillfully designed.
410 The plasmids Cre-Y2 and Cre-Y3 adopted two inversely oriented *lox* sites, and the Ura3
411 marker cannot be removed from the genome if the strain does not undergo integration
412 process as expected. Thus, the Ura^+ strain could be distinguished by isolating positive
413 colonies on the MD medium. It must be noted that except for the *cre* gene and *lox* sites,
414 all of the genetic tool elements are nonspecific and replaceable. Any DNA sequence can
415 be chosen as the homologous fragment as long as no gene is disrupted, and the length of
416 the homologous fragment is very flexible because of the excision of unnecessary
417 sequences. The *rml* gene in plasmids Cre-Y2 and Cre-Y3 can be replaced by
418 heterologous and endogenous genes of *Y. lipolytica* to accomplish repeated markerless
419 integration, and the plasmid construction process is simple and convenient. In addition,
420 the Cre/*lox*-based genetic tool can also be applied to other genes and loci if the
421 corresponding plasmid elements are available.

422 Recently, several genetic tools have been developed for markerless gene integration
423 of *Y. lipolytica* based on CRISPR-Cas9 system, Cre/*lox* system and Ura/5-fluoroorotic
424 acid resistance method (Supplemental Fig. S6) (Schwartz et al. 2017b; Gao et al. 2018;
425 Holkenbrink et al. 2018; Lv et al. 2019). As shown in Supplemental Table S1, the
426 integration efficiency markedly fluctuates with different genome loci and single-guide
427 RNAs (sgRNAs) for CRISPR-Cas9-mediated gene integrations (Schwartz et al. 2017b;
428 Holkenbrink et al. 2018). According to most of the existing Cre/*lox*-based methods
429 (Holkenbrink et al. 2018; Lv et al. 2019), the DNA fragment can be integrated into the
430 host genome via gene insertion or gene replacement. However, insertion event may

431 introduce unnecessary DNA segments into the genome, whereas replacement events,
432 which occur occasionally, can avoid this issue, but requires substantial screening of target
433 transformants. Furthermore, while the Ura/5-fluoroorotic acid resistance method (Ura
434 pop-in/pop-out method) can also accomplish markerless gene integration, reverse
435 mutation is difficult to avoid in the marker recovery process. In addition, new plasmids
436 carrying different sgRNA or homologous fragments must be constructed to repeatedly
437 realize gene integration in a specific genome locus via the three above-mentioned genetic
438 tools. These limitations can be completely overcome by the novel genetic tool designed
439 in the present study. The target gene is integrated into the host genome via efficient,
440 targeted, and repeatable gene insertion event, and the introduced non-target sequences
441 can be deleted by recombination reaction of the *lox* sites. Moreover, despite the
442 occurrence of inefficient insertion event in the integration process, a few recombinants
443 can be enriched via twice auxotroph screening. The existing methods based on CRISPR-
444 Cas9 and Cre/*lox* systems require two different selection markers and additional plasmid
445 recovery procedure (Schwartz et al. 2017b; Gao et al. 2018; Holkenbrink et al. 2018; Lv
446 et al. 2019). In contrast, only a single marker is required for the novel Cre/*lox*-based
447 genetic tool, and the process of gene integration, markers reuse, and plasmid recovery can
448 be combined and accomplished simultaneously, which can significantly reduce the
449 experimental period and workload. Overall, the novel genetic tool developed in this study
450 can overcome many limitations such as low HR rate, limited selection marker, random
451 integration site, and additional plasmid recovery.

452 In conclusion, a powerful and efficient Cre/*lox*-based genetic tool for repeated,
453 targeted, and markerless gene integration was developed in this study. In practice, only a

454 single selection marker can enable iterative integration of target gene into the genome,
455 which can facilitate genetic modifications of the host strains. Furthermore, in each round
456 of integration, all of the unnecessary segments are removed, the relative direction of each
457 target gene expression cassette is controllable, and the gene recombination on genome is
458 foreseeable. These characteristics can significantly decrease gene redundancy in the
459 engineering strain, making it convenient for the use of host strain in pharmaceutical, food,
460 and other health-related areas. Besides, the developed genetic tool is also suitable for
461 various other genes and loci. The findings of this study significantly broaden the
462 application of Cre/*lox* system and strengthen the capabilities for genome modification in
463 *Y. lipolytica*.
464

465 **Methods**

466 **Strains and media**

467 *Escherichia coli* TOP10 and TOP10 F' (Shanghai Weidi Biotechnology Co., Ltd,
468 Shanghai, China) were applied for plasmid amplification. The plasmid hp12d-*rml* (Zhou
469 et al. 2019) and auxotrophic *Y. lipolytica* Po1f (Madzak et al. 2000) and Po1h (Madzak et
470 al. 2004) were respectively utilized for plasmid construction and transformation. The T4
471 DNA ligase, Phanta Max Master Mix polymerase, Rapid Taq Master Mix polymerase,
472 and ClonExpress II One Step Cloning Kit were obtained from Vazyme Biotech Co., Ltd
473 (Nanjing, China) and used for gene amplification and genome identification. The *E. coli*
474 and *Y. lipolytica* strains were respectively cultured at 37 °C and 28 °C. All strains and
475 plasmids used in this study are listed in Supplemental Table S2. The LB, YPD, MD, and
476 BMSY media have been previously described (Zhou et al. 2019). The MO medium (20

477 mL/L oleic acid, 0.5 mL/L Tween 80, 13.4 g/L yeast nitrogen base with no amino acids,
478 and 0.4 mg/L biotin) was derived from MD medium, and leucine (final concentration,
479 262 mg/L) or uracil (final concentration, 22.4 mg/L) was added for auxotrophic strains.
480

481 **Plasmid construction**

482 Figure S7 illustrates plasmid Cre-Y1 construction. The pPOX2 promoter, *cre* gene,
483 tandem fragment of lip2t and *lox*71, and fusion segment of Upleu and *lox*71 were
484 respectively cloned via corresponding primer pairs (Pox2-F1/Pox2-R1, Cre-F1/Cre-R1,
485 lip2t-F1/lip2t-R1, and Upleu-F1/Upleu-R1). The four amplified products were assembled
486 by overlap extension PCR and then double-digested with *Nde*I and *Apa*I. Subsequently,
487 the digested product was inserted into *Nde*I/*Apa*I-digested plasmid hp12d-*rml* to
488 construct plasmid 1296-*cre*. Furthermore, the *ura3* gene (amplification using Ura-
489 F1/Ura-R1) was subcloned into *Nde*I-linearized 1296-*cre* by HR, generating Cre-Y1. All
490 the plasmids were confirmed by DNA sequencing by TsingKe Biological Technology
491 Co., Ltd. (Wuhan, China), and all the primers and *lox* sites used in this study are
492 presented in Supplemental Table S3.

493 As shown in Supplemental Fig. S8, the plasmids Cre-Y1 and hp12d-*rml* were
494 respectively double-digested using *Apa*I/*Nhe*I and *Apa*I/*Xba*I, and the two digestion
495 products with same cohesive ends were ligated to generate the plasmid hp12d-*rml*-*cre*.
496 Subsequently, the gene segment lip2t-*lox*66 was cloned by lip2t-F1/lip2t-R2 and
497 recombined into *Avr*II/*Mlu*I-digested hp12d-*rml*-*cre*, forming hp12d-*rml*-*cre*-*lox*66.
498 Furthermore, the fusion fragment of rc*lox*66 and partial *leu2* was amplified by leu-F2/leu-
499 R2 and recombined with *Nde*I/*Apa*I-opened hp12d-*rml*-*cre*-*lox*66 to generate plasmid

500 Cre-Y2. Similarly, the gene segment lip2t-rc*lox*71 was amplified by lip2t-F1/lip2t-R3 and
501 used for the construction of hp12d-*rml*-*cre*-rc*lox*71, and hp12d-*rml*-*cre*-rc*lox*71 was
502 recombined with *lox*71-partial *leu2* to obtain plasmid Cre-Y3 (Supplemental Fig. S9).
503 The *rml* gene of Cre-Y2 and Cre-Y3 was replaced with six different genes (*ire1*,
504 *kar2*, *pdi*, *sls1*, *hac1,* and *vgb*) to assemble the corresponding plasmids, and their
505 structures are presented in Supplemental Fig. S5A. Moreover, the homologous fragment
506 Upleu, *leu2,* and target gene *rml* were respectively substituted with Upaxp, *axp1-2,* and
507 *rol* to generate the plasmids Cre-axp1, Cre-axp2, and Cre-axp3 (Supplemental Fig. S5B).
508 The abovementioned procedures were achieved by conventional PCR amplification,
509 enzyme digestion, and ligation.
510
511 **Yeast transformation, screening and induction**
512 The integration process was performed as follows (Supplemental Fig. S10). The
513 *Sal*I-linearized plasmid Cre-Y1 was integrated into Po1f genome based on the previously
514 described method (Chen et al. 1997). Then, the transformants were screened twice in 5
515 mL of MD liquid medium supplemented with leucine (MD-Leu). Subsequently, the
516 derivatives were inoculated into MO liquid medium supplemented with leucine and uracil
517 (MO-Leu-Ura) for inducible expression of *cre* gene. To isolate single colony, the cell
518 suspension solution was streaked onto YPD plate, and the selected colonies were
519 respectively transferred onto YPD and MD-Leu plates for phenotype verification, where
520 the positive colonies could only grow on YPD medium. In addition, the genomic DNA of
521 positive colonies was extracted for further identification via PCR amplification with
522 appropriate primers and DNA sequencing by TsingKe Biological Technology Co., Ltd.

523 (Wuhan, China). Thus, the initial strain FY1 was obtained. The subsequent integration
524 was almost similar to the first-round integration; however, Cre-Y2 or Cre-Y3 was
525 linearized by *Apa*I, leucine was not required in MD or MO medium, and YPD medium
526 was replaced with BMSY medium containing 10 mL/L glyceryl tributyrate to select
527 colonies with high RML activity.
528
529 **Shaking flask culture and lipase activity determination**
530 Transformants harboring clear and large transparent halos were inoculated into 5 mL of
531 YPD for 24 h. Then, the cultures were transferred into 500-mL shake flasks containing 30
532 mL of BMSY medium and incubated for 120 h to produce RML. The RML activity in
533 cell culture supernatant was evaluated using previously described method (Zhou et al.
534 2019). All the experiments were performed in triplicate, and the results were analyzed by
535 ANOVA with Duncan's multiple range test at $P < 0.05$.
536
537 **Determination of gene copy number and transcriptional analysis**
538 Based on the protocol of ChanQ^{TM} Universal SYBR qPCR Master Mix (Vazyme Biotech
539 Co., Ltd, Nanjing, China), the *rml* gene copy numbers were determined by real-time
540 qPCR performed on StepOnePlus apparatus with StepOne software version 2.3 (Applied
541 Biosystems, Foster City, CA, USA). The endogenous *act1* was set as reference gene, and
542 the plasmids pMD19-*act1* and hp4d-*rml* (Zhou et al. 2019) were utilized for establishing
543 standard curves, respectively. The *rml* gene transcriptional level analysis was conducted
544 by TsingKe Biological Technology Co., Ltd (Wuhan, China). Each sample was collected
545 in triplicate.

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