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Modified protocol for quick and large-scale transformation of *Pichia pastoris*

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Running title:

Modified condensed protocol for *Pichia pastoris* transformation

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31 **Abstract**

32 Over the last couple of decades, methylotrophic yeast, *Pichia pastoris* emerges as an important
33 yeast species owing to its increasing application in industry and basic biological research.
34 Transformation of *Pichia pastoris* cells for the introduction of the gene of interest is common
35 practice for expression and purification of a heterologous protein(s). Presently available protocol
36 of *Pichia pastoris* transformation involves preparation of competent cells and followed by their
37 transformation. Preparation of competent cells requires growth of cells to certain cell density
38 which requires lots of resource, space, time and efforts. This limits the number of
39 transformations that can be performed by an individual at a time. In the present paper, I will
40 describe a modification in the available protocol which makes *P. pastoris* transformation hassle
41 free. The present procedure does not require growth of pre-culture or growth of cells to certain
42 cell density rather cells are grown in a patch on YPD plate(s) and rest procedure is performed in
43 small eppendrof tubes which allow a large number of transformations in quickest possible time
44 with minimal resource and efforts. In the end, I also compare various protocols in tabular form
45 which allows the user to choose best suitable procedure depending on the available resource,
46 time, number of transformations, requirement, and efforts. The present modified protocol does
47 not require big centrifuge and shaker which further makes this procedure more useful. I believe
48 that present protocol of transformation with its many unique features will be really helpful to
49 those working with *P. pastoris.*

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51 **Keywords:** *Pichia pastoris*, transformation, protocol

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64 **1 INTRODUCTION**

65 Methylotrophic yeast, *Pichia pastoris* is now an important model in both basic biological
66 research (Gasser et al., 2013) as well as an important industrial yeast species for expression of
67 heterologous proteins (Cereghino et al., 2000; Daly et al., 2005). Apart from that *P. pastoris* is
68 also used in health care industries where this yeast species is used for expression of
69 biopharmaceutical proteins (Daniel et al., 2013). Although budding yeast, *S. cerevisiae* is widely
70 used in basic science research but for many studies, *P. pastoris* remains a choice of model. For
71 example, processes like pexophagy and peroxisome biogenesis are best studied in *P. pastoris*
72 (Farré et al., 2008; Farré et al., 2017). Apart from that *P. pastoris* offers other benefits like the
73 low level of genetic redundancy, can grow on simple media with methanol as a sole carbon
74 source, culture can reach very high density, some of the genes which were absent in budding
75 yeast is present in *Pichia pastoris* (example Atg37) (Nazarko et al., 2014). Apart from that *P.*
76 *pastoris* processes many features which make it important industrial species. For example, as
77 mentioned above, it can grow to high cell density, can use methanol as a sole carbon source
78 (thereby checking the growth of other unwanted microbial species), glycosylation pattern of
79 proteins is closer to humans (in *S. cerevisiae* proteins are generally hyper glycosylated),
80 availability of many methanol inducive promoters, secretes the low level of endogenous proteins
81 (Cereghino et al., 2000), and more recently availability of more refined complete genome
82 sequence (Sturmberger et al., 2016) of this species.

83 Efforts were made to introduce DNA of interest for either introduction or deletion of the gene of
84 interest both from perspectives of basic science as well as industrial importance. As a result,
85 several protocols were devised for the introduction of DNA of interest in *P. pastoris* cells
86 including electroporation (Becker & Guarante, 1991), alkali cation method (Ito et al., 1983),
87 treatment of cell with polyethylene glycol (or PEG) (Dohmen et al., 1991) and method involving
88 spheroplast generation method (Cregg et al., 1985). Each of these methods has its own benefits
89 and shortcomings. But all the available procedure for *P. pastoris* transformation involves two
90 steps *viz* 1) preparation of competent cells and 2) transformation of competent cells. Although,
91 electroporation is quick, reliable and allows a large number of transformations, but the
92 preparation of competent cells is a long procedure which requires over-night pre-culture, dilution
93 of pre-culture followed by growth of cells to certain cell density (in all available methods pre-
94 culture is diluted to cell density ranging from OD_{600nm} 0.2-0.3 followed by growth of cells to

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95 OD_{600nm} 0.8-1.2) which may require 4 to 6 hours depending upon strain (Shixuan & Letchworth,
96 2004). Apart from that, each transformation requires handling of 50-100 mL of media which
97 limits the number of competent cells (different strain) which can be prepared by an individual at
98 a time. Although condensed protocol of *P. pastoris* transformation somehow makes procedure a
99 bit simple (Cereghino et al., 2005) but a modification which can still make the whole procedure
100 of *P. pastoris* transformation more simple, short, quick and reliable is always desired. In all
101 available protocols for *P. pastoris* transformation, preparation of competent cells still remains a
102 limiting and effort intensive step. Therefore, a protocol which does not required competent cell
103 preparation or allows preparation of a large number of competent cells in shortest possible time
104 with a minimum resource, space, time, efforts and does not involve handling of a large volume
105 of culture is always desirable.
106 In the present study, I am reporting a modification in available protocol for routine
107 transformation of *P. pastoris* which gives a significant number of colonies with a high rate of
108 positive transformants. The present procedure allows transformation without growing cells in
109 tubes and flasks which makes the present protocol simple, quick, reliable allowing
110 transformation of large numbers of samples which is not possible with the presently available
111 procedure for *P. pastoris* transformation. In the end, I also compare present protocol with the
112 already available procedure and discuss merits of the present procedure. The present modified
113 procedure with its unique way of competent cell preparation truly condensed the whole
114 procedure of *P. pastoris* transformation. Further, the present procedure has been tested on
115 different *P. pastoris* background several times over the span of six months. I believe that present
116 modified protocol will be really helpful for those working with *P. pastoris* and required a large
117 number of transformations on a daily or routine basis which is pretty common in industrial
118 settings.

119 **2 MATERIAL AND METHODS**

120 **2.1 Chemical and reagents**

121 DTT (from Roche), yeast extract (Difco), peptone (Difco), dextrose (Fisher), YNB (Bacto),
122 sorbitol (Fisher), restriction enzyme (NEB), electroporation cuvette (Genesee scientific), agarose
123 (Apex), 1 kb Plus DNA ladder (Thermo fisher), 6X DNA loading dye (NEB), pre stained protein
124 marker (Thermo fisher), nitrocellulose membrane (GE), ECL reagents (GE), Tris base (Sigma),
125 CSM-His (Sunrise Science), anti-GFP antibodies (Clonetech, JL-8), goat anti HRP conjugated

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126 secondary antibodies (Biorad), SDS (Fisher), NaOH (Fisher), glycerol (Calbiochem), TCA
127 (Sigma), methanol (Fisher), electroporation unit (BTX, ECM20, version 1.04) bench top
128 centrifuge (Eppendrof), antibiotics (sigma), UV-visible spectrophotometer (from Beckmann
129 Coulter), nonfat skimmed milk powder (Apex), X-ray film processor (from Knoica SRX-101A),
130 autoradiography films or sheets (Blue Devil). All other reagents were of either analytical or
131 molecular grade.

132 **2.2 Strain and plasmids**

133 All the *Pichia pastoris* strains used in the present study are isogenic to the PPY12h background.
134 Cartoon presentation of plasmid (showing essential elements) integrated into *P. pastoris* genome
135 for introducing gene(s) of interest using the protocol described in present manuscript is shown in
136 figure 2.1.

137 **2.3 Media**

138 YPD media (2 % yeast extract, 1 % peptone and 2 % dextrose). SD+CSM-His (0.17 % YNB
139 without amino acid and ammonium sulfate, 0.5 % ammonium sulfate, 0.08 % CSM-His, 2 %
140 agar, 2 % dextrose), YPD + Zeocin (YPD + 100 μg/mL Zeocin). All cultures were grown at 30
141 °C, 250 rpm.

142 **2.4 Protein extraction**

143 Transformants were patched on fresh selection plate(s) and plates were incubated at 30 °C till
144 patches grow (generally 1-2 days). One mL YPD media were inoculated using cells from the
145 patch. Tubes were incubated at 30 °C, 250 rpm. Next day OD_{600nm} of cell suspension was
146 checked and cell suspension equal to one OD was transferred into fresh 1.5 mL eppendrof tube
147 and TCA (Trichloroacetic acid) was added to the tube such that final concentration of TCA was
148 around 12.5 %. Tubes were incubated at -80 °C for one hour. After one-hour tubes were taken
149 out from deep freezer, thawed at room temperature and vortexed for 30 second. The tube was
150 then centrifuged at 18000 g for 8 min and the supernatant was discarded. The resulting pellet was
151 resuspended in 1 mL of chilled 100 % acetone (using water bath). Tube(s) were again
152 centrifuged as above, and the supernatant was discarded carefully without disturbing or losing
153 protein pellet. The protein pellet was air dried and resuspended in 100 μL Laemmli buffer
154 (Laemmli, 1970).

155 **2.5 Immunoblotting**

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156 Whole cell lysates (extracted above) were resolved on 10 % SDS-PAGE and proteins were
157 transferred onto the nitrocellulose membrane as described elsewhere (Towbin et al., 1979).
158 Efficiency and quality of transfer were checked by staining the blots with Ponceau S stain just
159 before incubating the blots in blocking solution (5 % nonfat skimmed milk powder in TBST)
160 (Romero-Calvo et al., 2010). Proteins were detected by using monoclonal anti-GFP and HRP
161 conjugated goat anti-mouse as secondary antibodies respectively. Blots were developed using
162 chemiluminescence (from GE).

163 **2.6 Fluorescence microscopy**

164 Images were captured and analyzed using a fluorescence microscope and software as described
165 elsewhere (Wang et al., 2017).

166 **2.7 Transformation protocol**

167 The detailed protocol for *Pichia pastoris* transformation is described below. On a fresh YPD
168 plate, a patch of required strain(s) was prepared, and plate(s) were incubated at 30 °C. Patch size
169 of around 2 cm by 1.5 cm (length x breadth) was sufficient for two transformations (giving
170 around 15-18 OD_{600nm} cells). After 18-24 hour of incubation, cells from the patch(s) can be used
171 for transformation. Transfer 1 mL of YPD in 1.5 mL sterile Eppendrof tube. Add 40 µL of DTT
172 (from a stock of 1 M, prepared from DDT powder from Roche) and 40 µL HEPES-NaOH buffer
173 (from a stock of 1 M pH8). Scrap the cells from the patch with help of 200 µl sterile tips or blunt
174 ended sterile toothpicks and transfer them in a tube having YPD with DTT and HEPES buffer.
175 Make sure that cells are resuspended completely. Shake the tube gently for 15 min at 30 °C.
176 After 15 min of gentle shaking at 30 °C, wash the cells twice with sterile water. During washing
177 steps, cells should be pelleted down at 3000 g for 3 min. After completing washing steps,
178 incubate the tube on ice for 3-5 min, mix with DNA (PCR product or digested plasmid) and
179 gently mix the content of tube. Transfer the content of tube in pre-labeled electroporation
180 cuvettes which were already kept on ice. After transfer content in cuvette give the electric pulse
181 to the cells at the following settings (Voltage: 1500 VH, Resistance: 200 Ω, Capacitor: 0025 µF
182 using BTX, ECM20, version 1.04). Just immediately after the electric pulse, add 1 mL ice-cold 1
183 M sorbitol (as recovery medium) into the electroporation cuvette and mix well. If the selection is
184 on an antibiotic plate, incubate the cuvette with cells at 30 °C for 2-3 hour and if a selection is on
185 dropout plate, plate the content of tube just after the addition of recovery medium. Note that

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186 steps which can be modified in present modified procedure are discussed in the discussion
187 section.

188 **3 RESULTS**

189 **3.1 Need for a new or modified protocol**

190 As mentioned in the introduction that, all the available protocol for *P. pastoris* transformation
191 involved over-night pre-culture (source of inoculum), dilution of pre-culture followed by growth
192 of cells to OD_{600nm} close to 1.2 which take around 5-6 hour depending upon strain(s) (table 1
193 showing comparison of various available protocol) (Ito et al., 1983; Dohmen et al., 1991; Cregg
194 et al., 1995; Cereghino et al., 2005; Shixuan & Letchworth, 2004; Hinnen et al., 1978). Further,
195 steps involved in preparing competent cells again require 1-2 hour depending upon the number
196 of strains handled at a time. And if the selection is on an antibiotic plate, it again increases the
197 time till final plating of cells on plate. This means whole day is required to complete the
198 transformation experiment. Apart from that present protocols involve growing cells in 50-100
199 mL media which again limit the number of flasks which can be handled by an individual at a
200 time. In short transformation of *P. pastoris* is a lengthy process requiring a lot of efforts and
201 resource. Basic steps of different protocols for *Pichia pastoris* transformation are compared in
202 table 1.

203 Therefore, a protocol which is short, reliable and robust which can reduce the efforts, time,
204 resource and allows transformation of a large number of strains will be important. Importantly, a
205 protocol which does not require growth of cells in flasks or handling of bulk culture i.e. skip
206 culturing step is always desirable. Therefore, in the present study, I will be describing a protocol
207 for *P. pastoris* transformation which does not require culturing the cells and allows a large
208 number of transformations with minimum efforts and also requires less resource in terms of use
209 of lab equipment like shaker, big centrifuge, and other lab reagents.

210 **3.2 Basic workflow**

211 A detailed procedure of *Pichia pastoris* transformation is described in materials and methods
212 section. Here only the basic workflow of the protocol is described, and comparison was drawn
213 between the protocol described in the present study and other available protocols (Figure 1).
214 Basic elements of plasmid integrated in *P. pastoris* using present modified protocol is shown in
215 figure 2A. The present protocol does not require growth of pre-culture and growth of cells in big
216 flasks. A patch of around 2 cm by 1.5 cm is sufficient for two transformations (Fig. 2B). This

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217 saves lots of media used in pre-culture and growing cells. This step also reduces use of plastic
218 wares (culture tubes) and proved to be more economical. Required strain(s) are patched properly
219 on fresh YPD plate and plate(s) were incubated at 30 °C for 18-24 hour. This step allows
220 transformation even in absence or non-availability of big shakers. The appearance of a fine layer
221 of cells in the patched area suggested that patch is ready for transformation. Transfer 1 mL of
222 YPD with 40 mM DTT, 40 mM HEPES buffer pH8 in a required number of sterile eppendrof
223 tubes. Using a sterile 200 mL tips or toothpick scrap the cells and resuspended in YPD. Make
224 sure cells are dispersed properly. One can vortex the tube to disperse cells to get uniform cell
225 suspension. Gently shake the tube for 15 min at 30 °C. After completion of this step, cells were
226 washed twice with sterile water. Each time cells were pelleted down by centrifugation at 3000 *g*
227 for 3 min. This allows preparation of competent cell more quick, easy and cost effective as it
228 does not require big tubes (50 mL tubes) to harvest cells, washing of cells in different buffers.
229 The supernatant was discarded, and the cell pellet was resuspended in such a way that total
230 volume of cell suspension was around 50-70 μL and a required DNA (intact plasmid, digested
231 plasmid or PCR product) was mixed properly with cell suspension and the cell suspension was
232 transferred into an electroporation cuvette. Electric pulse or shock was given using settings
233 described in material and methods. After electric shock 1 mL recovery medium (1 M sorbitol or
234 2 % glucose) was added to cuvette and cells were mixed well. The content of cuvette was
235 transferred into eppendrof tube and cells were pellet down by centrifugation at 3000 *g* for 3 min.
236 The supernatant was discarded, and the cell pellet was resuspended in sterile water such that final
237 volume is no more than 100 μL. Cells were plated on a required plate.
238 Number of transformants that appeared on transformant plate vary significantly and represented
239 image of one of the transformant plate is shown in figure 2C. Cost effectiveness of present
240 modified procedure for *P. pastoris* transformation is shown through table 2.

241 **3.3 Integration and expression of a gene of interest**

242 Working of the protocol was checked by introducing PpCUE5-GFP and PpPGK1-GFP. Pgk1 is a
243 cytosolic enzyme involved in glycolysis and gluconeogenesis (Hitzeman et al., 1980; Blake and
244 Rice, 1981) while Cue5 is a cytosolic ubiquitin binding protein (Shih et al., 2003; Lu et al.,
245 2014). Both fusion proteins were checked by detecting C-terminal GFP tags using anti-GFP
246 antibodies (Fig. 3A). Western blot image also shows that the expression of PpCue5-GFP and
247 PpPgk1-GFP was similar in all the transformants checked by western blot. Protein loading was

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248 shown by Ponceau S stained blot image which clearly showed the amount of protein loaded in
249 each well were similar (Fig. 3B). Out of six colonies checked by western blot, five were positive
250 and was negative for both PpPgk1-GFP and PpCue5-GFP. In both the cases colonies were
251 selected randomly for verification by western blot. Plasmids (with PpCUE5 and PpPGK1 under
252 their native promoter and C-terminal GFP tag) was integrated at *his* locus (Fig. 2A) after
253 linearizing plasmid by *Eco*NI. Introduction and expression of a gene introduced using the present
254 modified procedure of *P. pastoris* transformation were also checked and confirmed by detecting
255 GFP in cells using fluorescence microscopy (Fig. 3C and 3D for PpPgk1-GFP and PpCue5-GFP
256 respectively). Taken together with data of western blot and microscopy data showed that gene(s)
257 introduced by a modified procedure for *P. pastoris* transformation were integrated properly into
258 the genome at required locus or position and able to express properly.
259 Both western blot as well as microscopy data showed significant difference in abundance of
260 Pgk1 and Cue5 and our present data is accordance with previous studies (Kulak et al., 2014)
261 suggesting that observed difference in protein abundance is not due to experimental artifact. Note
262 in both the case gene is under its endogenous promoter. Mass of fusion protein was 65.7 kDa and
263 71.6 kDa for Cue5-GFP and Pgk1-GFP respectively.

264 **3.4 Factors affecting transformation efficiency**

265 The efficiency of transformation depended on several factors including the physiological state or
266 age of cells or culture, way the competent cells are prepared, nature of recovery medium, the
267 number of cells or cell density, amount of DNA. It is important to study how different factors(s)
268 affect the efficiency of transformation. Therefore, in the present study, I check the effect of age
269 of patch on YPD plate, use of DDT, HEPES buffer during competent cell preparation and
270 recovery medium after giving an electric shock to cells. Presently available data showed that age
271 of patch on YPD plate affect transformation efficiency significantly (Fig. 4A). After 3 days I
272 could get only a few colonies and after five days I could not get any colony on transformants
273 plate (data not shown). Further, it was observed that application of DTT and HEPES pH8 at a
274 final concentration of 40 mM improve the efficiency of transformation significantly (Fig. 4B, C
275 respectively). Just like previous reports DTT and HEPES at 40 mM concentration gives best
276 results, DTT and HEPES more than 40 mM does not increase transformation efficiency
277 significantly. Application of chilled 1 M sorbitol or 2 % YPD as recovery medium does not
278 affect transformation efficiency significantly (fig. 4D). Thus, some factors hardly have any effect

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279 on transformation efficiency while others are crucial. Factors like cell density, amount of DNA
280 added to competent cells were not investigated in present study as these factors were already
281 investigated by other lab (Shixuan & Letchworth, 2004) and I believe these may behave
282 similarly in present study. Only those factors were investigated which were unique to present
283 procedure like age of patch on YPD plate. Although it was observed that for high efficiency
284 transformation plasmid should be linearized by digestion from middle of marker used during
285 transformation.

286 **4 DISCUSSIONS**

287 Based on the results and comparison of already published protocol for *P. pastoris* transformation
288 with the procedure described in this paper, it can be said that present procedure got a clear
289 advantage of being short, simple, economical and well suited for large-scale transformation. By
290 getting rid of steps which include overnight pre-culture, dilution of pre-culture and then further
291 growth of cells to certain cell density, the present procedure allows a large number of
292 transformation with minimum efforts, resource and time. Since patches were made on plates
293 which were incubated at 30 °C and the rest steps were carried out in small 1.5 mL eppendrof
294 tubes means the present protocol does not require big shaker and centrifuge which is itself a big
295 advantage especially early days of lab establishment. By simplifying the step of competent cells
296 preparation, the present procedure makes sure that a large number of transformation can be
297 carried out in quickest possible time and added much needed high throughput element in *P.*
298 *pastoris* transformation which is very important especially in industries where a large number of
299 transformations are carried out on a daily basis. The procedure described in the present paper is
300 also very economical. One patch of 2 cm X 1.5 cm (length X width) is enough for two
301 transformations and one can prepare at least 6 patches of this size means 10-12 transformation
302 can be performed using a single plate with 23-25 mL media compare other available procedure
303 which requires growing of cells in 50-100 mL media for each transformant. Apart from saving
304 on media per transformation, there is a huge saving on other plastic ware, buffers if a large
305 number of transformations are required. Technical improvements in *P. pastoris* transformation
306 are already described by Cai et al. (2001) certainly make *P. pastoris* transformation more robust.
307 I believe that present modified protocol for expression of gene of interest is simpler compared to
308 commercial kits (https://tools.thermofisher.com/content/sfs/manuals/easys_elect_man.pdf) and
309 this may enhance the utility of present procedure.

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310 It is important to mention that efficiency of transformation is affected by nature of plates. It is
311 highly recommended to avoid old plates and does not allow patch grows too thick. It was
312 observed that transformation efficiency falls dramatically as the thickness of patch increases or
313 patch become older. I find that patch generally of 18-24 hour old is most suitable for preparing
314 cells for transformation. It is also advised that one should prepare patch using healthy growing
315 cells. Patch prepared for old dying cells significantly reduces transformation efficiency. Apart
316 from that described procedure will also of great importance for those who do not have access to
317 large shakers and centrifuges. It is important to mention that addition of reducing agent (example
318 DDT) dramatically affects transformation efficiency and present observation is in accordance
319 with the previously published protocol (Cereghino et al., 2005). It was also observed that use of
320 1 M sorbitol as a recovery medium is not essential and one can also use 2 % dextrose if selection
321 will be made on dropout media lacking required amino acid. But if selection will be made of
322 antibiotic plate, even YPD is good enough as a recovery medium. It is important to mention that
323 number of colonies on transformant plates is highly dependent upon nature of DNA (plasmid or
324 PCR product), gene locus for integration of DNA, nature of selection, number of homologous
325 residues in DNA, whether the transformation is for gene deletion or introduction of gene and so
326 on. It is important to mention that apart from introduction of gene of interest present modified
327 protocol was also suitable for deletion of endogenous gene or ORF (dada not shown).
328 The present protocol has been tested on different *P. pastoris* background including GS200,
329 GS115, PPY12h and PPY12m over a period of six months. Apart from introducing gene through
330 integrating plasmid at gene locus (generally selection marker like *his, arg*), I was able to
331 integrate the cassette within ORF (of the gene of interest) for N-terminal tagging of protein.
332 Incubation of cells in water with DTT and HEPES pH8 at a final concentration of 40 mM in
333 place of YPD is equally good. Further, in present procedure all steps can be carried out at room
334 temperature without requiring refrigerated centrifuge or ice and incubation of cuvettes after
335 mixing DNA with cells does not affect transformation in a significant way and same goes with
336 the addition of 1 M sorbitol as recovery medium. Although I have not checked the effect of
337 freezing competent cells prepared by present modified procedure, I believe that present
338 procedure makes freezing of competent cells irrelevant and save space in the deep freezer. In the
339 end, it can be said that present procedure makes the transformation of *P. pastoris* simpler, quick,
340 economical, and less exhaustive and may become choice of method both in labs and industries.

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348 **References**

349 1. Becker, D. M., & Guarante, L. (1991). High-efficiency transformation of yeast by
350 electroporation. *Methods Enzymol,* 194, 182-187.
351 2. Blake, C.C., & Rice, D.W. (1981). Phosphoglycerate kinase. *Philos Trans R Soc Lond B*
352 *Biol Sci* 293(1063):93-104
353 3. Cai, C.Q., & Fang, R.X. (2001). Technical improvements in genetic manipulation of
354 *Pichia pastoris* and their application in hirudin expression. *Sheng Wu Gong Cheng Xue*
355 *Bao,* 17(2),155-60.
356 4. Cereghino LJ, Cregg JM. Heterologous protein expression in the methylotrophic yeast
357 *Pichia pastoris*. FEMS Microbiol. Rev. 2000; 24:45-66.
358 5. Cereghino, L.J., Wong, W.W., Xiong, S., Giang, W., Luong, L.T., Vu, J., Johnson, S.D.,
359 & Lin-Cereghino, G.P. (2005). Condensed protocol for competent cell preparation and
360 transformation of the methylotrophic yeast *Pichia pastoris*. *Biotechniques*, 38(1), 44-48.
361 6. Cregg, J.M., Barringer, K.J, Hessler, A.Y., & Madden, K.R. (1985). *Pichia pastoris* as a
362 host system for transformations. *Mol Cell Biol*, 5(12),3376-85.
363 7. Daly, R., & Hearn, M.T. (2005). Expression of heterologous proteins in *Pichia pastoris:*
364 a useful experimental tool in protein engineering and production. *J Mol Recognit,*18(2),
365 119-38.
366 8. Daniel Weinacker, Claudia Rabert, Andrea B. Zepeda, Carolina, A. Figueroa, Adalberto
367 Pessoa, & Jorge, G. Farías. (2013). Applications of recombinant Pichia pastoris in the
368 healthcare industry. *Braz J Microbiol*, 44(4), 1043–1048.
369 9. Dohmen, R.J., Strasser, A.W.M., Höner, C.B., & Hollenberg, C.P. (1991). An efficient
370 transformation procedure enabling long-term storage of competent cells of various yeast
371 genera. *Yeast*, 7, 691-692.

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372 10. Farré, J.C., Carolino, K., Stasyk, O.V., Stasyk, O.G., Hodzic, Z., Agrawal, G., Till, A.,
373 Proietto, M., Cregg, J., Sibirny, A.A., & Subramani, S. (2017). A New Yeast Peroxin,
374 Pex36, a Functional Homolog of Mammalian PEX16, Functions in the ER-to-Peroxisome
375 Traffic of Peroxisomal Membrane Proteins. *J Mol Biol*, 429(23):3743-3762.
376 11. Farré, J.C., Manjithaya, R., Mathewson, R.D., & Subramani, S. (2008). PpAtg30 tags
377 peroxisomes for turnover by selective autophagy. *Dev Cell,* 14(3), 365-76.
378 12. Gasser, B., Prielhofer, R., Marx, H., Maurer, M., Nocon, J., Steiger, M., Puxbaum, V.,
379 Sauer, M., & Mattanovich, D. (2013). *Pichia pastoris:* protein production host and model
380 organism for biomedical research. *Future Microbiol*, 8(2),191-208.
381 13. Hinnen, A., Hicks, J. B., & Fink, G. R. (1978). Transformation of yeast. *Proc. Natl.*
382 *Acad. Sci. USA* 75, 1929–1934.
383 14. Hitzeman, R.A., Clarke, L., & Carbon, J. (1980). Isolation and characterization of the
384 yeast 3-phosphoglycerokinase gene (PGK) by an immunological screening technique. *J*
385 *Biol Chem* 255(24):12073-80
386 15. Ito, H., Fukuda, Y., Murata, K., & Kimura, A. (1983). Transformation of intact yeast
387 cells treated with alkali cations. *J. Bacteriol*, 153:163-168.
388 16. Klebe, R. J., Harriss, J. V., Sharp, Z. D., & Douglas, M. G. (1983). A general method for
389 polyethylene-glycol-induced genetic transformation of bacteria and yeast. *Gene* 25, 333-
390 341.
391 17. Kulak, N.A., Pichler, G., Paron, I., Nagaraj, N., & Mann, M. (2014). Minimal,
392 encapsulated proteomic-sample processing applied to copy-number estimation in
393 eukaryotic cells. *Nat Methods* 11(3), 319-24
394 18. Laemmli, U.K. (1070). Cleavage of structural proteins during the assembly of the head of
395 bacteriophage T4. *Nature,* 227,680-685.
396 19. Lu, K., Psakhye, I., & Jentsch, S. (2014). Autophagic clearance of polyQ proteins
397 mediated by ubiquitin-Atg8 adaptors of the conserved CUET protein family. *Cell*
398 158(3),549-63
399 20. Nazarko, T.Y., Ozeki, K., Till, A., Ramakrishnan, G., Lotfi, P., Yan, M., & Subramani,
400 S. (2014). Peroxisomal Atg37 binds Atg30 or palmitoyl-CoA to regulate phagophore
401 formation during pexophagy. *J Cell Biol,* 204(4), 541-57.

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402 21. Romero-Calvo, I., Ocón, B., Martínez-Moya, P., Suárez, M.D., Zarzuelo, A., Martínez-
403 Augustin, O., & de Medina, F.S. (2010) Reversible Ponceau staining as a loading control
404 alternative to actin in Western blots. *Anal Biochem,* 401(2), 318-20.
405 22. Shih, S.C., Prag, G., Francis, S.A., Sutanto, M.A., Hurley, J.H., & Hicke, L. (2003). A
406 ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE
407 domain. *EMBO J* 22(6),1273-81
408 23. Shixuan, Wu., & Letchworth, G.J. (2004). High efficiency transformation by
409 electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol.
410 *Biotechniques*, 36(1),152-4.
411 24. Sturmberger, L., Chappell, T., Geier, M., Krainer, F., Day, K.J., Vide, U., Trstenjak, S.,
412 Schiefer, A., Richardson, T., Soriaga, L., Darnhofer, B., Birner-Gruenberger, R., Glick,
413 B.S., Tolstorukov, I., Cregg, J., Madden, K., & Glieder A. (2016). Refined *Pichia*
414 *pastoris* reference genome sequence. *J Biotechnol,* 235:121-31.
415 25. Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from
416 polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl*
417 *Acad Sci U S A,* 76(9), 4350-4.
418 26. Wang, W., Xia, Z.J., Farré, J.C., & Subramani, S. (2017). TRIM37, a novel E3 ligase for
419 PEX5-mediated peroxisomal matrix protein import. *J Cell Biol,* 216(9), 2843-2858.

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428 **Legends**

429 **Figure**

430 **Figure 1. Schematic showing basic workflow of present modified protocol for Pichia**
431 **pastoris transformation.**

432 **Figure 2. Transformation of *P. pastoris* using modified protocol.** (A) Basic features of
433 plasmid integrated in *P. pastoris* gene using present procedure. (B) YPD plate showing size of
434 patch used for transformation. (C) Image of one of the transformant plate. Note number of
435 transformants (colonies) on transformant plate vary 3-5 times compared to one shown in figure
436 2C.

437 **Figure 3. Integration of gene of interest in *P. pastoris* genome.** (A) Western blot showing
438 positive transformants for PpPgk1-GFP (first 1-6 lane, left side) and PpCue5GFP (last 1-6 lane,
439 right side) Expected bands are pointed by arrow towards them. *Fragmented or nonspecific
440 bands. Mass of both fusion proteins are mentioned in main text. (B) Protein loading is shown by
441 Ponceau S stained blot. Both the fusion proteins were detected at expected size range. Western
442 blot results were also confirmed by detecting GFP signal (C) PpPgk1-GFP and (D) PpCue5-GFP.
443 Scale bar represent 5 μM.

444 **Figure 4. Factors affecting transformation efficiency.** Effect of (A) age of patch on YPD
445 plate, (B) effect of reducing agent (DTT), (C) recovery medium and (D) HEPES buffer in on
446 transformation efficiency. Note these parameters were checked on different background *P.*
447 *pastoris* although data is shown only for PPY12h background.

448 **Table**

449 **Table 1. Comparison of present modified protocol with previously published protocols**
450 **form economic point of view.**
451 **Table 2. Comparison of various published protocol for *P. pastoris* transformation with**
452 **present modified procedure**
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464 **Table 1. Comparison of various published protocol for *P. pastoris* transformation with**
465 **present modified procedure**
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Heat Shock (Dohmen et al., 1991) [A]	**Alkali Cation** (Ito et al., 1983) [B]	**PEG** (Klebe et al., 1983) [C]	**Electroporation** (Becker & Guarante, 1991) [D]	**Condensed Protocol** (Cereghino et al., 2005) [E]	**Present study (modified electroporation)**
Growth of preculture	Growth of preculture	Growth of preculture	Growth of preculture	Growth of preculture	Making of patch on plate
Dilution of preculture	Dilution of preculture	Dilution of preculture	Dilution of preculture	Dilution of preculture	Scrap the cells from patch and resuspend them in 1 mL YPD with DTT and HEPES pH8
Growth of cells to desired OD_{600nm} (0.6 to 1.0)	Growth of cells to desired OD_{600nm}	Growth of cells to desired OD_{600nm}	Growth of cells to desired OD_{600nm}	Growth of cell to desired OD_{600nm}	Incubate the cells at 30 C, for 15 min in gentle shaking condition
Harvesting culture and washing with solution/buffer	Harvesting culture and washing with solution/buffer	Harvesting culture and washing with solution/buffer	Harvesting culture and washing with solution/buffer	Harvesting culture and washing with solution/buffer	Wash the cells twice with sterile 1 mL water each time.
Resuspend cells in 0.02 volume of above same solution	Incubate the cells with buffer for 1 h at 30°C.	Resuspend cells in buffer	Suspend the cells in 100 mL of YPD medium plus HEPES, add 2.5 mL of 1 M DTT and gently mix	Resuspending of cells in 9 mL BEDS solution supplemented with 1 mL 1.0 M DTT	Discard supernatant
Freeze cells or proceed for transformation	Mix vector DNA with cells	Add DMSO and freeze or proceed for transformation	Incubate at 30°C for 15 min	Incubate the cells for 5 min, 100 rpm, 30 °C	Mix DNA with cells
Mix DNA with cells	Mix carrier DNA with cells	Mix DNA with cells	Bring to 500 mL with cold water	Incubate the cells for 5 min, 100 rpm, 30 °C	Incubate cells on ice for 3-5 min
Add 40% polyethylene glycol (PEG),	Incubate samples at 30°C for 30 min	Mix carrier DNA with cells	Again, wash the cells	Harvest the cells at 500 g, 5 min, RT and resuspend them in 1 mL BEDS solution without DTT	Give electric pulse to cells
Incubate for 60 min at 30°C	Add 0.7 mL of PEG + LiCl solution, and briefly vortex to mix	Incubate samples in a 37°C water bath for 5 min.	Freeze at -70 C or proceed for transformation	Freeze at -70 C or proceed for transformation	Either plate the cells or incubate depending upon selection
Heat shock at 42°C for 10 min	Incubate samples at 30°C for 30 min	Add another solution and mix well	Mix DNA with cells	Mix DNA with cells	Mix the cells with recovery medium

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Pellet cells	Heat-shock at 37°C for 5 min	Incubate tubes in a 30°C water bath for 1 h	Incubate on ice	Incubate on ice	Incubate for 2-3 hour or spread on desired plate
Resuspend cells in buffer	Centrifuge samples at 2000g and resuspend in 0.1 mL of H2O	Pellet down cell and resuspend in another buffer (repeat this again)	Giving electric pulse to cells	Giving electric pulse to cells	
Repeat last two steps	Centrifuge samples at 2000g and resuspend in 0.1 mL of H2O	Again, pellet down cell and resuspend in another buffer	Mix the cells with recovery medium	Mix the cells with recovery medium	
Incubate for 2-3 hour or spread on desired plate	Incubate for 2-3 hour or spread on desired plate	Incubate for 2-3 hour or spread on desired plate	Incubate for 2-3 hour or spread on desired plate	Incubate for 2-3 hour or spread on desired plate	

467 Note: In this table only basic steps are compared without describing fine details of each step(s), buffer used and their
468 composition. For this readers are advised to check original proper (reference provided).

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482 **Table 2. Comparison of present modified protocol with previously published protocols**
483 **form economic point of view.**

Present procedure	**Other published protocol** {A to E}*	**Comments**
No requirement of culture tube for pre-culture	Require culture tube for preculture	Present procedure is cost effective
45-50 mL YPD agar is sufficient for 10 transformations	Relatively more media is required	Present procedure is cost effective
Require incubator	Require big shaker, more electric power	Present procedure is cost effective
Cell preparation is performed in 1.5 mL tube	May require bigger tubes (50 mL, 10 tubes)	Present procedure is cost effective
Small benchtop centrifuge is sufficient	Since high volume of media and buffer handling is required which necessitates big centrifuge {	Present procedure is cost effective
No special buffer for cell washing as washing is done with water	Require special buffer depending upon procedure, may require different buffers at different washing steps	Present procedure is cost effective
Less requirement of power for different steps	More power is required at different steps	Present procedure is cost effective
Relatively less investment in terms of big shaker and centrifuge	Big investment in terms of big shaker and centrifuge	Present procedure is cost effective

484 ***Note:** In this table comparison is made based on requirement on different steps in previously published protocols
485 (A to E in table 1).

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(A)

AmpR

Ori

Selection
marker eg His

C terminal tag

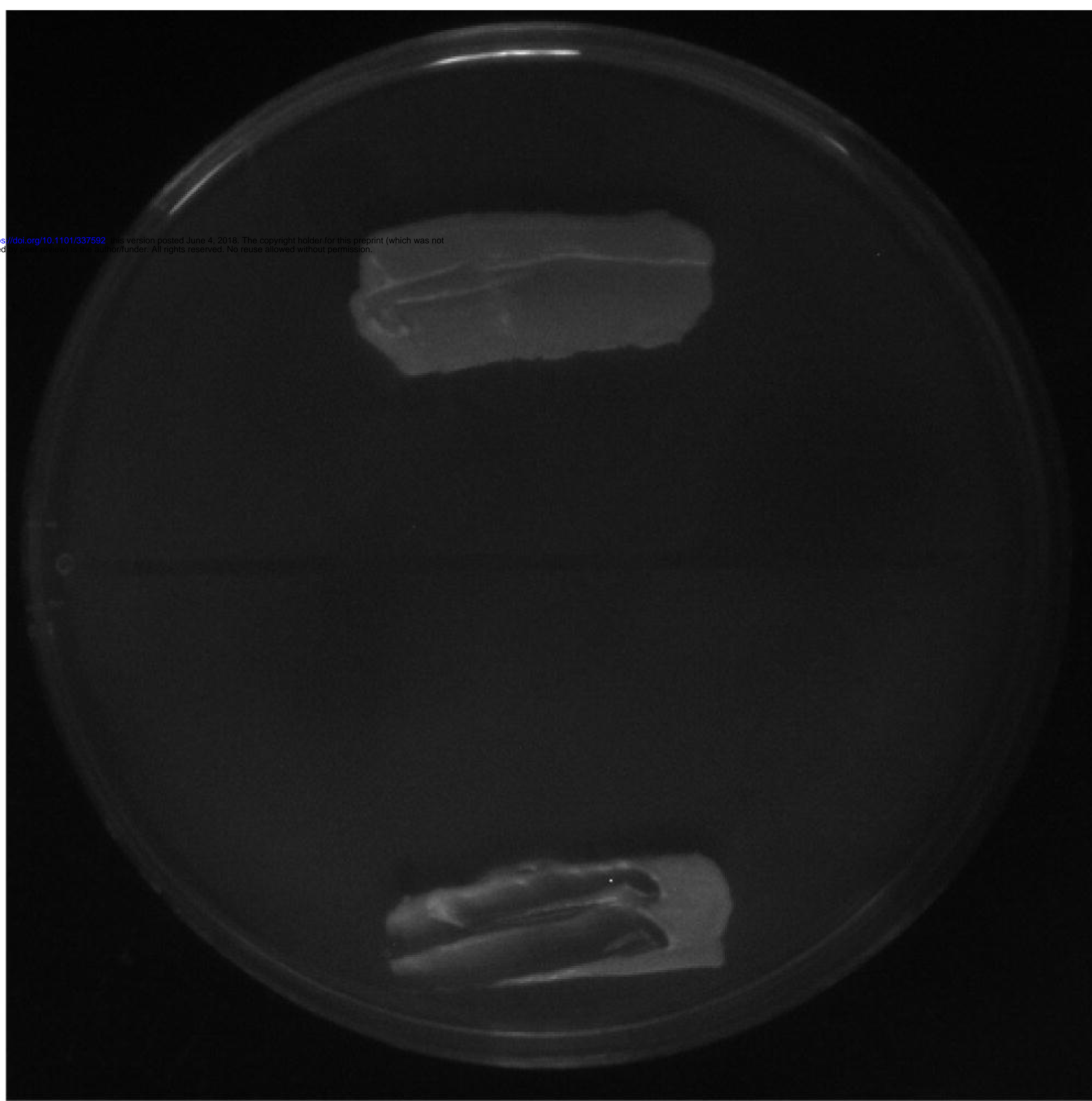
Gene of interest

Promoter

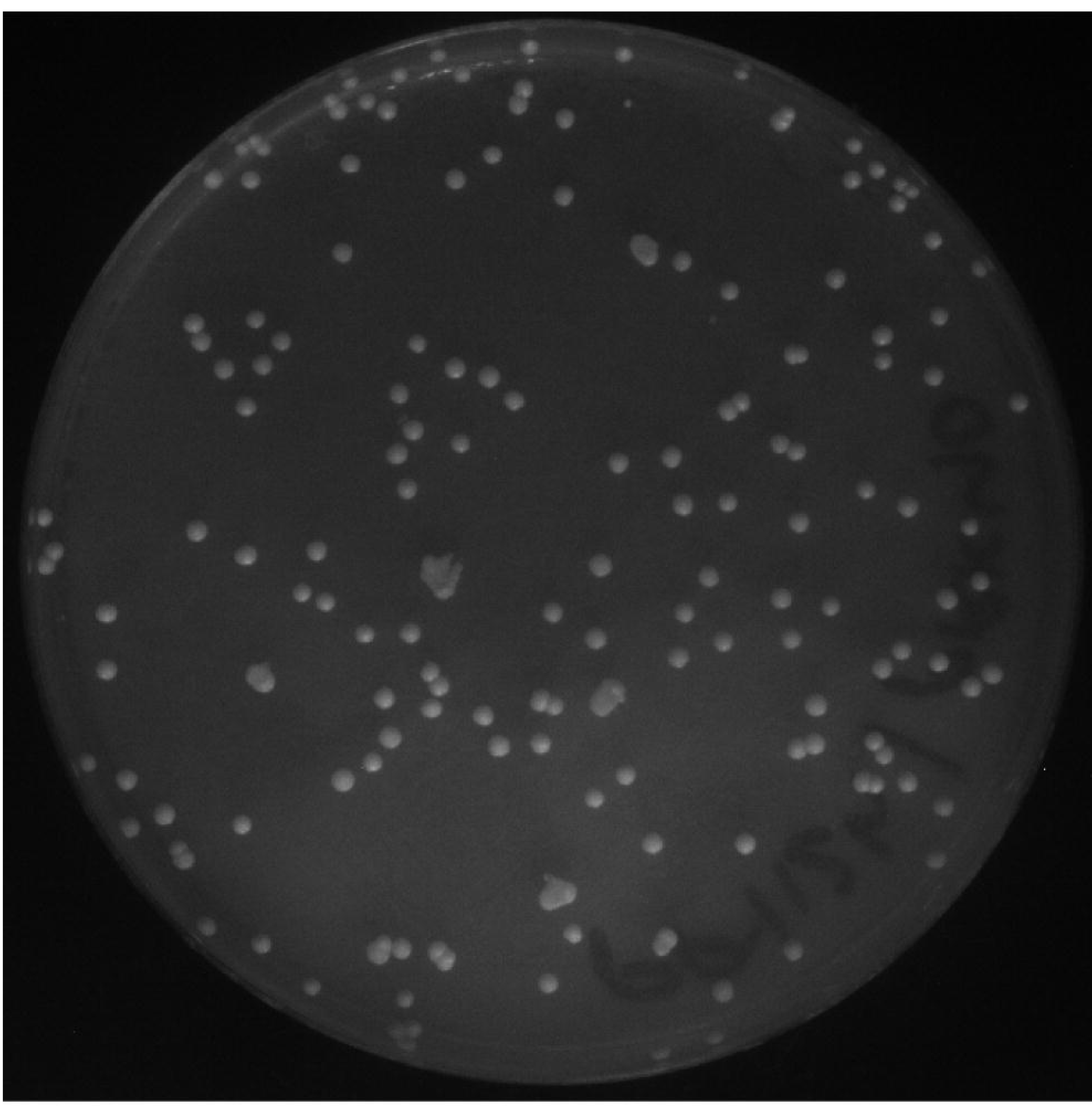
Restriction endonuclease
cut within marker

Linear plasmid integrating in genome

(B)



(C)



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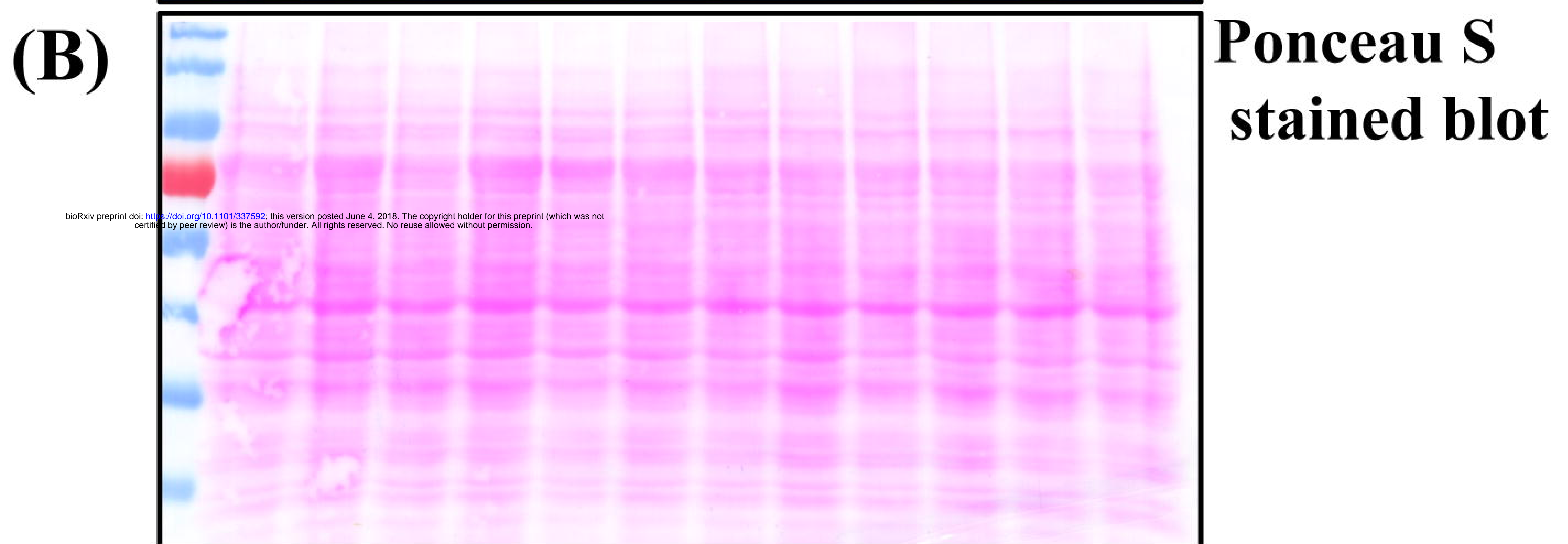
Pgk1-GFP Cue5-GFP

(A) 1 2 3 4 5 6 1 2 3 4 5 6

Western blot

*

(B)



Ponceau S stained blot

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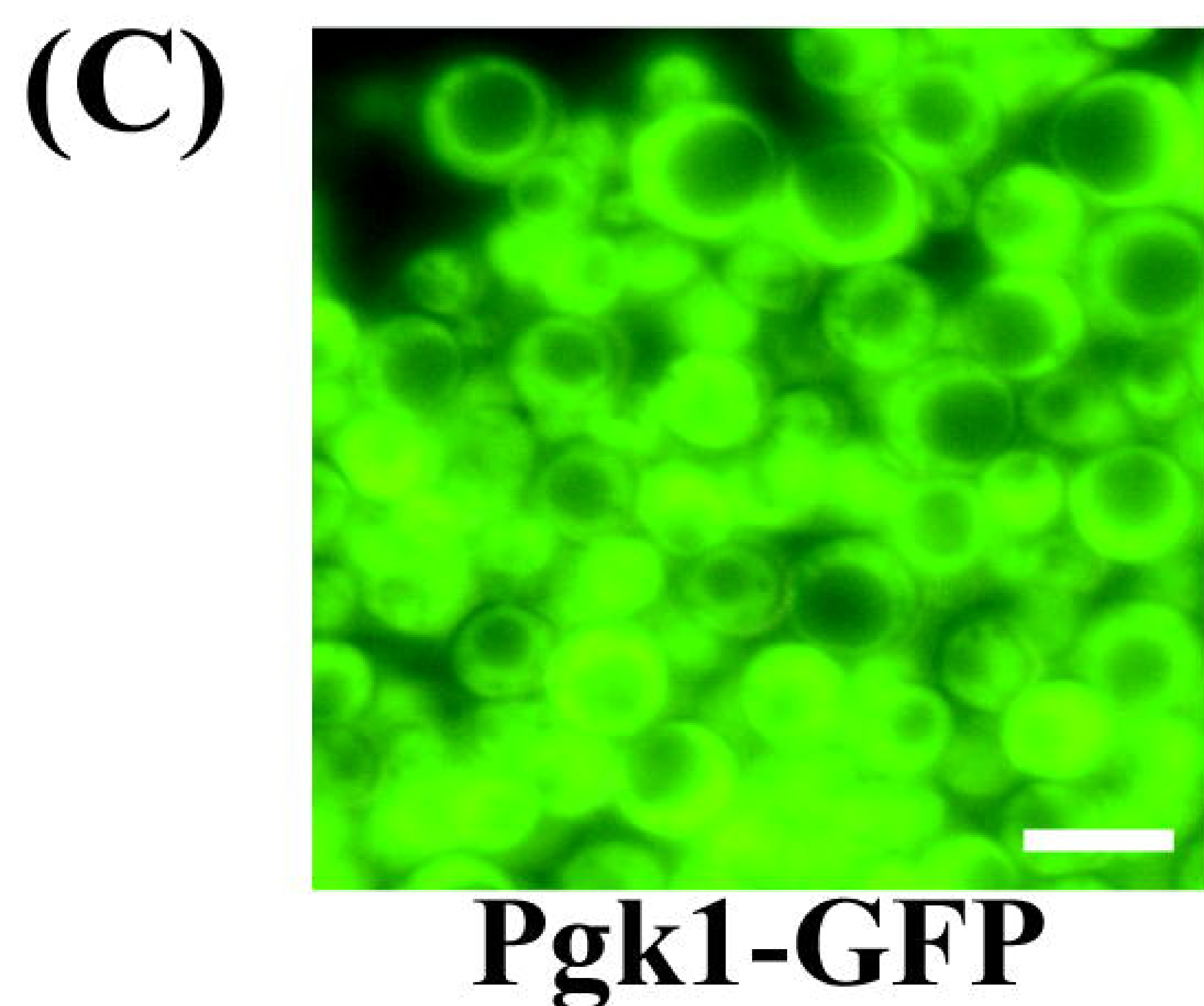
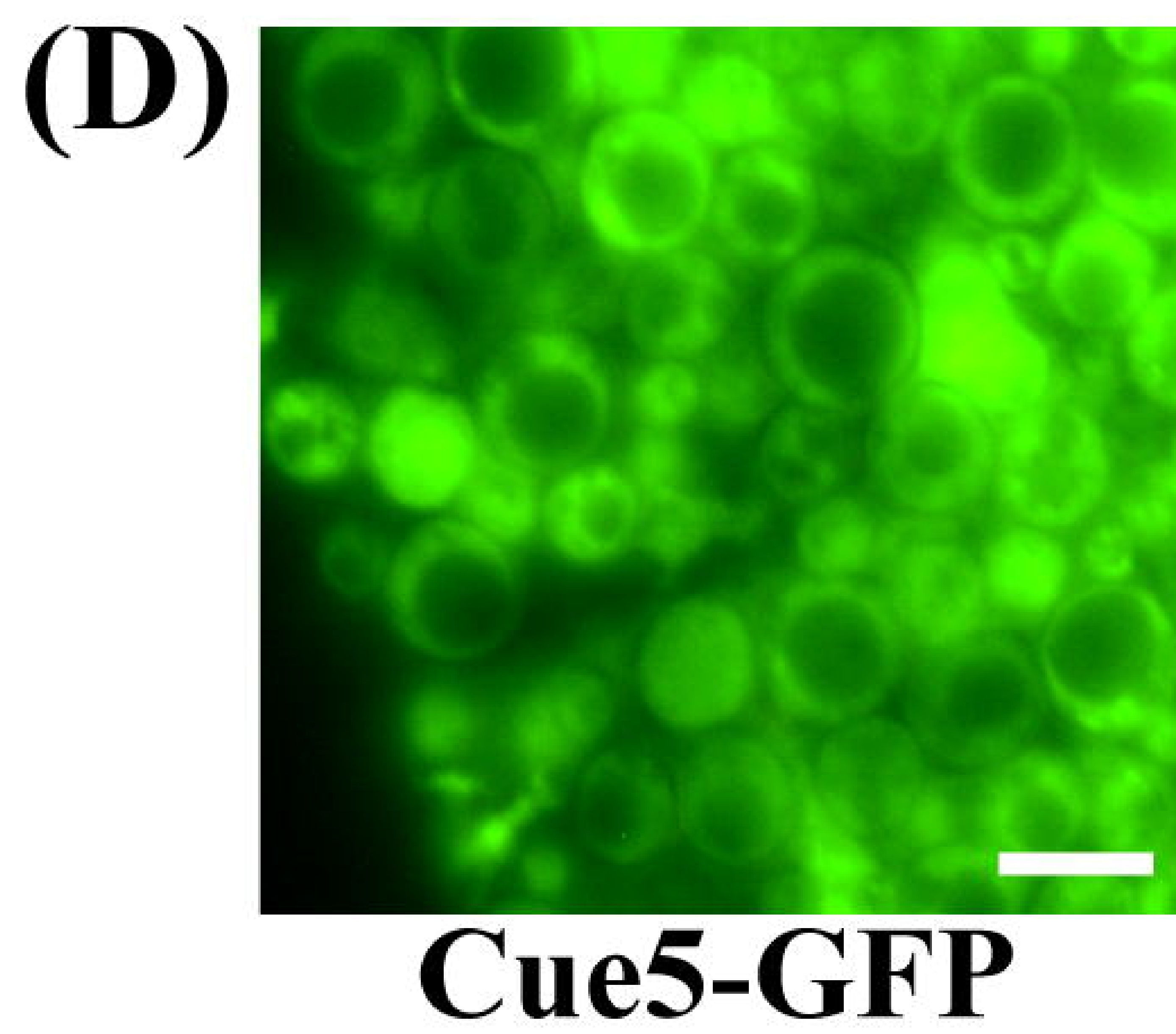
(C)

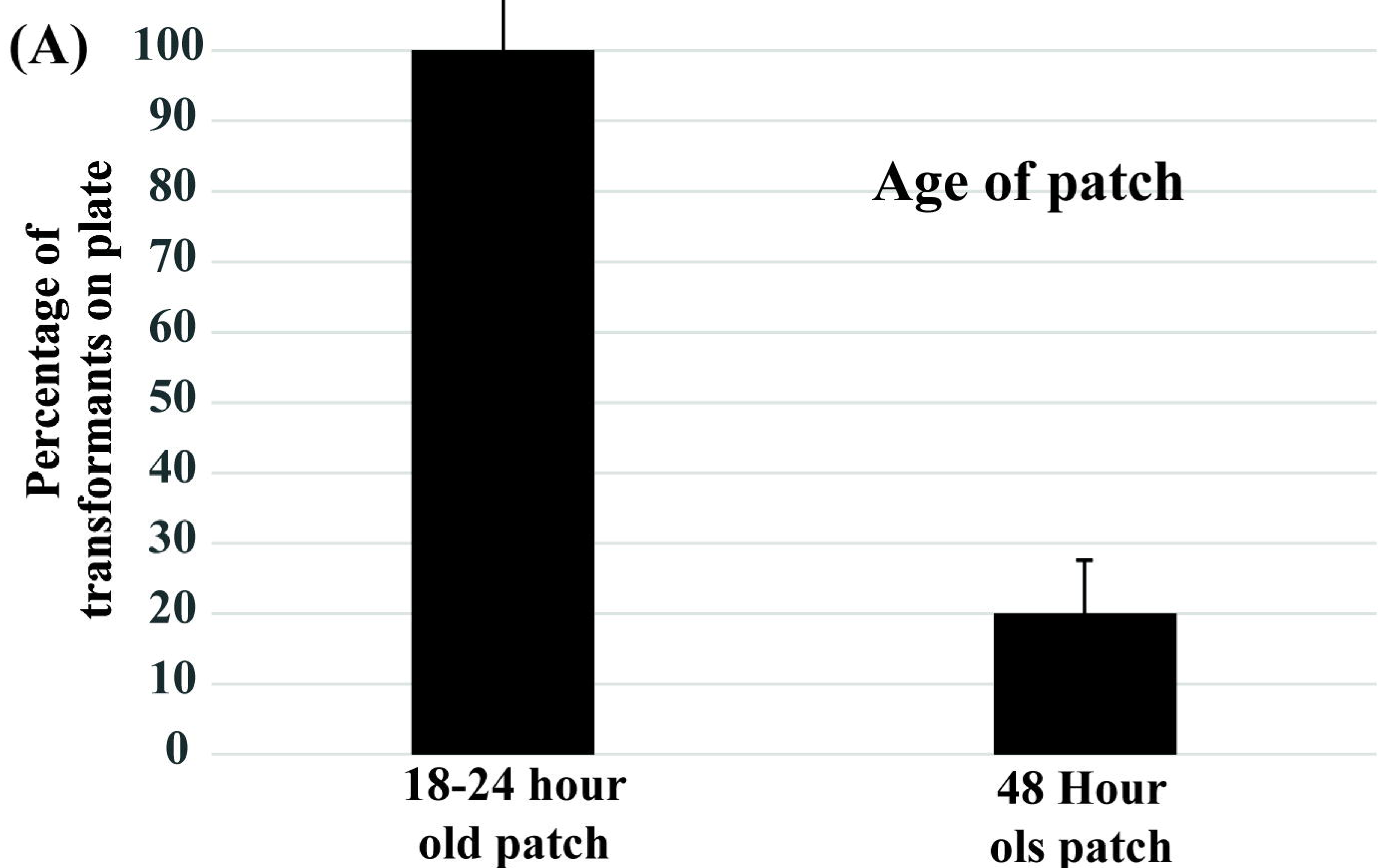


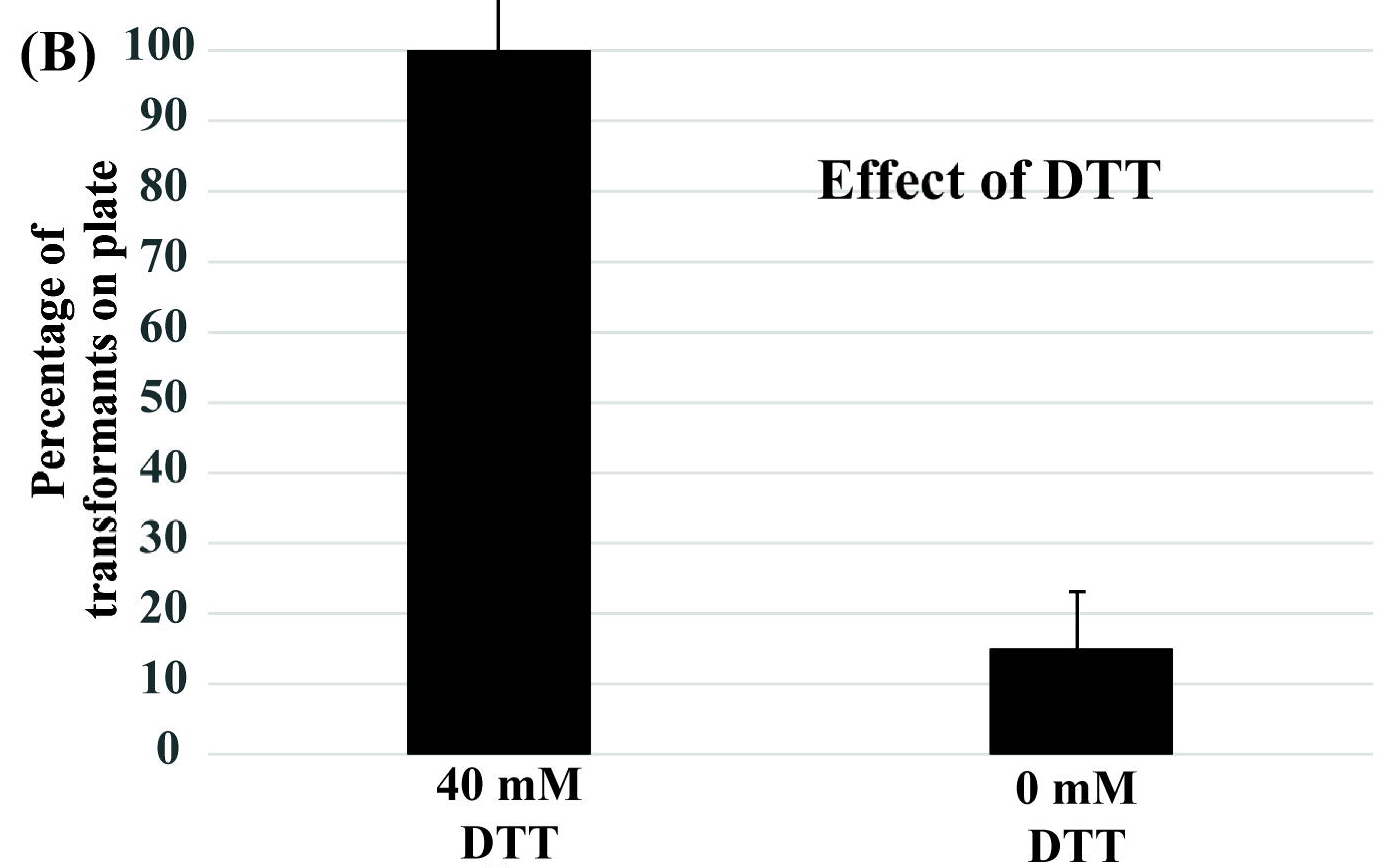
Pgk1-GFP

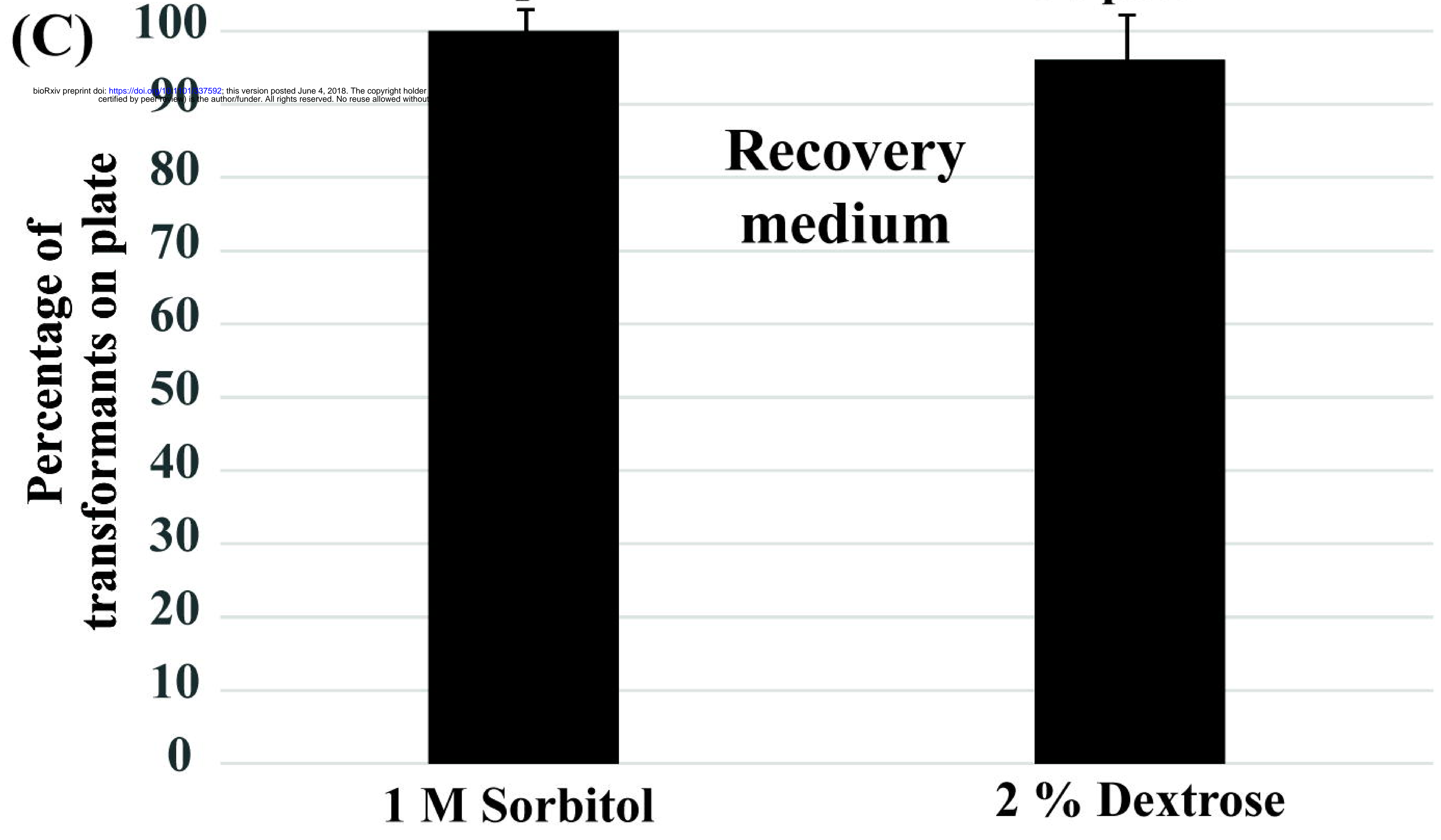
(D)



Cue5-GFP







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