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Single-cell cloning enables the selection of more productive *Drosophila melanogaster* S2 cells for recombinant protein expression

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ABSTRACT

The generation of monoclonal cell lines is an important early process development step for recombinant protein production. Although single-cell cloning is an established method in mammalian cell lines, straightforward protocols are not yet available for insect cells. We describe a new method for the generation of monoclonal insect cells without using fetal bovine serum and/or feeder cells pretreated by irradiation or exposure to mitomycin. Highly productive clones of *Drosophila melanogaster* S2 cells were prepared in a two-step procedure, comprising the establishment of a polyclonal population and subsequent single cell isolation by limiting dilution. Necessary growth factors were provided by co-cultivation of single transformants with untransfected feeder cells, which were later removed by antibiotic selection. Enhanced expression of EGFP and two target peptides was confirmed by flow cytometry and dot/western blotting. Highly productive clones were stable, showed a uniform expression profile and typically a sixfold to tenfold increase in cell-specific productivity.

1. Introduction

Stably transformed *Drosophila melanogaster* S2 cells (rS2) have emerged as a key platform for recombinant protein expression, and several related products have already entered clinical trials [1,2]. Like other frequently used expression systems based on mammalian cell lines or baculovirus vectors, rS2 cell lines must undergo comprehensive optimization during process development [2]. This not only includes the optimization of transfection conditions [3,4], but also the selection of highly productive subpopulations [5–7] or clonal derivatives [8–10]. Although single-cell cloning is the state of the art in mammalian cell lines [11–13], the same approach in stably transformed S2 cells is controversial, as highlighted by the following statements in the literature:

> "[. . .] the additional effort for cloning does not seem worthwhile, because the levels of expression reported for high-expressing clones [. . .] appear to be similar to those expected from a polyclonal population [. . .] "[14]
> "[. . .]One can, with a little more effort, clone the selected cells; the resulting clonal lines are generally more nearly homogeneous, and individual clones may differ sufficiently in their properties that the experimenter can choose a clone most suitable for his purposes.[. . .]" [15]

Despite the ongoing discussion, several protocols for single-cell cloning are available for insect cell lines (Table 1). The methods differ in terms of the separation technology (limiting dilution or plating in soft agar) and the method used to provide essential autocrine growth factors, for example by adding fetal bovine serum (FBS), conditioned medium or pre-treated feeder cells. The latter aspect is important because S2 cells cease proliferation when seeded at low density, which reflects their demand for high concentrations of autocrine growth factors [16]. Non-transfected,

Abbreviations: AMP, antimicrobial peptide/protein; BR021, *Harmonia axyridis* antimicrobial peptide BR021; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; GmGlv, *Galleria mellonella* antimicrobial peptide Gloverin; GMP, good manufacturing practice; OD_{600}, optical density at 600nm; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; RMCE, recombinase mediated cassette exchange; rS2, recombinant *Drosophila melanogaster* Schneider 2 cells; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Sf9, clonal isolate of *Spodoptera frugiperda* Sf21 cells; SFM, serum free medium.

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Table 1
Summary of previously reported techniques for the generation of monoclonal insect cell lines; [(a)] indicates the use of fetal bovine serum (FBS) in the medium during cloning, [(b)] indicates the use of serum free medium (SFM).

Method	Source of growth factors	Cell lines	Ref.
Limiting dilution	Conditioned medium	*Drosophila melanogaster* S2,	[18][(a)]
		*Spodoptera frugiperda Sf*9	[19][(a)]
	Feeder cells - mitomycin C	*D. melanogaster* S2	[20] [(a)]
	Feeder cells - irradiated	*D. melanogaster* S2	[21,17,22][(a,b)]
	Feeder cells - spatially separated	*D. melanogaster* imaginal disc	[23][(a)]
	Feeder cells - untreated, living	*D. melanogaster* S2	[8,9,10,24,25,26][(a,b)]
Soft agar	Conditioned medium	*D. melanogaster* S2	[18,27][(a)]
	Feeder cells - irradiated	*D. melanogaster* S2	[17,28][(a)]

irradiated feeder cells have often been used to support the growth of S2 cells [17] but the necessary X-ray or γ sources are not readily available in every cell culture laboratory and irradiation requires extensive empirical testing to ensure the complete inactivation of the feeder cells (typically wild-type S2 cells) while maintaining their viability. The same restrictions apply when using mitomycin C to chemically block cell division. Although all the methods shown in Table 1 can successfully yield monoclonal[1] rS2 lines, the specific protocols are often cumbersome or inefficient. We encountered difficult handling when using soft agar and found only a poor growth support when working with conditioned or FBS-supplemented medium instead of feeder cells. To overcome this challenge, we combined the limiting dilution of polyclonal transformants and co-cultivation with untreated feeder cells with a second round of antibiotic selection. This simple approach has been used before, but was only reported peripherally in the context of other research [9,10]. Within the previous works either only a small number of clones was generated (e.g. n = 8 in [9]) or a comparison between different clones and the parental population was missing. Here we provide a comprehensive analysis of the protocol to confirm that it offers a simple, robust and broadly available replacement for traditional single-cell cloning methods. In contrast to most of the older methods, the new protocol enabled the preparation of monoclonal cell lines in a completely serum-free environment, which is highly desirable in context of good manufacturing practice (GMP)-compliant cell line development.

2. Materials and methods

2.1. Construction of expression plasmids for the generation of recombinant S2 cells

The recombinant S2 cells were generated either by the transfection with a single plasmid containing an expression cassette and a selection cassette or by co-transfection with two separate plasmids (Fig. 1). Both systems are reliable for the stable transformation of S2 cells [17,29] and were used here to produce different proteins. Enhanced green fluorescent protein (EGFP) was used as a fluorescent reporter for the establishment and investigation of the limiting dilution assay, whereas the antimicrobial peptides (AMPs) *Galleria mellonella* gloverin (GmGlv) [8,30] and BR021 [31] were used as representative target molecules.

2.1.1. Plasmid construction by Golden Gate assembly

The Golden Gate (GG) assembly of expression plasmids for cell lines 1, 2 and 4 was conducted as previously described [32]. Corresponding donor and acceptor plasmids were synthesized by GenScript (Piscataway, New Jersey, USA) or were already part of an existing plasmid library [32]. The reaction volume was 20 μL, comprising 40 fmol of each plasmid, 20 U T4 DNA ligase (Promega, Mannheim, Germany), 2 μL of the corresponding T4 DNA ligase buffer (Promega) and 10 U BsaI (NEB, Frankfurt am Main, Germany). The GG mix was incubated in a PCR cycler (PEQLAB, Erlangen, Germany) at 37 °C for 15 min, followed by 30 cycles at 37 °C (2 min) and 16 °C (5 min). Subsequently, the enzymes were heat-inactivated at 50 °C for 15 min and 65 °C for 5 min. Finally, 5 μL of the GG mix was introduced into chemically competent *E. coli* NEB 10-β cells (NEB) as described in Section 2.1.3.

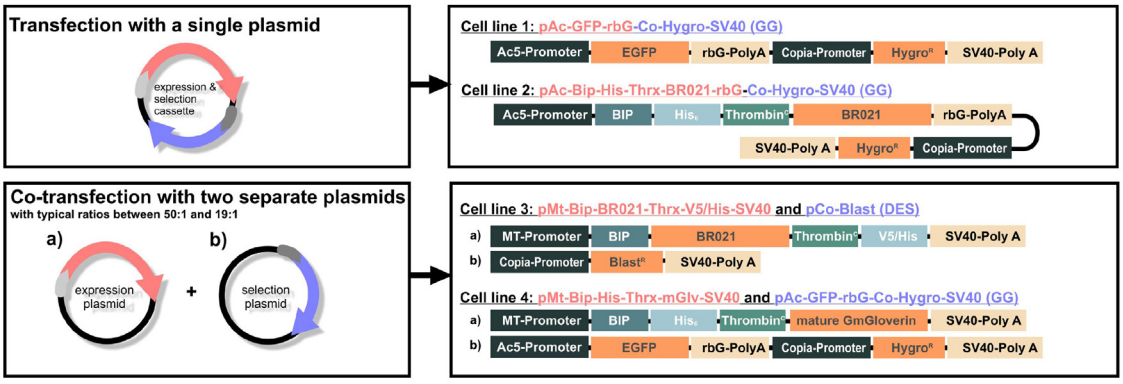
2.1.2. Plasmid construction by classical restriction-ligation cloning

For cell line 3, we used the commercially available DES® plasmids pMT/BiP/V5-His B and pCoBlast (Thermo Fisher Scientific, Darmstadt, Germany). The BR021 sequence was amplified by PCR using primers to introduce a C-terminal thrombin cleavage site as well as BglII and XhoI restriction sites (all primer sequences are provided in Supplementary Material 1). Following digestion with BglII and XhoI (NEB) and agarose gel electrophoresis of the backbone, the insert and the backbone were purified using the GeneJet Gel Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequent ligation was carried out with Instant Sticky End Ligase Mix (NEB) using 100 ng of the backbone and 23 ng of the insert.

2.1.3. Plasmid propagation, verification and purification

Following the construction of the plasmids, 5 μL of the resulting DNA solution was added to 80 μL of lysogeny broth (LB) medium containing chemically competent *E. coli* NEB 10-β cells. The mixture was incubated for 15 min on ice, before a heat shock (42 °C for 60 s) to facilitate DNA uptake. After incubation on ice for a further 5 min, we added 250 μL LB medium. The cells were allowed to recover at 37 °C for 1 h, while shaking at 1100 rpm in a thermomixer (Eppendorf, Hamburg, Germany). The transformants were plated on selective LB agar containing either 10 μg/mL bleomycin (GG-plasmids) or 100 μg/mL ampicillin (DES® plasmids). After incubation for 1 day at 37 °C, colonies were picked and propagated in 3 mL selective LB medium to enable small-scale plasmid isolation with the NucleoSpin Plasmid EasyPure kit (Macherey–Nagel, Düren, Germany). The resulting plasmid stocks were digested and sequenced by Microsynth Seqlab (Göttingen, Germany) to confirm the correct integration of the gene of interest. Raw material for large-scale plasmid purification was generated by culturing the transformed *E. coli* stains in 200-mL shake flask cultures. Plasmids were recovered by alkaline lysis followed by purification using the NucleoBond® Xtra Midi-Kit (Macherey–Nagel) according to the manufacturer's instructions. The pure DNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in sterile water at a final concentration of 1 μg/μL, as determined by absorption at 260/280 nm using a Cytation 3 spectrophotometer (BioTek, Bad Friedrichshall, Germany).

[1] In the present work, the term *monoclonal cell line* designates a group of identical cells derived from a single parental cell.



	Cell line 1	Cell line 2	Cell line 3	Cell line 4
Expression system	One-plasmid system	One-plasmid System	Two-plasmid system	Two-plasmid system
Origin of plasmids	Golden Gate assembly	Golden Gate assembly	Restriction-Ligation (DES®)	Golden Gate assembly
Cell suspension	3 mL in SF 900II	5 mL in ExCell 420	3 mL in ExCell 420	3 mL in ExCell 420
Grace insect medium	300 µL	500 µL	300 µL	300 µL
Expression plasmid	3 µg	5 µg	2.85 µg	10 µg
Selection plasmid	-	-	0.15 µg	0.2 µg
Transit-IT®-Insect	6 µL	10 µL	6 µL	20 µL
Final products	EGFP (intracellular)	His_6-Thr^C-BR021 (secreted)	BR021-Thr^C-V5/His_6 (secreted)	His_6-Thr^C-Gloverin (secreted) EGFP (intracellular)

Fig. 1. Overview of techniques and corresponding plasmids for the generation of recombinant S2 cell lines (upper panel). Abbreviations: MT: *Drosophila melanogaster* metallothionein promoter, Ac5: *D. melanogaster* actin 5C promoter, Copia: promoter from *D. melanogaster copia* LTR-retrotransposon, BIP: Bip-secretion signal, EGFP: enhanced green fluorescent protein, rbG: rabbit beta-globulin polyadenylation signal, SV40: Simian virus 40 polyadenylation signal, $Thrombin^C$/Thr^C: thrombin cleavage site, His_6: polyhistidine tag, $Hygro^R$: hygromycin B resistance, $Blast^R$: blasticidin S resistance. Overview of corresponding transfection conditions (lower panel).

2.2. Maintenance of D. melanogaster S2 cells

The *D. melanogaster* S2 cells were grown at 27 °C in ExCell 420 (Sigma-Aldrich, Taufkrichen, Germany) or Sf-900 II serum free medium (Thermo Fisher Scientific) containing 8–10 mM L-glutamine (Biochrom, Berlin, Germany). For strain maintenance, the cells were split every 3–4 days to a density of $1.5 \cdot 10^6$ cells/mL. Unless otherwise stated, stable rS2 cells were handled under selection pressure by the addition of 10–25 µg/mL blasticidin S or 300 µg/mL hygromycin B (both supplied by Invivogen, Toulouse, France). Long-term preservation was achieved by freezing $1.5 \cdot 10^7$ cells in 1 mL of a 1:1 mixture of spent and fresh medium with 7.5% dimethyl sulfoxide (DMSO) and subsequent storage in liquid nitrogen. Clones were not weaned off antibiotics before being frozen. However, we omitted antibiotics in the freezing medium and for the first passage after thawing to allow cell recovery. DMSO was removed after thawing by pelleting the cells and resuspending them in DMSO free medium.

2.3. Transfection of S2 cells and construction of polyclonal cell lines

Wild-type S2 cells were transfected using the TransitIT®-Insect reagent according to the manufacturer's specifications (Mirus Bio LCC, Madison, Wisconsin, USA). Briefly, the cells were seeded 18–24 h before transfection at a density of $1.5 \cdot 10^6$ cells/mL. Plasmid DNA was mixed with Grace's Insect medium (Sigma-Aldrich) and pre-warmed TransitIT®-Insect at the ratios indicated in Fig. 1. The resulting transfection mixture was incubated for 30 min at room temperature to allow complex formation, before drop-wise distribution throughout the culture vessel. According to the observed viability, the cells were allowed to recover for 3–7 days without antibiotics. Selection was then started by adding blasticidin S or hygromycin B.

2.4. Single-cell cloning by limiting dilution

Once stable expression of the polyclonal population was verified by His_6-specific western blot or flow cytometry (3–7 weeks after transfection), single-cell cloning was initiated with standard growth medium containing 10–15 transfected cells/mL and $5 \cdot 10^5$ untreated, living S2 feeder cells/mL. For limiting dilution, 100 µL/well was transferred to 10 96-well suspension plates (Eppendorf) in order to seed statistically 1–1.5 cells per well. Undisturbed co-cultivation for 1–3 days allowed the cells to proliferate before the second round of selection commenced by the addition of 30 µL growth medium supplemented with hygromycin B or blasticidin S (final concentration 300 µg/mL or 25 µg/mL, respectively). Clonal colonies formed on the decaying layer of feeder cells over the next few weeks. Colonies > 1 mm in diameter (confirmed by microscopy) were picked by pipetting and transferred to another 96-well plate containing 50 µL fresh medium. Clonal cell lines were subsequently scaled up according to the conditions shown in Table 2, tested for protein expression and finally preserved in liquid nitrogen.

2.5. Analysis of EGFP expression by flow cytometry

EGFP expression was analyzed using an easyCyte HT flow cytometer and the corresponding InCyte software (Merck Millipore, Darmstadt, Germany). During analysis, dead cells were excluded by counterstaining with 5 mg/L propidium iodide (Carl Roth, Karlsruhe, Germany).

Table 2
Scale up during single-cell isolation. Note that recommended volumes are plate-dependent and may be adjusted according to the equipment used. Desiccation was prevented by ensuring a humidified environment and subsequent re-feeding. † The lower ratio between $V_{cells\ and}$ V_{medium} was chosen to ensure proper surface oxygenation.

Culture vessel	Culture method	$V_{cells\ from\ previous\ step}$ [μL]	V_{medium} [μL]	V_{total} [μL]
96-well plate	static	100	50†	150
48-well plate	static	150	150	300
24-well plate	static	300	300	600
12-well plate	static	600	600	1200
6-well plate	static	1200	1200	2400
T25 flask	dynamic	2400	600–2600	3000–5000

2.6. Analysis of AMP expression by dot blot screening and western blot analysis

The expression of GmGlv and BR021 was analyzed after 3 days of cultivation at the 24-well scale. Supernatants from clonal derivatives of cell line 2 were used directly, because protein expression was driven by the constitutive *Ac5* promoter. Protein expression in clones originating from cell lines 3 and 4 was driven by the metallothionein promoter, which had to be induced with $CuSO_4$. Therefore, 75 μL of the cell suspension was mixed with 75 μL fresh medium to yield a final concentration of 600 μM $CuSO_4$. Induced cells were cultivated for 3 days in a 96-well plate, before the supernatants were collected. Before harvest, the optical density at 600 nm (OD_{600}) was determined as a measure of the current cell density using a Cytation 3 spectrophotometer (Biotek). Protein expression was detected by vacuum-assisted dot blots specific for the His_6 tag using a VWR dot blot device (Darmstadt, Germany). We spotted 50 μL of the collected supernatants onto an Amersham Protan 0.2 μm nitrocellulose membrane (GE Healthcare, Freiburg, Germany) allowing a binding phase of 30 min. Membranes were blocked with phosphate-buffered saline (PBS) containing 5% bovine serum albumin (BSA), stained overnight with a His_5-HRP antibody conjugate (Qiagen, Hilden, Germany) diluted 1:5000 in PBS containing 0.05% Tween-20, and washed three times with PBS containing 0.1% Tween-20. The target was detected by enhanced chemiluminescence using the ChemiDoc™ system and Clarity Western ECL substrate (Bio-Rad, Munich, Germany). For cell line screening, we calculated the quotient of the dot blot intensity and OD_{600}. Supernatants from promising clones were also analyzed by western blot to confirm the correct expression of the target protein. Therefore, proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on CriterionXT 4–20% polyacrylamide gradient gels and detected using stain-free technology (Bio-Rad). Proteins were then transferred to polyvinylidene difluoride membranes (PVDF Trans-Blot® Turbo™ Biorad, 7 min at 25 V and 2.5 A, Trans-Blot® Turbo™) and detected according to the dot blot staining protocol. Finally, the relative cell-specific productivity, defined as western blot intensity/OD_{600}, was calculated to compare selected clones and the parental cell line.

2.7. Analysis of genomic DNA by Southern blot hybridization

In order to identify the plasmid integration pattern of one EGFP-expressing clone, we used the North2South™ Biotin Random Prime Labeling Kit and the North2South™ Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific). Based on the EGFP expression plasmid and a specific set of primers (Supplementary Material 1), we used a Q5 polymerase (NEB) for PCR to construct a biotinylated Southern blot probe specific for the EGFP sequence. Genomic DNA from rS2 cells was isolated using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. We digested 9 μg of purified DNA overnight at 37 °C using 100 U of EcoRI-HF in CutSmart buffer (NEB) in a total reaction volume of 400 μL. The digest was precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol for 2 h at 80 °C. The DNA was then pelleted by centrifugation for 20 min at 16,100 *g* and 4 °C. The resulting pellet was air dried and resuspended in 15 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The mixture was resolved by 0.8% agarose gel electrophoresis at 35 V for 5.5 h. In order to fragment large DNA molecules before blotting, the DNA in the gel was depurinated for 10 min in 0.25 M HCl. For neutralization, the gel was then incubated for 30 min in neutralization buffer (0.5 M Tris–HCl pH 7.0, 1.5 M NaCl). Single-stranded DNA was produced by two denaturing steps (each 15 min, 1.5 M NaCl, 0.5 M NaOH). For the capillary blot, the blotting reservoirs were filled with transfer buffer (20x SSC: 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and the gel was placed together with blotting paper below a Biodyne B Nylon membrane (Thermo Fisher Scientific). DNA was allowed to migrate overnight, before the membrane was collected, washed in 5x SCC buffer and DNA was crosslinked to the membrane by 15 min exposure to UV light. After a final drying step for 3 h, we detected DNA fragments containing the EGFP sequence by staining with the biotinylated Southern blot probe according to the North2South Chemiluminescent Hybridization and Detection Kit (Thermo Fisher Scientific).

2.8. Bioreactor-scale production

The best clones were cultivated at the 1-L bioreactor scale (Labfors 5, Infors HT, Bottmingen, Switzerland) as previously described [8]. For batch cultivation, $1.5 \cdot 10^6$ cells/mL were seeded and cultivated without antibiotics at 27 °C, pH 6.4 and dO_{2set} 40%. If necessary, cells were induced at the mid-exponential growth phase using 600 μM $CuSO_4$. Harvest commenced at the onset of the stationary phase and was timed using a capacitive biomass sensor (Incyte Arc View system, Hamilton Bonaduz, Switzerland). The target products were quantified by SDS-PAGE against purified protein standards.

3. Results

3.1. Setup of the cloning protocol

In the course of establishing the transfection and cloning protocol, we generated dose-response curves for the selection antibiotics (Supplementary Material 2), which revealed different kinetics of feeder cell death for each antibiotic. Blasticidin S builds up a higher selection pressure compared to hygromycin B, achieving the faster removal of untransfected feeder cells. However, despite the differences in selection speed, both antibiotics induced the growth of clonal colonies and led to growth arrest and decay of the wild-type feeder cells. Microscopically, the first colonies were visible within the first 2 weeks of selection. After 3–4 weeks, well growing colonies had a diameter of about 1 mm, yielding enough cells for further scale up (Fig. 2).

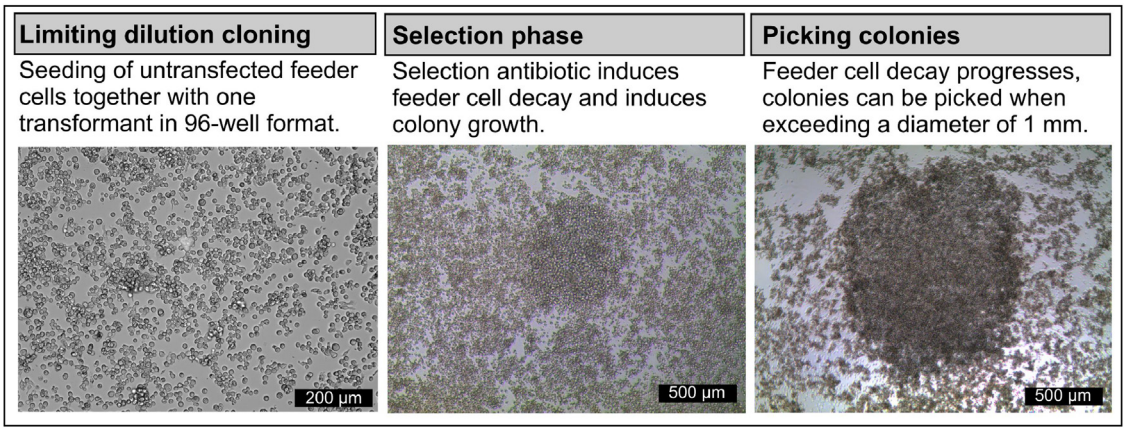


Fig. 2. The process of single-cell colony growth, illustrated with representative microscopy pictures for the different process stages.

3.2. Intracellular EGFP expression in monoclonal rS2 cells

The generation of polyclonal cell line 1 resulted in clearly detectable but very heterogeneous EGFP expression as determined by flow cytometry (Fig. 3a, small panel). The shape of the corresponding histogram can be interpreted as the superimposition of multiple expression patterns resulting from different subpopulations with varying copy numbers or integration loci. Consequently, subsequent single-cell cloning led to the isolation of 19 clones with considerably varying EGFP expression and the low, moderate and high producers were clearly distinguishable (Fig. 3b). A detailed analysis of the related expression profiles revealed that the clones showed more homogeneous fluorescence and a sharper fluorescence distribution than the original parental culture (Fig. 3a, main panel), indicating the successful segregation of the different genotypes. The fluorescence signal in the high producers was up to tenfold higher than the polyclonal population. To verify stable EGFP expression in the single-cell clones even without the sustained addition of hygromycin B, one of the monoclonal lines was cultured in the presence and in the absence of antibiotics for 3 weeks. Under selection pressure, almost all of the cells remained EGFP-positive, but even if the antibiotic was omitted, the proportion of EGFP-positive cells remained well above 95%, indicating stable integration (Supplementary Material S3).

Because the integration of long tandem arrays of the donor transgene is a known reason for high-level expression in *Drosophila* S2 cells [17], we analyzed the highly productive cell line 19 (Fig. 3b) by Southern blot hybridization. To gain more insight into the nature of transgene integration, isolated cellular DNA was digested with EcoRI, a single cutter in the expression cassette, allowing the characterization of the integration pattern (Fig. 4a and b). The prominent band at 8.4 kb revealed that multiple copies of the expression plasmid were integrated in a head-to tail fashion (Fig. 4c).

3.3. Expression of antimicrobial peptides in monoclonal rS2 cells

Following the general setup of the cloning procedure, we analyzed its impact on the co-expression of EGFP and GmGlv (cell line 4). For the polyclonal population, even 70 days of permanent selection was not sufficient to achieve a homogeneous EGFP expression profile and nonproductive cells were still present. In contrast, monoclonal lines isolated within the same time interval showed a unimodal EGFP distribution (Fig. 5). However, although they were EGFP positive, the polyclonal and most of the monoclonal lines produced almost undetectable levels of GmGlv (Fig. 6c). This may reflect the silencing of the GmGlv expression cassette or that integration of the selection plasmid is favored over integration of the expression plasmid. Because of the missing correlation between EGFP and GmGlv, screening for the target peptide by dot blot was necessary. After cloning and dot blot screening, a highly productive clone was identified, which, in contrast to the parental polyclonal population, was suitable for further scale up (Table 3).

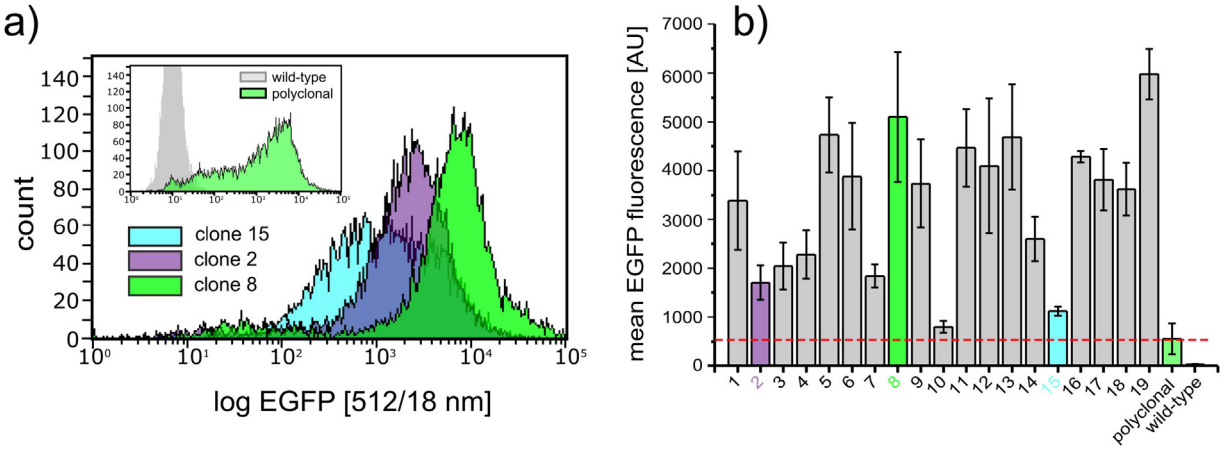


Fig. 3. Analysis of EGFP-expressing cell lines by flow cytometry. **(a)** EGFP expression profile of wild-type and polyclonal S2 cells (small panel) and three representative monoclonal cell lines representing low, moderate and high producers (indicated by different colors). **(b)** Comparison of EGFP expression (mean ± SD) in 19 different monoclonal cell lines and the parental polyclonal culture.

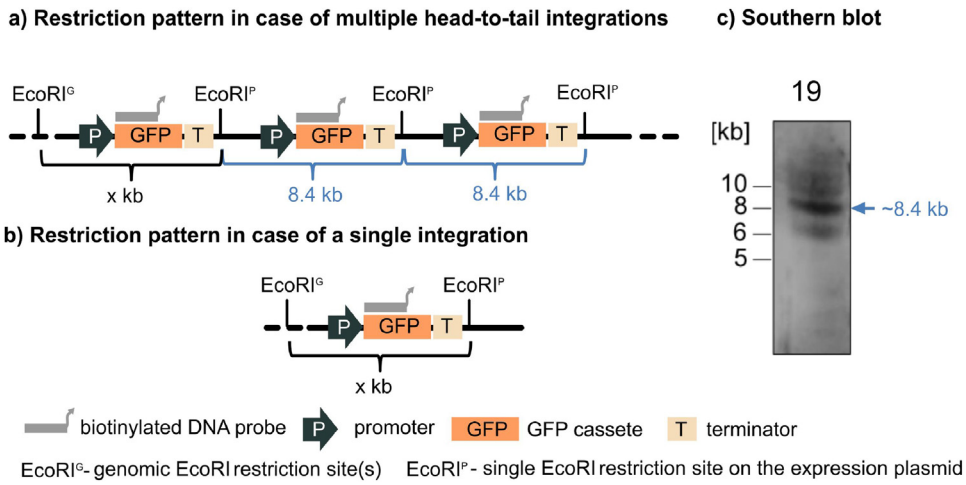


Fig. 4. Southern blot analysis of the EGFP-expressing monoclonal cell line 19. Digestion with the single-cutter EcoRI should yield **(a)** 8.4-kb fragments if there are tandem head-to-tail repeats of the integrated transgene ($EcoRI^P$) but **(b)** a single fragment whose size depends on the position of the next genomic EcoRI site ($EcoRI^G$) if there is a single-copy transgene. **(c)** The presence of an 8.4-kb band in the Southern blot confirms the integration of multiple cassettes in tandem.

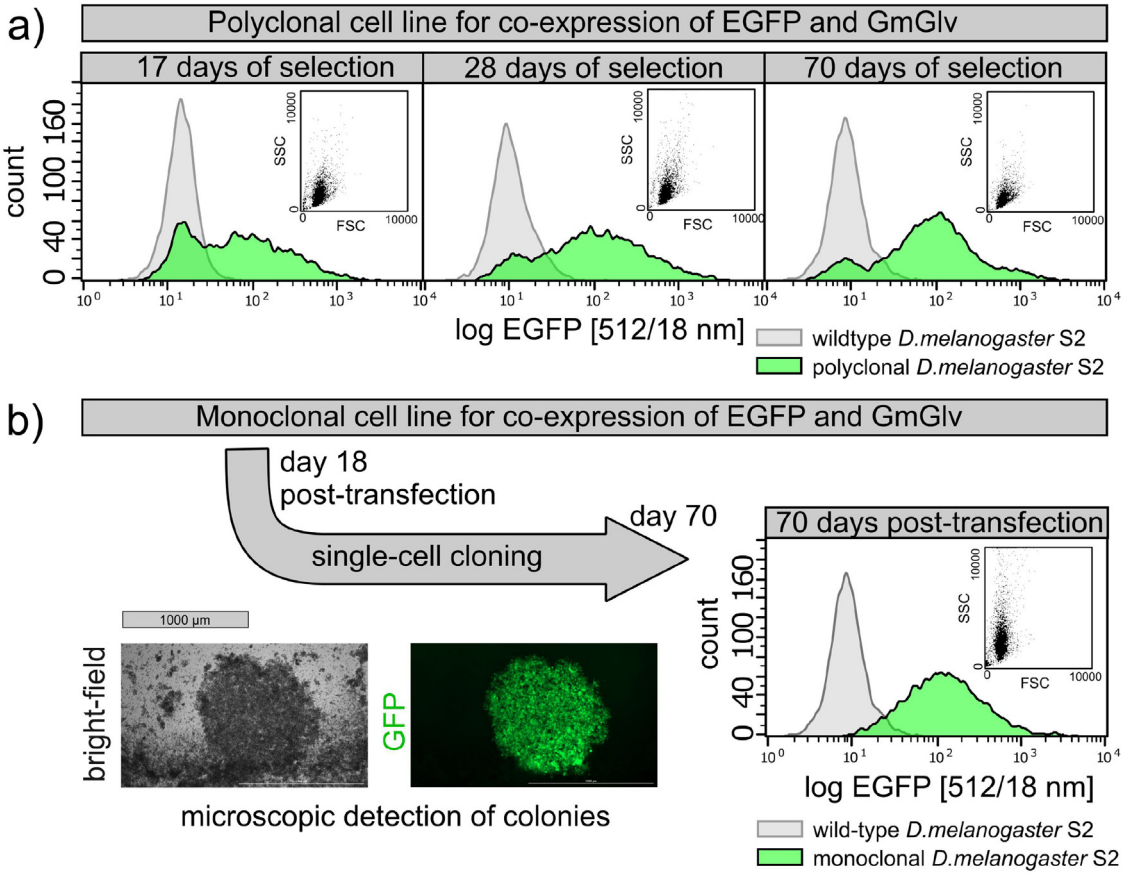


Fig. 5. Co-expression of EGFP and the antimicrobial protein GmGlv. **(a)** The establishment of a polyclonal population and subsequent strain maintenance under selection pressure did not lead to a uniform expression pattern. **(b)** Single-cell clones can easily be distinguished from the decaying feeder cells due to the intracellular EGFP fluorescence. Isolated monoclonal cell lines show a uniform EGFP expression pattern.

To further demonstrate the benefit of single-cell cloning, two additional monoclonal cell lines expressing another peptide (BR021) were generated (cell lines 2 and 3). Because cell line 4 showed that the expression level of the fluorescent reporter protein did not predict the expression level of the target peptide, no reporter was used in those two cell lines. Compared to the GmGlv-producing cell line the number of high producers was significantly higher and 10–20% of the picked clones proved suitable for scale up (Table 3). In comparison with the corresponding polyclonal lines, the best clones showed a sixfold to sevenfold increase in cell-specific productivity, as assessed by western blot analysis (Fig. 6, right panel). During subsequent production at the bioreactor scale, we recovered 17–26 mg/L of the corresponding peptide. The supernatants were used as basis for BR021/GmGlv purification and an activity assay (Supplementary Material 4).

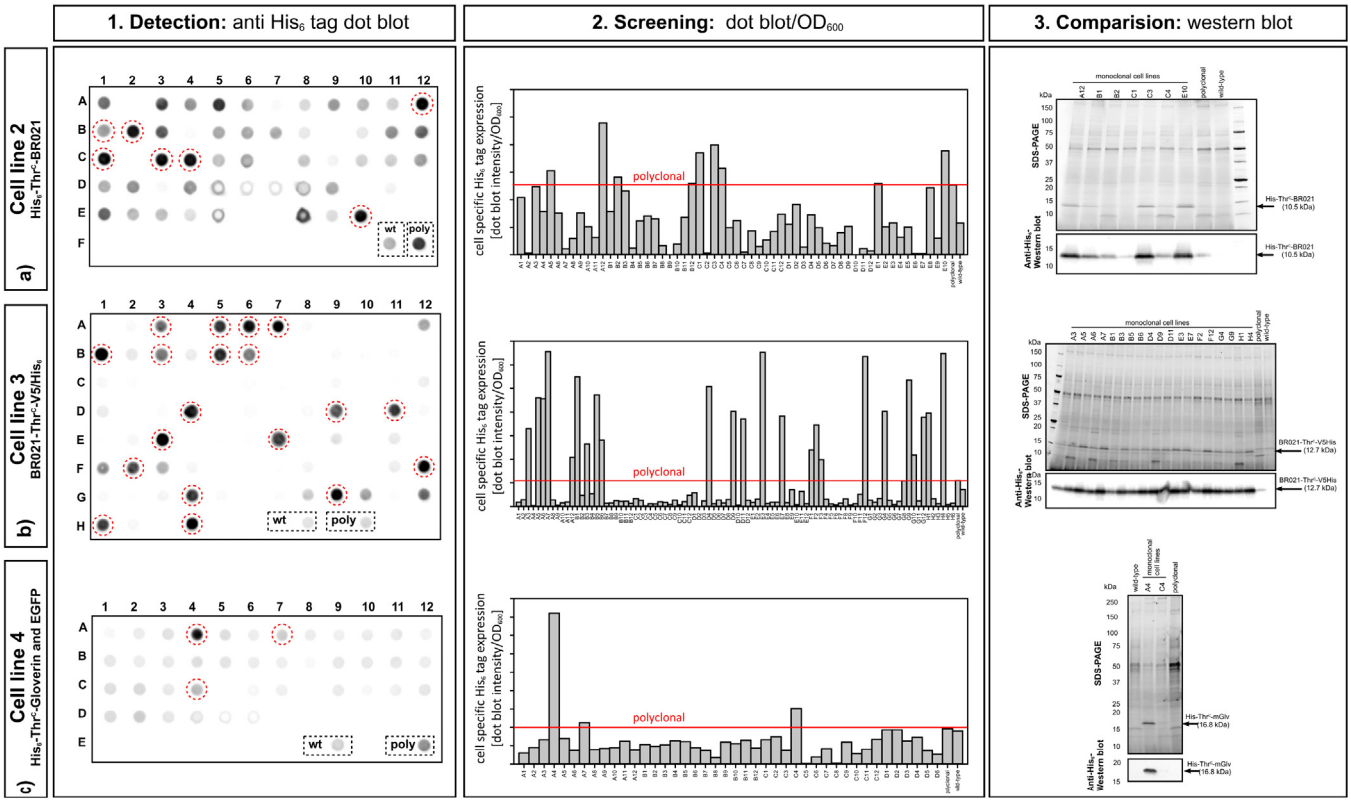


Fig. 6. Expression analysis during the preparation of monoclonal cell lines for the production of **(a)** His_6-Thr^C-BR021, **(b)** BR021-$Thrc^C$-V5/His_6 and **(c)** His-Thr^C-Gloverin. Dot blot screening of selected clones (analyzed after reaching the 24-well scale) together with a polyclonal (poly) and a wild-type (wt) control (left and middle panel). Western blot analysis to compare the identified high producers with the polyclonal population (right panel).

Table 3
Summary of the single-cell cloning experiments for cell lines 2–4.

Cell line	Expressed protein	Picked monoclonal cell lines	Putative high producers (dot blot)	Verified high producers (western blot)	Max. protein titer during batch culture	Growth inhibition of *E. coli* at 10 μM
2	His_6-Thr^C-BR021	58	9 (15.5%)	6 (10.3%)	19 mg/L	Not tested
3	BR021-Thr^C-V5/His_6	90	24 (26%)	19 (21%)	17 mg/L	Yes
4	His_6-Thr^C-Gloverin	42	3 (7.1%)	1 (2.4%)	26 mg/L	Yes

4. Discussion

4.1. General considerations on the heterogeneity of the S2 cell line

The need for single-cell cloning can be justified by considering the origin of the S2 cell line and the nature of transgene insertion into the host cell genome. The wild-type S2 line is heterogeneous because it was established from 100 to 300 late-stage *D. melanogaster* embryos before hatching [33]. According to the German Collection of Microorganisms and Cell Cultures (DSMZ, ACC 130), S2 cells were originally diploid with 5–10% having an XY karyotype, but become now 60–80% tetraploid and exclusively XX. This diversity is also reflected in the fact that three different isolates of the original S2 line showed vastly differing behavior in transcriptomic studies, demonstrating the substantial divergence caused by long-term subculture in different laboratories [15,34].

Plasmid-based transformation also introduces heterogeneity because it results in the integration of multiple copies of the transgene at a random genomic location [17] (see also Appendix A). High-copy-number transgenic loci at transcriptionally active sites are generally beneficial for maximizing protein expression [7]. However, this causes an additional metabolic burden, which may inhibit cell growth and proliferation. Consequently, the long-term cultivation of polyclonal populations can reduce protein yields because highly-productive cells become over-populated by faster-growing but less productive neighbor cells [2,35]. Especially in continuous bioprocesses, where homogeneous cell populations are needed to maintain a stable and controllable operation [36], single-cell cloning is necessary to reduce the adverse effects of polyclonality. However, it should be stressed that a complete genetic homogeneity cannot be achieved even with clonal cell lines. This is because of probable recombination events within the multiple head-to-tail array [15]. Furthermore, it is also conceivable that the long, repeated sequences can form heterochromatic chromatin structures, which interfere with protein expression [15]. Despite the lack of complete homogeneity, clonal cell lines still provide a better starting point for process development as they were derived from a single cell, with certain desirable properties in terms of growth and protein expression.

4.2. Single-cell cloning overcomes cell line heterogeneity and improves product yield

Consistent with the statements above, our polyclonal cell lines showed variable cell-specific expression levels. This is in agreement with previous reports concerning other polyclonal rS2 cells expressing EGFP [37] as well as polyclonal Sf9 cells expressing GFP-tagged virus-like particles [38]. To overcome the limitations of polyclonality, we isolated monoclonal lines by limiting dilution, which not only unified the expression profile but also achieved a multifold increase in cell-specific productivity. High-level expression in *D. melanogaster* and *Aedes* spp. cell lines is usually associated with the integration of multi-copy head-to-tail transgene arrays [17,18,39,40]. We also observed such arrays in our monoclonal line producing high levels of EGFP, increasing the fluorescence signal by tenfold compared to the polyclonal line. The sixfold to sevenfold increase in BR021 levels we observed, agreed with an earlier study in which a comparable limiting dilution protocol achieved a fivefold increase in productivity for the expression of an antibody [10]. In our GmGlv-producing cell line, an even greater increase in productivity was observed; indeed, protein production was only possible using the monoclonal cell line, whereas polyclonality resulted in negligible expression levels and no product recovery. The overall proportion of highly productive clones varied between 2.4% and 21% in our assay, which is comparable to the 7% reported for a protocol based on mitomycin C [20]. All of our clones that were scaled up to production level were exceptionally stable in terms of their expression profile, even in the absence of selection pressure (e.g. during the 5–7 days of expression at the bioreactor scale). This behavior can be attributed to the inherent homogeneity of the monoclonal population. However, as reported earlier, not all clones with high productivity perform well during scale up and it is important to screen for the best combination of growth properties, robustness and productivity [10]. The AMP titers of 17–26 mg/L we achieved in our batch culture are competitive with those from other expression systems used for the production of insect derived AMPs, which typically generate titers of 0.5–68 mg/L [41,42]. Further process intensification should be possible by changing from batch to continuous perfusion culture, which has already enabled the production of hundreds to thousands of milligrams of target protein using comparable rS2 cells [10,43].

Our study strongly supports arguments in favor of single-cell cloning for insect cell lines and confirms that the proposed limiting dilution approach with untransfected S2 feeder cells can be used as a routine method. Highly productive monoclonal cell lines were successfully prepared regardless of whether one or two plasmids were used in the initial transfection mixture. The likelihood of isolating superior clones is usually low, so an appropriate number of clones must be screened. Future automation of the process will therefore be advantageous because this allows a considerable increase of the number of clones that can be examined.

4.3. Limiting dilution compared to other techniques for population enrichment

Although limiting dilution is broadly applicable, the limitations of the method include the time consuming work and the statistical nature of cell separation, which requires additional microscopic validation of monoclonality after seeding [44]. Therefore, other groups have evaluated less cumbersome methods to obtain homogeneous and highly-productive rS2 cells. The simplest method involves the repeated treatment of a polyclonal population with high concentrations of the selection antibiotic in order to enrich for a high-producer subpopulation [6]. Cell lines expressing surface proteins can also be isolated using immuno-magnetic selection [5,45,46]. However, in both cases, the corresponding subpopulations remain polyclonal and are therefore still genetically inhomogeneous, possibly reducing the stability of protein expression over time due to differences in growth kinetics. Fluorescence activated cell sorting (FACS) is an alternative method for the controlled separation of single cells based on expression-related fluorescence intensity. FACS can be used for the simple enrichment of productive subpopulations or even for the verified isolation of single cells, and has already been successfully applied to Sf9 and rS2 cells [46,47]. After separation by FACS, single rS2 cells are expanded using essentially the same protocol as for limiting dilution [48]. FACS is useful, especially in the context of cell line generation by targeted gene insertion using recombinase mediated cassette exchange (RMCE), because here the fluorescent tagging cassette is later exchanged for a targeting cassette and consequently there is a direct relationship between the preliminary fluorescence signal and the final product expression level [38,48,49]. However, unless the reporter is exchanged by RMCE or directly fused to the target protein, the use of fluorescence for selection is problematic. A general drawback of antibiotic or fluorescence-driven enrichment is that it does not necessarily address the expression of the actual target protein, as it only selects for cells with high antibiotic resistance or reporter fluorescence. Furthermore, the reporter usually does not resemble the target protein in terms of size, stability and other physicochemical properties. In this context, cell line 4 showed that the expression level of the target peptide did not match that of the co-expressed EGFP, and only limiting dilution combined with direct screening by dot blot allowed the identification of a high producer.

In conclusion, limiting dilution is a universally applicable and cost-efficient method for the generation of monoclonal rS2 cell lines. Using the protocol described herein, limiting dilution does not require expensive equipment (unlike FACS), involves no radiation, gives reproducible results and is easy to automate. As a future perspective, the combination of limiting dilution with upstream enrichment techniques may achieve an increase in the proportion of highly productive clones. Furthermore, the main aspects of our protocol are also compatible with microfluidic single-cell printing, which offers a more controlled single-cell isolation and ensures the presence of single cells in each cavity of the microtiter plate [44].

5. Author contributions

Jan Zitzmann conceived, designed and performed all experiments with cell lines 2, 3 and 4. He wrote the manuscript and coordinated the creation of the paper. Christine Schreiber designed and performed all steps for the generation of cell line 1 and the corresponding experiments. Additionally, she prepared the plasmid set for cell line 3 and revised the manuscript. Joel Eichmann helped to set up the Golden Gate assembly and revised the manuscript. Roberto Otmar Bilz performed the purification and activity assay. Tobias Weidner and Denise Salzig helped to draft and revise the manuscript. Peter Czermak helped to draft and revise the manuscript, and supervised the research. All authors have given their approval for this final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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Appendix A.

Linearizing the expression plasmids prior to transfection. An additional tool to improve homogeneity?

Transfection is usually performed with non-linearized plasmids. However, during cell line establishment the transfected circular plasmid is randomly linearized within the cell, leading to heterogeneity and possibly destroying important elements such as the resistance gene or the gene of interest [50]. Linearization of the plasmid prior to transfection by a restriction enzyme with a single recognition site in a non-coding region preserves the integrity of all sensitive parts and may be therefore beneficial. Although linear DNA provides a well-defined and putatively superior starting point for cell line generation, there are some issues that need to be addressed when using this method. As an example, the Lipofectamine- and PEI-mediated transfection of HeLa-cells with linearized plasmid-DNA led to decreased protein yields and enhanced cytotoxicity compared to standard circular plasmids [51]. The same was observed for the liposome-mediated transfection of Vero cells [52]. Both studies indicate changes in the morphology of the transfection complex as a reason for the deterioration in transfection efficiency. While circular DNA led to compacted and roughly spherical shaped complexes, linear DNA results in a worm like and apparently cytotoxic structure [51,52]. This cytotoxicity has to be prevented by augmenting the transfection mixture with cationic amphiphiles [52]. Despite these issues, linearization of the plasmids can be superior, as demonstrated for the transfection of mammalian neuronal cell lines, where it yielded a three-fold increase in the number of stable colonies [50]. However, the efficiency of stable clone generation and gene expression was highly dependent on the restriction site selected for linearization [50].

For *D. melanogaster* S2 cells and their associated transfection reagents (e.g. TransitIT®-Insect), no comprehensive investigation on the influence of plasmid linearization is available yet. As a future perspective the regarding interdependencies should be examined in a structured way, ideally through statistically designed experiments.

Appendix B. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2018.e00272.

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