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Supplementary materials

Proteostasis Engineering of Glutamine Synthetase for Stringent, Drug-Free Selection in Chinese Hamster Ovary Cells

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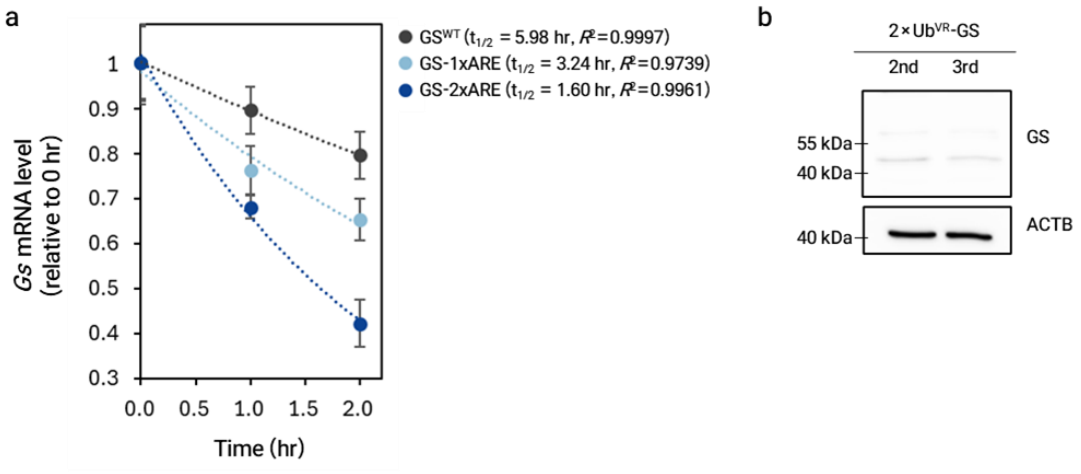
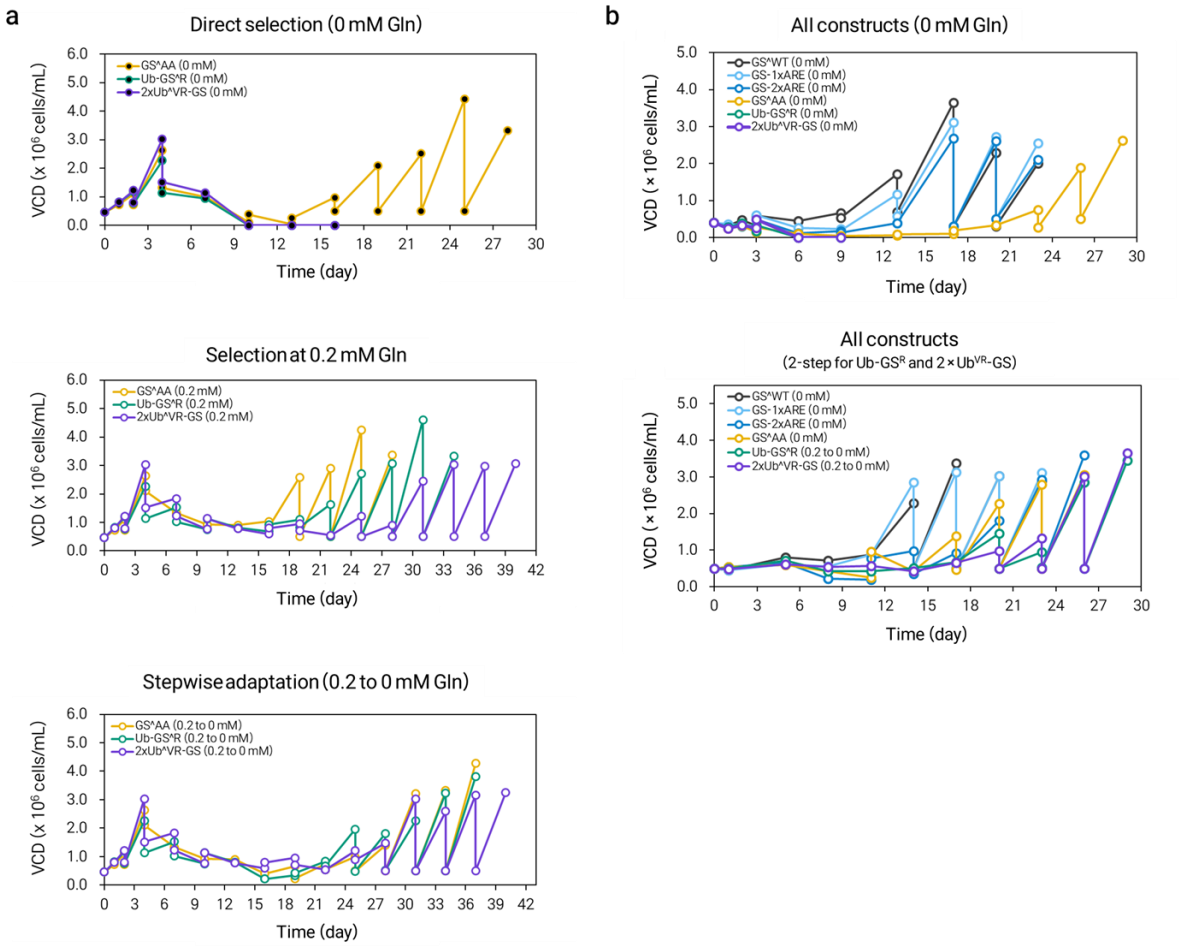
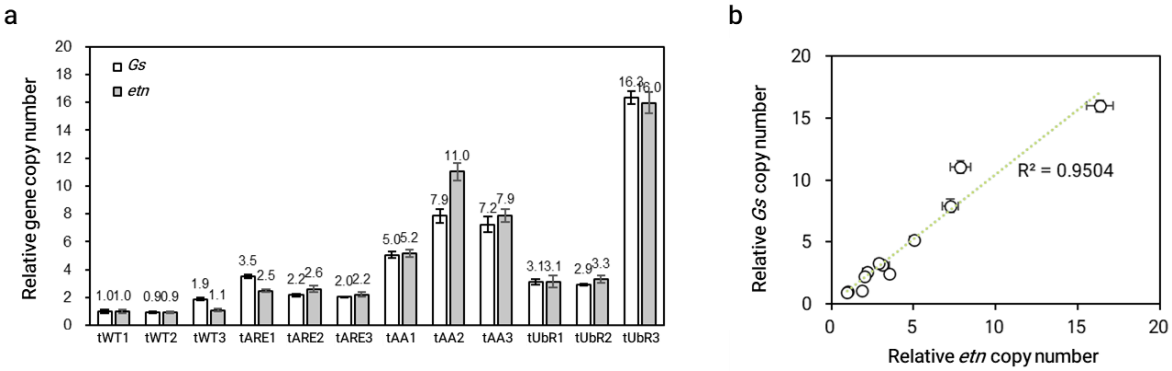


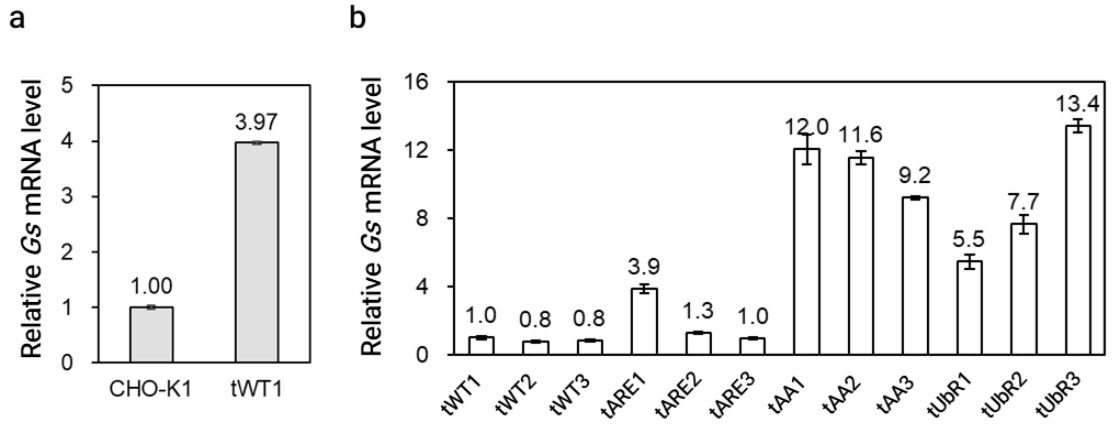
Supplementary Figure S1. Functional validation of control vectors and 2×Ub^{VR}-GS expression (a) mRNA half-life analysis of GS-ARE constructs. Stable pools of GS^{WT}, GS-1×ARE, and GS-2×ARE were treated with 5 μg/mL ActD. *Gs* mRNA levels were quantified by RT-qPCR at 0, 1.0, and 2.0 hours post-treatment and normalized to the 0-hour time point. Data are presented as mean ± standard deviation (n = 3). Calculated half-lives were 5.98 h (GS^{WT}), 3.24 h (GS-1×ARE), and 1.60 h (GS-2×ARE). (b) Analysis of 2×Ub^{VR}-GS protein expression. Two additional independently generated stable mini-pools (labeled 2nd and 3rd on the blot) were harvested without CHX treatment and analyzed by western blotting. No distinct band was detected at the predicted full-length size (~60 kDa). β-Actin was used as a loading control. GS, glutamine synthetase; ACTB, β-Actin; ARE, AU-rich element; ActD, actinomycin D; RT-qPCR, real-time quantitative polymerase chain reaction; CHX, cycloheximide.



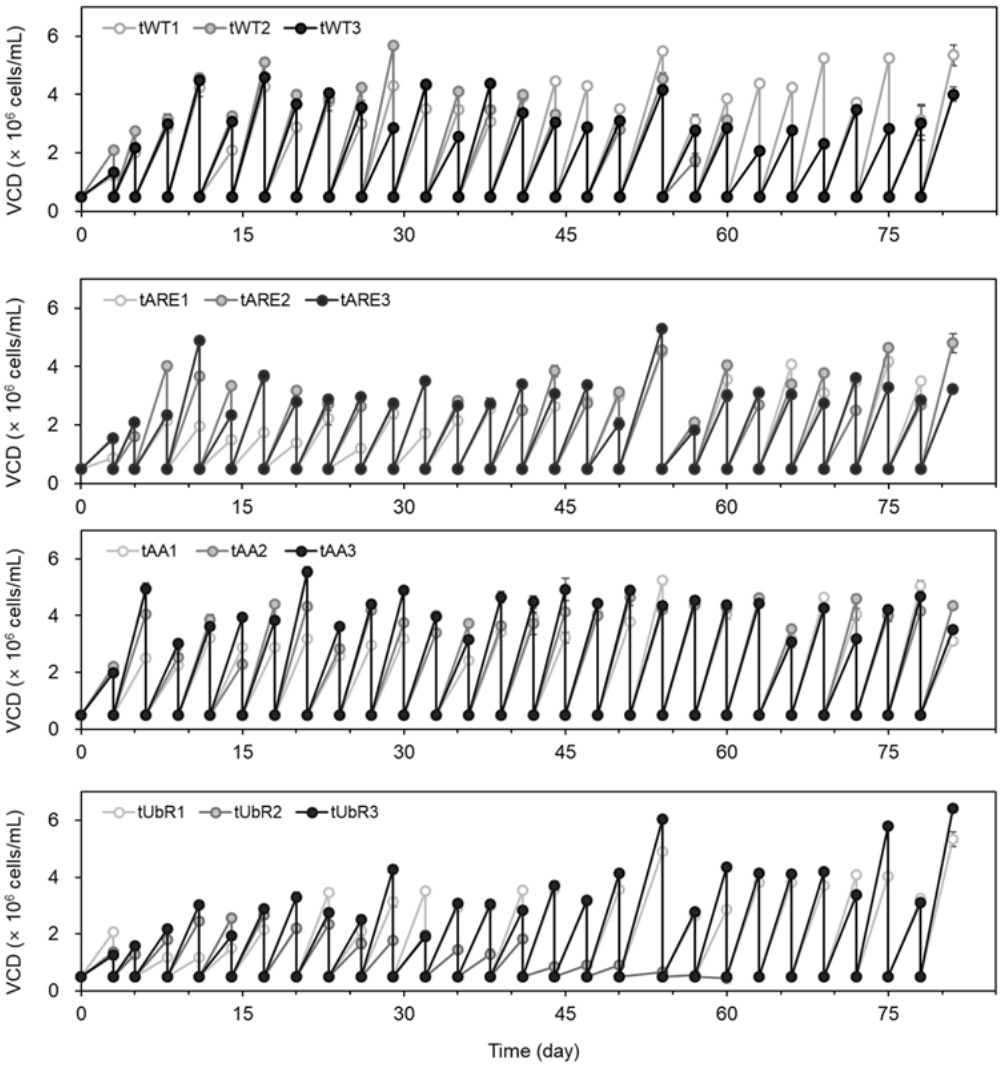
Supplementary Figure S2. Pool establishment outcomes under different glutamine selection schemes (a) VCD profiles during bulk pool establishment for GS^{AA}, Ub-GS^{R}, and 2×Ub^{VR}-GS under three selection regimens: direct glutamine-free selection (0 mM Gln), selection initiated at 0.2 mM Gln, and stepwise adaptation from 0.2 mM to 0 mM Gln. GS^{AA} established expandable pools under direct 0 mM selection, whereas Ub-GS^{R} and 2×Ub^{VR}-GS failed to recover to an expanding state. Initiating selection at 0.2 mM Gln enabled pool establishment for Ub-GS^{R} and 2×Ub^{VR}-GS, and established pools could subsequently be transitioned to 0 mM Gln. (b) Comparison across all constructs. Top: direct glutamine-free selection (0 mM Gln), under which Ub-GS^{R} and 2×Ub^{VR}-GS did not establish viable pools. Bottom: selection initiated at 0.2 mM Gln; Ub-GS^{R} and 2×Ub^{VR}-GS pools were subsequently transitioned to 0 mM Gln (2-step), enabling expansion alongside GS^{WT}, ARE-based, and GS^{AA} constructs. GS, glutamine synthetase; VCD, viable cell density; Gln, glutamine; ARE, AU-rich element.



Supplementary Figure S3. Stable co-integration of *Gs* and *etn* expression cassettes Relative gene copy number of the *Gs* marker and the *etn* gene of interest were quantified by qPCR for the 12 top-producing mini-pools from Table 1. (a) Bar chart comparing the relative gene copy number of *Gs* (white bars) and *etn* (grey bars) for each clone. Data are normalized to tWT1 (*Gs* = 1.0, *etn* = 1.0). Error bars represent the standard deviation of technical replicates (n = 3). (b) Correlation analysis between relative *Gs* gene copy number and relative *etn* gene copy number. The strong linear correlation (R^2 = 0.9504) validates the stable co-integration (and/or co-amplification) of the two expression cassettes across all selected clones. GS, glutamine synthetase; ETN, Etanercept; qPCR, quantitative polymerase chain reaction.



Supplementary Figure S4. Transcriptional landscape of *Gs* mRNA across selection systems (a) Comparison of *Gs* mRNA levels between the host CHO-K1 cell line and the stable GS^{WT} mini-pool (tWT1). Transcript levels were quantified by RT-qPCR and normalized to CHO-K1 (set as 1). (b) Compensatory upregulation of *Gs* mRNA in dGS-selected mini-pools. Relative *Gs* mRNA levels in the top three stable mini-pools from each selection system were normalized to the tWT1 pool (set as 1). Data represent mean ± standard deviation (n = 3). GS, glutamine synthetase; RT-qPCR, real-time quantitative polymerase chain reaction.



Supplementary Figure S5. Long-term culture stability of top three producers from GS variants Growth profiles during continuous maintenance culture without selective agents. The top-producing mini-pools from each group were subcultured every 3 or 4 days to monitor growth stability over time. GS, glutamine synthetase; VCD, viable cell density.

Supplementary Table S1. Summary of glutamine synthetase variants and engineering strategies

Construct name	Engineering strategy	Mechanism of action	References
GS^{WT}	None (Control)	Expression of unmodified GS from *Cricetulus griseus*	Park et al., 2016
GS-1×ARE	mRNA destabilization	Insertion of a single ARE (TTATTTATTTATTATTTATTTATT) in the 3′ UTR to promote mRNA decay	Koh et al., 2019
GS-2×ARE	mRNA destabilization	Insertion of tandem AREs in the 3′ UTR to enhance mRNA degradation efficiency beyond the single-ARE construct	
GS^{AA}	CRBN-dependent degradation	Mutation of N-terminal lysine residues (K11A, K14A) to mimic the acetylated state recognized by Cereblon E3 ligase complex	Van Nguyen et al., 2016
Ub-GS^{R}	N-end rule pathway	Fusion of a cleavable ubiquitin moiety to GS, exposing an N-terminal arginine after cleavage to trigger N-end rule degradation	Chassin et al., 2019
2×Ub^{VR}-GS	Proteasomal degradation	Fusion of tandem non-cleavable ubiquitin moieties (G76VR mutation) to the N-terminus	

All constructs are driven by the SV40 promoter. GS, glutamine synthetase; ARE, AU-rich element; UTR, untranslated region; CRBN, cereblon.

Supplementary Table S2. Primer list used in this study

Primer name	Description	Sequence (5′–3′)
Ub_F	Ubiquitin extraction from CHO-K1 (forward)	tccattttcgccaccatgcagatcttcctgaaaacc
UbM1R_R	Ubiquitin extraction from CHO-K1 and GS M1R mutation (reverse)	tgctgaggtggccatcccaaccctgaggc
UbVR_R	Ubiquitin extraction from CHO-K1 and G76VR mutation (reverse)	tgctgaggtggccatccgaacaaccctgaggcg
GS_qF	Glutamine synthetase qPCR primer for copy number and mRNA level quantification (forward)	ctgtgagcccaagtgtgtag
GS_qR	Glutamine synthetase qPCR primer for copy number and mRNA level quantification (reverse)	tgctcaccatgtccattatcc
ETN_qF	Etanercept qPCR primer for copy number and mRNA level quantification (forward)	gagcagtacaacagcacgta
ETN_qR	Etanercept qPCR primer for copy number and mRNA level quantification (reverse)	gacctggttcttggtcatctc
Actb_qF	*beta-actin* qPCR primer for copy number standardization (forward)	gatctggctttccggctatt
Actb_qR	*beta-actin* qPCR primer for copy number standardization (reverse)	catggaggctcggtttatgt
Akr1a1_qF	*Akr1a1* qPCR primer for mRNA level standardization (forward)	aagcatcactccttctcgtatc
Akr1a1_qR	*Akr1a1* qPCR primer for mRNA level standardization (reverse)	ctcttcccatccaccgtaatc

Ub, ubiquitin; GS, glutamine synthetase; ETN, Etanercept; Actb, *beta-actin*; Akr1a1, *aldo-keto reductase family 1 member A1*; qPCR, quantitative polymerase chain reaction.

Supplementary Table S3. Comprehensive growth and productivity data of all stable mini-pools evaluated in batch culture

Mini pool				Growth				Productivity			
				Day 0-3	Day 6	Day 9	Day 12	Day 9		Day 12	
Selection		No.	Name	Growth rate (day^{-1})	PVCD ($\times 10^6$ cells/mL)	IVCD ($\times 10^6$ cells*day/mL)		Titer (mg/L)	Q_P (pg/cell/day)	Titer (mg/L)	Q_P (pg/cell/day)
GS^{WT}	Priority	1	WH1	0.67	8.37	37.13	N/A	70	1.89	N/A	N/A
		2	WH2	0.61	5.63	32.18	N/A	3	0.09	N/A	N/A
		3	WH3	0.67	8.51	37.41	N/A	33	0.88	N/A	N/A
		4	WH4 (= tWT3)	0.48	6.11	30.68	N/A	72	2.35	N/A	N/A
		5	WH5	0.61	10.07	40.38	N/A	5	0.12	N/A	N/A
		6	WH6 (= tWT1)	0.69	9.20	47.03	N/A	81	1.72	N/A	N/A
	Random	1	WR1	0.64	8.25	43.31	N/A	5	0.12	N/A	N/A
		2	WR2	0.59	9.29	37.41	N/A	4	0.11	N/A	N/A
		3	WR3	0.18	2.70	15.14	22.7	9	0.59	14	0.62
		4	WR4 (= tWT2)	0.52	6.98	36.17	N/A	81	2.24	N/A	N/A
		5	WR5	0.55	10.92	41.39	N/A	3	0.07	N/A	N/A
		6	WR6	0.54	9.38	36.35	N/A	4	0.11	N/A	N/A
		7	WR7	0.47	5.70	27.71	N/A	24	0.87	N/A	N/A
		8	WR8	0.57	5.84	34.64	N/A	3	0.09	N/A	N/A
		9	WR9	0.47	2.44	15.90	N/A	3	0.19	N/A	N/A
		10	WR10	0.57	10.02	39.11	N/A	25	0.64	N/A	N/A
GS-2×ARE	Priority	1	2AUH1 (= tARE1)	0.37	2.00	13.80	N/A	212	15.37	N/A	N/A
		2	2AUH2	0.72	11.73	49.04	N/A	4	0.08	N/A	N/A
		3	2AUH3 (= tARE2)	0.67	6.38	36.62	N/A	201	5.49	N/A	N/A
		4	2AUH4	0.56	4.41	25.81	N/A	3	0.12	N/A	N/A
		5	2AUH5	0.68	10.47	43.76	N/A	19	0.43	N/A	N/A
	Random	1	2AUR1	0.53	5.59	29.84	N/A	30	1.01	N/A	N/A
		2	2AUR2	0.57	7.74	37.16	N/A	5	0.13	N/A	N/A
		3	2AUR3	0.49	2.15	15.17	N/A	84	5.54	N/A	N/A
		4	2AUR4 (= tARE3)	0.53	4.94	22.98	N/A	96	4.18	N/A	N/A
		5	2AUR5	0.58	3.84	26.01	N/A	85	3.27	N/A	N/A
		6	2AUR6	0.60	7.49	38.05	N/A	4	0.11	N/A	N/A
		7	2AUR8	0.51	7.65	37.16	N/A	3	0.08	N/A	N/A
		8	2AUR9	0.54	6.24	31.62	N/A	82	2.59	N/A	N/A
GS^{AA}	Priority	1	AAH1 (= tAA1)	0.52	3.59	21.57	24.53	870	40.33	791	32.25
		2	AAH2 (= tAA2)	0.69	4.88	31.95	N/A	522	16.34	N/A	N/A

		3	AAH4	0.52	4.88	27.53	N/A	87	3.16	N/A	N/A
		4	AAH5 (= tAA3)	0.62	9.35	45.15	N/A	399	8.84	N/A	N/A
	Random	1	AAR1	0.58	7.96	41.01	N/A	3	0.07	N/A	N/A
		2	AAR2	0.5	4.54	24.62	N/A	179	7.27	N/A	N/A
		3	AAR3	0.61	5.80	32.52	N/A	260	8.00	N/A	N/A
		4	AAR4	0.58	7.54	35.26	N/A	333	9.44	N/A	N/A
		5	AAR5	0.46	2.95	19.37	N/A	87	4.49	N/A	N/A
		6	AAR6	0.5	3.06	20.24	N/A	85	4.20	N/A	N/A
		7	AAR7	0.46	3.77	21.14	N/A	111	5.25	N/A	N/A
		8	AAR9	0.58	7.26	31.13	N/A	213	6.84	N/A	N/A
		9	AAR10	0.53	4.46	21.42	N/A	334	15.59	N/A	N/A
		10	AAR11	0.6	6.94	34.46	N/A	82	2.38	N/A	N/A
Ub-GS^R	Priority	1	RH1	0.48	8.19	31.71	N/A	4	0.13	N/A	N/A
		2	RH2	0.4	6.30	30.30	36.02	376	12.41	396	11.00
		3	RH3	0.32	4.79	23.80	30.98	136	5.71	161	5.20
		4	RH4 (= tUbR1)	0.49	3.20	20.27	23.7	712	35.12	665	28.06
		5	RH5 (= tUbR2)	0.46	3.56	20.96	25.72	698	33.3	826	32.12
		6	RH6	0.28	1.18	8.88	N/A	52	5.86	N/A	N/A
	Random	1	RR1	0.35	3.72	20.11	25.79	149	7.41	249	9.65
		2	RR2	0.52	6.71	32.18	N/A	112	3.48	N/A	N/A
		3	RR3	0.49	3.76	22.28	28.31	272	12.21	352	12.44
		4	RR4	0.58	6.63	29.27	N/A	90	3.07	N/A	N/A
		5	RR5	0.39	7.32	32.39	43.37	251	7.75	262	6.04
		6	RR6 (= tUbR3)	0.41	6.36	31.21	40.49	392	12.56	422	10.42
		7	RR7	0.53	7.33	35.87	41.68	372	10.37	381	9.14
		8	RR8	0.32	6.06	26.91	35.99	226	8.40	269	7.47
		9	RR9	0.37	3.50	19.40	26.49	82	4.23	N/A	N/A

PVCD, peak viable cell density; IVCD, integral viable cell density; Q_P, specific productivity.

Supplementary Table S4. Comparison of growth and productivity metrics between batch and fed-batch cultures for selected dGS mini-pools at PDL 60

Mini-pool (PDL 60)	Specific growth rate (μ, day^{-1})		PVCD (× 10^6 cells/mL)		IVCD (× 10^6 cells·day/mL)		Max titer (mg/L)		Q_P (pg/cell/day)	
	Batch	Fed-batch	Batch	Fed-batch	Batch	Fed-batch	Batch	Fed-batch	Batch	Fed-batch
tWT3	0.51	0.54 ± 0.01	4.7	13.5 ± 0.4	25.8	88.5 ± 0.4	88	172 ± 5	3.6	1.9 ± 0.1
tAA1	0.62	0.63 ± 0.01	9.7	17.6 ± 0.6	39.2	145.9 ± 1.3	647	3084 ± 136	16.4	21.1 ± 0.8
tAA2	0.74	0.59 ± 0.02	7.2	14.1 ± 0.3	38.9	142.5 ± 2.2	548	3869 ± 129	14.3	27.1 ± 0.5
tUbR1	0.70	0.53 ± 0.01	9.3	15.5 ± 0.7	40.8	108.6 ± 0.4	563	2626 ± 73	14.5	24.2 ± 0.6

Fed-batch experiments were performed using biological triplicates, and values are presented as mean ± SD. Batch culture data represent the average of technical duplicates. The specific growth rate (μ) was calculated based on viable cell density data collected during days 0–3. PVCD, peak viable cell density; IVCD, integrated viable cell density; Q_P, specific productivity; SD, standard deviation.