Supporting Information

BiCistronic Design-based continuous and high-level membrane protein production in *Escherichia coli*

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Methods

Strains and culture conditions

E. coli strains (**Supplementary Table 1**) were cultured in liquid Lysogeny Broth (LB) or LB-agar with appropriate antibiotics, kanamycin (50 µg/mL) and/or chloramphenicol (35 µg/mL). For rhodopsin production, all-trans retinal (Sigma-Aldrich) was added to cultures from a 20 mM ethanol stock to a final concentration of 20 µM and re-added once after 2-4 hours of cultivation or induction to compensate for degradation of the unstable pigment. Long-term stability experiments were performed over 72 hours by diluting 4 replicate cultures 1:50 every 24 hours.

E. coli BL21(DE3) was used to express all the BCD constructs. Tube cultures were performed in 10 mL LB medium in 50 mL Greiner tubes. 1% or 2% overnight pre-cultures were used to start the cultures and these were grown for 22 hours at 250 rpm, at 30°C for AraH-GFP and YidC-GFP, and 37°C for GR and TR. Pre-cultures (100 µL/well) and expression cultures (500 µL/well) for library screening of *E. coli* BL21(DE3) were performed in MASTERBLOCK® Deep 96-Well plates (Greiner). Plates were kept in a plastic bag with a humidified atmosphere to prevent evaporation and grown for 22 hours at the defined temperature and 900 rpm.

E. coli Lemo21(DE3) was used to produce YidC, as well as GR and TR. Fresh colonies were used to inoculate pre-cultures, as the use of re-streaked glycerol stocks was reported to lead to severely reduced recombinant protein production in LEMO[1]. Overnight pre-cultures were used to inoculate 1%-2% in 10 mL LB medium in 50 mL Greiner tubes with different L-rhamnose concentrations (0, 50, 100, 500, 1000 and 2000 µM). At an OD_{600} of 0.35-0.45, cells were induced with IPTG (isopropyl β-D-1-thiogalactopyranoside) at a concentration 0.4 mM, and for rhodopsin production all-trans retinal was added as well, and re-added 2-4 hours after induction. After induction, cells were grown for 22 hours (30°C for YidC-GFP/AraH-GFP 37°C for rhodopsins, 250 rpm) and then harvested for production-level analysis.

E. coli BL21(DE3) pLysS was used for the production of AraH-GFP from pET28(+)-opt-AraH-GFP-his. The strain was cultured as previously published[2]. In short, 2% overnight pre-culture was inoculated in 10 mL LB in 50 mL tubes, and incubated at 37°C, 180 rpm until OD_{600} of 0.25-0.35 was reached. Then the cultures were induced with 1.0 mM IPTG and incubated for 5 and 22 hours at 25°C or 30°C at 180 rpm.

Supplementary Table 1. *E. coli* strains used in this study.

Strain	***Description***	***Origin***
E. coli DH5α	*F⁻ λ⁻ fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
E. coli BL21(DE3)	*F⁻ λ⁻ fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS*	NEB
E. coli Lemo21(DE3)	*F– λ– fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS/* pLemo(CamR)	NEB
E. coli BL21(DE3) pLysS	*F⁻ λ⁻ fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS/*pLysS (CamR)	Promega

Plasmid construction

Plasmids used for our study can be found in Supplementary Table 2, oligos used are in Supplementary Table 3. PCR products were generated using either Phusion polymerase (ThermoFisher) or Q5 polymerase (NEB) according to manufacturer's protocols. Assemblies were mostly performed using type IIS restriction enzymes BsaI or BbsI (NEB) and subsequent ligation by T4 ligase (NEB). When appropriate PCR samples were treated with DpnI (NEB) to digest template DNA. For the construction of some plasmids Gibson Assembly was performed using the NEBuilder® HiFi DNA Assembly (NEB) according to the manufacturer's protocol. Agarose gel DNA purifications and DNA purifications were respectively performed using ZymocleanTM Gel DNA Recovery Kit and DNA Clean & Concentrator (Zymo Research). Assemblies were generally confirmed by Sanger sequencing (GATC Biotech).

For construction of the BIOFAB-P14 backbone plasmid for YidC-GFP, *yidc-gfp* was amplified from pET28a(+)-YidC-GFP (BG7342,BG7343) and assembled into a BIOFAB-P14 vector pFAB3913 (amplified from pFAB3913 by BG7338,BG7339) via Gibson Assembly. pFAB3913 was a kind gift of Drew Endy and obtained from Addgene (Plasmid #47816)[3]. For the construction of the BIOFAB-P14 assembly with AraH-GFP, *arah-gfp-his* was amplified from pET28a(+)-AraH-GFP-his (BG8448,BG7937), and together with one BCD oligo pair, introduced in the PCR amplified BIOFAB-P14 backbone from

pFAB3913 (BG7933, BG7934) by three part ligation. pET28a(+)-AraH-GFP was a kind donation of Daniel Daley[4]. The gene encoding GR was codon-optimized for *E. coli* (Supplementary data 1) and synthesized by GeneArt for insertion, initially for another study together with an N-terminal GFP fusion, into BglBrick vector pBbE0A (Addgene # 35372)[5]. GR-GFP was then PCR amplified from pBbE0A-GR-GFP (BG5971, BG5972) for introduction into BIOFAB standard plasmids pFAB3913 by Golden Gate assembly. As the GFP fusions at the C-terminus of GR were hampering proper retinal incorporation into GR, those were removed from the constructs using phosphorylated primers BG6162 and BG6163 and subsequent plasmid recircularization with T4 ligase. TR was codon-harmonized using our public Codon Harmonizer Tool (http://codonharmonizer.systemsbiology.nl/) [6,7] (Supplementary data 1), subsequently synthesized and cloned into pGFPe by GeneArt. For construction of the BIOFAB-P14 plasmid for TR, TR was amplified from pGFPe-TR (BG7340, BG7337) and introduced in a BIOFAB-P14 backbone (pFAB3913 amplified from BG7339, BG7341) by Gibson assembly.

For the construction of the BCD variant libraries, the above constructed BIOFAB-P14 plasmids were PCR amplified to introduce BsaI or BbsI type IIs restriction sites (BG7784, BG7785 for YidC-GFP and BG8448, BG7934 for AraH-GFP, BG7335, B7336 for GR and BG7505, BG7506 for TR). For the BIOFAB-P14 plasmid, YidC-GFP BbsI was used, as BsaI gave digestion issues. The BsaI/BbsI-digested PCR products were subsequently assembled one by one with the annealed, phosphorylated oligo pairs (BG7291-BG7334) for each of the BCD variants (YidC-GFP, AraH-GFP), or with the complete pool of all BCD variants (GR, TR). The oligos used hereto were first phosphorylated using T7-polynucleotide kinase (NEB) according to the manufacturer's protocol. Then, pairs of oligos for each BCD variants were annealed, by heating to 95°C for 3 min and gradual cooling to RT in 30 min. The annealed oligo pairs were stored for long-term usage, both individually and pooled. 0.5 µl of individual or pooled annealed oligos (10 ng/µL) was ligated with ~100 ng BsaI/BbsI-digested, dephosphorylated PCR products. Those ligation mixes were transformed to directly to *E. coli* BL21(DE3) (YidC-GFP, AraH-GFP) or first through *E. coli* DH5α for TR and GR. For AraH-GFP clones with BCD21 were not obtained in the first attempts, and hence not further pursued as this low-strength BCD is probably not so relevant. For YidC-GFP and ArH-GFP at least three colonies for each BCD variant were picked and sequence verified, for some more colonies had to be picked to obtain at least three correct replicate clones.

The pET28a(+)-AraH-GFP vector with an optimized sequence at the 5'UTR:CDS junction was reconstructed based on the optimal junction found sequence before[2] by PCR amplification of pET28(+)-AraH-GFP by phosphorylated primers (BG8565,BG8566) and recirculation by T4 ligase.

For validating membrane protein production of GR and TR in *E. coli* Lemo21(DE3), pET28a(+)-expression vectors were generated. GR and TR genes were subcloned into the pET28a(+)-derived pGFPe and the C-terminal GFP fusion was removed by PCR amplification with phosphorylated primers (BG6696, BG6697 for GR, BG6696, BG8454 for TR) and recircularization by T4 ligase.

Supplementary Table 2. Plasmids used in this study.

Plasmid name	***Antibiotic marker***	***Origin of replication***	***Important components***	***Reference***
pFAB-P14-BCD#-YidC-GFP	Kan	p15a	P14, varying BCDs, YidC-GFP-his	This work
pFAB-P14-BCD#-AraH-GFP-his	Kan	p15a	P14, varying BCDs, AraH-GFP-his	This work
pFAB-P14-BCD#-GR-his	Kan	p15a	P14, varying BCDs, GR-his	This work
pFAB-P14-BCD#-TR-his	Kan	p15a	P14, varying BCDs, TR-his	This work
pET28a(+)-YidC-GFP	Kan	pBR322	P_{T7}, YidC-GFP fusion	[8]
pET28(+)-opt-AraH-GFP-his	Kan	pBR322	P_{T7}, opt junction, AraH-GFP-his	Reconstructed as published[2]
pET28a(+)-GR-his	Kan	pBR322	P_{T7}, GR-his	This work
pET28a(+)-TR-his	Kan	pBR322	P_{T7}, TR-his	This work
pFAB3913	Kan	P15a	P14,BCD9,RFP	[3]
pLemo	Cam	P15a	PrhaBAD, lysY	[9]
pGFPe	Kan	pBR322	pET28a(+) derived for N-term GFP-his fusions	[10]
pGFPe-AraH (pET28(+)-AraH-GFP-his)	Kan	P15a	P_{T7}, AraH-GFP-his fusion	[4]
pBbE0A-GR-GFP	Amp	colE1	GR-GFP-his fusion protein	This work

pGFPe-GR	Kan	pBR322	P_{T7}, GR-GFP-his fusion	[6]
pGFPe-TR	Kan	pBR322	P_{T7}, TR-GFP-his fusion	GeneArt/this work

Supplementary Table 3. Oligos used in this study.

Oligo nr.	***Sequence***	***Purpose***
BG5971	TTTTTTGGTCTCAAATGCTGATGACCGTTTTT	GR-GFP Fw amplification, BsaI
BG5972	TTTTTGGTCTCCTCAGTGGTGGTGGTGGTG	GR-GFP Rv amplification, BsaI
BG6162	TGAGGATCGGTTGTCGAGTA	GFP deletion from GR-GFP fusion vectors Fw
BG6163	GCTAATCAGGCTACCACCGC	GFP deletion from GR-GFP fusion vectors Rv
BG6696	CACCACCACCACCACTGAC	pET-delGFP fw
BG6697	GTGCTCGAGTGCGGCCGCGCTAATCAGGCTACCACCGC	pET-GR-delGFP-rv
BG7291	AACATCTTAATCATGCACAGGAGACTTTCT	BCD1-fw
BG7292	CATTAGAAAGTCTCCTGTGCATGATTAAGA	BCD1-rv
BG7293	AACATCTTAATCATGCTAAGGAGGTTTTCT	BCD2-fw
BG7294	CATTAGAAAACCTCCTTAGCATGATTAAGA	BCD2-rv
BG7295	AACATCTTAATCATGCAGGGGAGGGTTTCT	BCD5-fw
BG7296	CATTAGAAACCCTCCCCTGCATGATTAAGA	BCD5-rv
BG7297	AACATCTTAATCATGCGCCGGAGGTTTTCT	BCD6-fw
BG7298	CATTAGAAAACCTCCGGCGCATGATTAAGA	BCD6-rv
BG7299	AACATCTTAATCATGCTGGGGAGGGTTTCT	BCD7-fw
BG7300	CATTAGAAACCCTCCCCAGCATGATTAAGA	BCD7-rv
BG7301	AACATCTTAATCATGCATCGGACCGTTTCT	BCD8-fw
BG7302	CATTAGAAACGGTCCGATGCATGATTAAGA	BCD8-rv
BG7303	AACATCTTAATCATGCAGAGGAGTCTTTCT	BCD9-fw
BG7304	CATTAGAAAGACTCCTCTGCATGATTAAGA	BCD9-rv
BG7305	AACATCTTAATCATGCGGAGGATCGTTTCT	BCD10-fw
BG7306	CATTAGAAACGATCCTCCGCATGATTAAGA	BCD10-rv
BG7307	AACATCTTAATCATGCGGGGGAGTGTTTCT	BCD11-fw
BG7308	CATTAGAAACACTCCCCCGCATGATTAAGA	BCD11-rv
BG7309	AACATCTTAATCATGCTGCGGAGGGTTTCT	BCD12-fw
BG7310	CATTAGAAACCCTCCGCAGCATGATTAAGA	BCD12-rv
BG7311	AACATCTTAATCATGCAATGGAGGCTTTCT	BCD13-fw
BG7312	CATTAGAAAGCCTCCATTGCATGATTAAGA	BCD13-rv
BG7313	AACATCTTAATCATGCGGTGGAGGGTTTCT	BCD14-fw
BG7314	CATTAGAAACCCTCCACCGCATGATTAAGA	BCD14-rv
BG7315	AACATCTTAATCATGCGGGGGAGTCTTTCT	BCD15-fw
BG7316	CATTAGAAAGACTCCCCCGCATGATTAAGA	BCD15-rv
BG7317	AACATCTTAATCATGCTTAGGAGTCTTTCT	BCD16-fw
BG7318	CATTAGAAAGACTCCTAAGCATGATTAAGA	BCD16-rv
BG7319	AACATCTTAATCATGCGGAGGAGGGTTTCT	BCD17-fw
BG7320	CATTAGAAACCCTCCTCCGCATGATTAAGA	BCD17-rv
BG7321	AACATCTTAATCATGCGACGGAGCGTTTCT	BCD18-fw
BG7322	CATTAGAAACGCTCCGTCGCATGATTAAGA	BCD18-rv
BG7323	AACATCTTAATCATGCTATGGAGGTTTTCT	BCD19-fw
BG7324	CATTAGAAAACCTCCATAGCATGATTAAGA	BCD19-rv
BG7325	AACATCTTAATCATGCTGAGGAAAGTTTCT	BCD20-fw
BG7326	CATTAGAAACTTTCCTCAGCATGATTAAGA	BCD20-rv
BG7327	AACATCTTAATCATGCGAGGGATGGTTTCT	BCD21-fw
BG7328	CATTAGAAACCATCCCTCGCATGATTAAGA	BCD21-rv
BG7329	AACATCTTAATCATGCCTAGGAAGTTTTCT	BCD22-fw
BG7330	CATTAGAAAACTTCCTAGGCATGATTAAGA	BCD22-rv
BG7331	AACATCTTAATCATGCGAAGGACAGTTTCT	BCD23-fw
BG7332	CATTAGAAACTGTCCTTCGCATGATTAAGA	BCD23-rv
BG7333	AACATCTTAATCATGCGATGGACGGTTTCT	BCD24-fw
BG7334	CATTAGAAACCGTCCATCGCATGATTAAGA	BCD24-rv
BG7335	ATTATAGGTCTCAAATGCTGATGACCGTTTTTAGCA	pFAB-P14-GR-fw
BG7336	TAATTAGGTCTCATGTTTCAGTACGAAAATTGCTTTCATT	pFAB-P14-GR-rv
BG7337	GATCCTTAGTGGTGGTGGTGGTGCTCTAACGCCGCTTTAGCTTCC	TR-rv, Gibson
BG7338	GATGAGCTCTACAAATAAGGATCGGTTGTCGAGTAAG	pFAB-P14-fw2, Gibson
BG7339	TGGCTACTTATAGGTCTCTTGTTTCAGTACGAAAATTGCTTTCATT	pFAB-P14-rv, Gibson
BG7340	CGTACTGAAACAAGAGACCTATAAGTAGCCAGGTCTCAAATGCGGATGTTACCCGAA	TR-fw, Gibson

BG7341	CACCACCACCACCACT	pFAB-P14-fw, Gibson
BG7342	AAACAAGAGACCTATAAGTAGCCAGGTCTCAAATGGATTCGCAACGCAATC	YidC-GFP fw, Gibson
BG7343	CGATCCTTATTTGTAGAGCTCATCCATGCC	YidC-GFP rv, Gibson
BG7505	TATATTGGTCTCAAATGCGGATGTTA	pFAB-P14-TR fw, BsaI
BG7506	CTTATAGGTCTCTTGTTTCAGTACG	pFAB-P14-TR rv, BsaI
BG7784	TATATTGAAGACTTAATGGATTCGCAACGCA	pFAB-P14-YidC-GFP fw, BbSI
BG7785	TTATTAGAAGACTATGTTTCAGTACGAAAATTGCTTTC	pFAB-P14-YidC-GFP rv, BbSI
BG8454	GTGCTCGAGCGCCGCTTTAGCTTCCACAC	pET-TR-delGFP-rv
BG8565	ATTACTATGATGTCGTCTGTTTCTACATCGGGGTCTGGC	Construction of optimized junction from pET28a-AraH-GFP Fw
BG8566	TCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCC	Construction of optimized junction from pET28a-AraH-GFP rv
BG7933	TATTATGGTCTCAGGATCCGAAAACCTGTACTTCCAG	pFAB-P14-fw, BsaI
BG7934	TAATTAGGTCTCATGTTTCAGTACGAAAATTGCTTTCATTGT	pFAB-P14-fw, BsaI
BG7937	TTTATTGGTCTCTATCCGACAGTGCGTTTCGCTTTTTG	AraH-GFP rv, Golden gate
BG8448	TATTATGGTCTCTAATGATGTCTTCTGTTTCTACATCGGG	AraH-GFP fw, BsaI, Golden Gate

Whole-cell GFP quantification

Production of YidC-GFP and AraH-GFP was estimated using whole-cell GFP fluorescence as described before[11]. In short, 1 mL of culture (or 0.5 mL from deep-well cultivations) was spun down (10 min, 13,000xg, 4ºC) and the pellet was resuspended in ice-cold 100 µL PBS (phosphate buffer saline) and incubated at 4°C for at least 1 hour for further maturation of GFP. After this, suspensions were centrifuged (10 min, 13,000xg, 4ºC) and resuspended in 100 µL PBS, then they were transferred to a black 96-well plate with transparent bottoms (Greiner). Fluorescence was directly measured using excitation at 485 nm and emission at 512 nm at a constant gain value (75) (BioTEK SynergyMX).

In-gel fluorescence assay

To validate if the GFP signal originated from full-length fusions of YidC-GFP and AraH-GFP, an in-gel fluorescence assay was performed. Cultures were centrifuged for 5 minutes at 13,000xg and the pellets were stored at -20°C. After thawing, pellets were resuspended to an estimated final concentration of 5 µg protein/µL in 50 mM kPi buffer (pH 7.5) (assuming 150 mg protein/L for OD_{600} of 1). This buffer was supplemented with 1 mM $MgSO_4$, 10% glycerol, 1 mM EDTA, 0.1 mg/mL DNase and 10 mg/mL lysozyme. Cells were lysed for 1 hour at 300 rpm at room temperature and stored at -20°C for later analysis. 4x Laemmli buffer (Biorad) was added to the cell lysate, incubated for 5 minutes at 37°C (and not higher to prevent denaturation of folded GFP). After incubation and right before loading in gel, the samples were shortly sonicated with three 0.1 ms pulses (Bandelin SONOPLUS HD 3100) to reduce sample viscosity. Twenty-five µL of diluted sample (containing 6.25 µg protein in all cases except for AraH-GFP dry blotting, where 93.5 µg were used) were loaded and run on a 10% Mini-PROTEAN® TGX™ protein gel (Biorad) in Tris-Glycine-SDS buffer (25 mM, 250 mM and 0.1% respectively). In-gel fluorescence was then imaged with a Syngene G-box using a 525nm filter.

Western blot

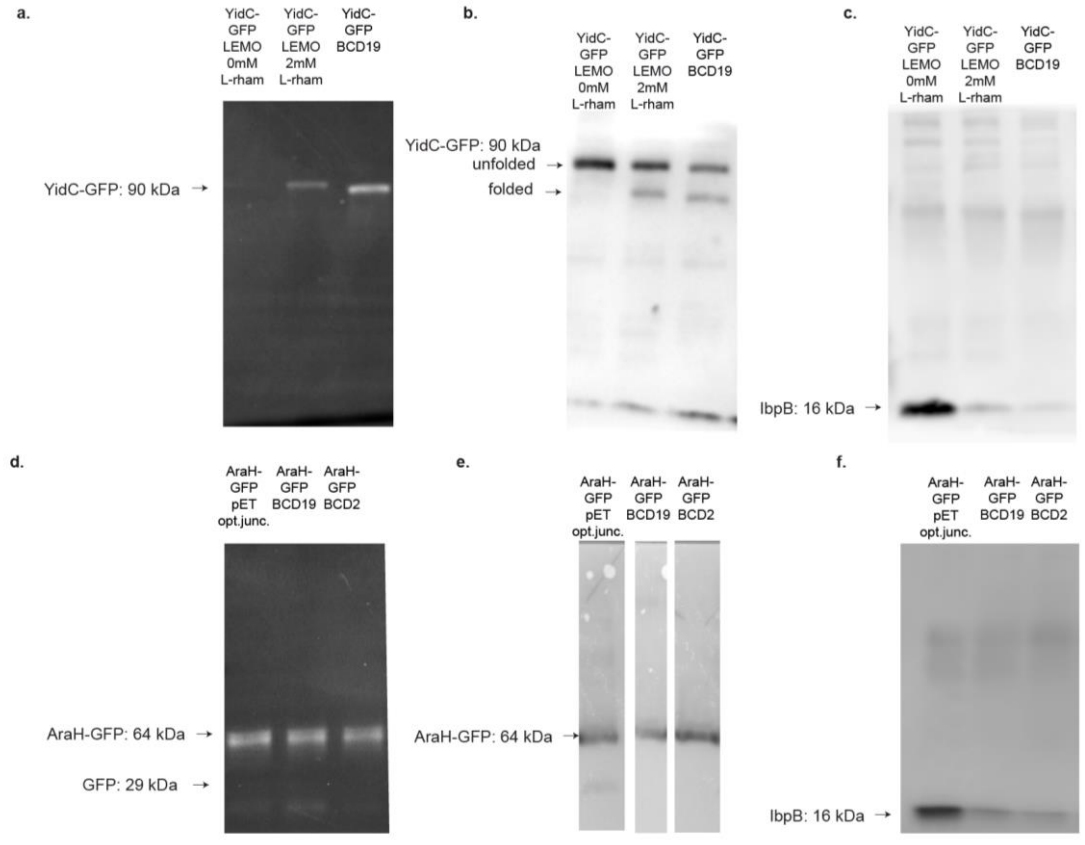
After imaging in-gel fluorescence, the proteins were transferred overnight from the gel to 0.2 micron PVDF membranes by wet transfer in a tank blotting system (Bio-Rad) at 70 mA, or for AraH-GFP blotting with anti-his by dry transfer (iBlot2® Dry Blot system, ThermoFisher) using standard settings. After transfer, the membranes were blocked in PBST (3% BSA) for 1 hour, washed twice in PBST, incubated with 6x-His Tag Mouse Monoclonal Primary Antibody (3D5) (ThermoFisher) or IbpB rabbit antiserum (1/5000)[8,9] for 2 hours, washed three times in PBST and finally incubated for 1 hour with either Goat anti-Mouse IgG (H+L) Secondary Antibody-HRP or Goat anti-Rabbit IgG (H+L) Secondary Antibody-HRP (ThermoFisher) (1/20000), respectively. Blots were developed using SuperSignal West Pico PLUS Substrate (ThermoFisher) following manufacturer's instructions and imaged in a Syngene G-box.

Flow cytometry

Samples for flow cytometry were washed in PBS, diluted 10,000 times in 1 mL PBS (<10^6 cells per mL), supplemented with 2 µL of 0.2 mM FM4-64 dye (ThermoFisher) to stain all cells and discern them from debris, and left on ice for 30 minutes, as performed before.[12] Single-cells were analyzed for GFP fluorescence, capturing 10,000 events for each sample by an BD Accuri C6 Flow Cytometer. Data were processed using BD Accuri C6 software.

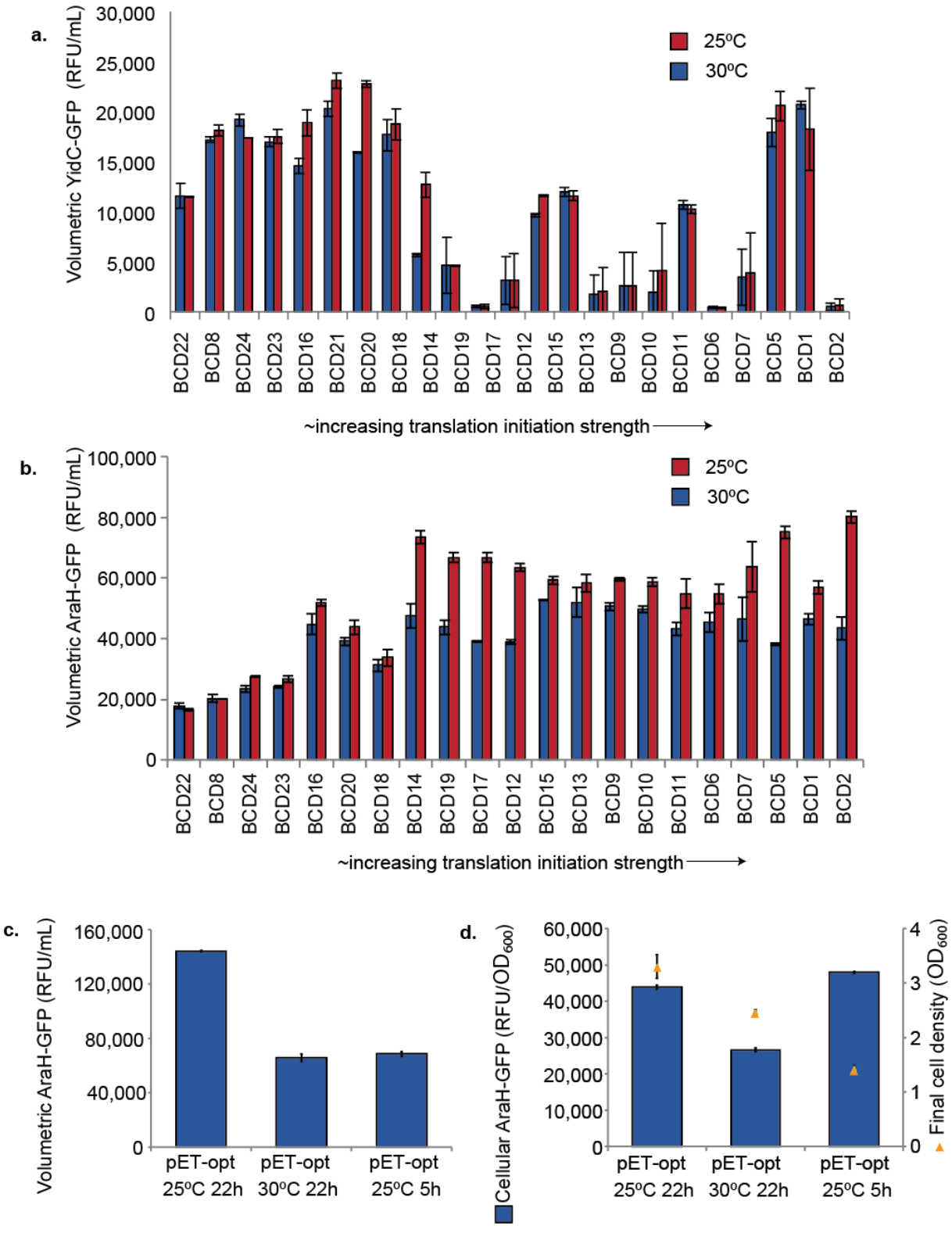
Rhodopsin quantification

Rhodopsin quantification was adapted from a previous method[13]: 10 mL of culture were resuspended in 295 µL extraction buffer, and frozen for at least 1 hour to increase lysis efficiency. Cells were thawed and additional 295 µL extraction buffer was added, supplemented with 6 mg/mL lysozyme and 0.4 mg/mL DNase. For cell lysis, this suspension was incubated at room temperature for 30 minutes. Rhodopsins were extracted from the crude cell extract by addition of 2.5% (w/v) dodecyl-maltoside (DDM, Sigma) and incubation at 180 rpm for 24-48 hours in the dark. The extraction for GR was performed at room temperature, and to increase the extraction efficiency of TR, its extraction was performed at 65°C. After extraction the mixture was spun down to check for the color of the pellet, and if a colorless pellet was obtained the supernatant fraction was used for spectroscopic quantification. 200 µL of supernatant was transferred to a transparent flat bottom 96-well plate (Greiner) and the absorption spectrum (300-700 nm) was measured (Synergy MX BioTek). Next, 0.1 M hydroxylamine was added to bleach the retinal from the rhodopsin for 1 hour in dark at room temperature with gentle shaking. Then, the absorption spectrum was measured again. The difference absorption spectrum could be generated, and from differential absorption at 540 nm (GR) or 525 nm (TR), the molar rhodopsin concentration was determined, assuming an extinction coefficient of 50,000 (M cm^{-1}) for both rhodopsins, and was converted to mg/L based on rhodopsin molecular weights.

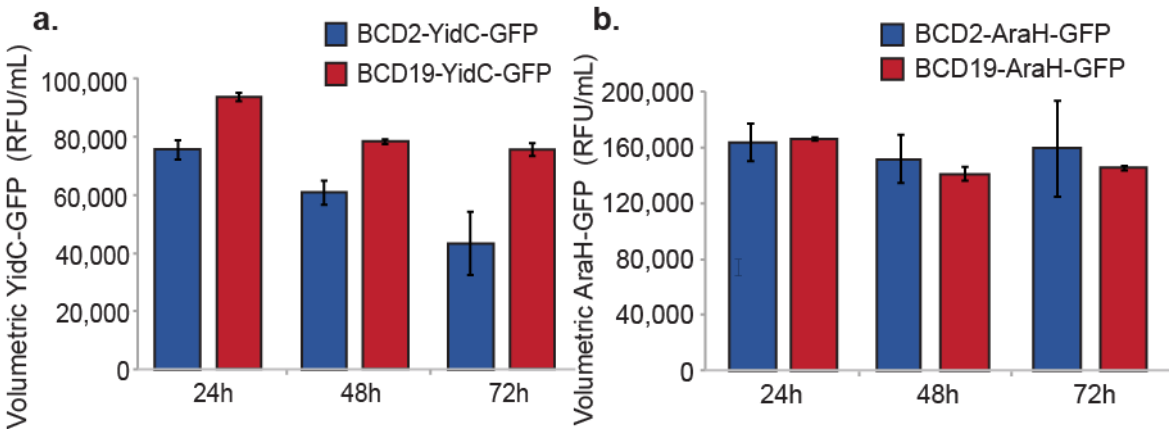


Supplementary Figure S1. In-gel fluorescence and complete Western blots. (a) The fluorescent signal from YidC-GFP cultures completely originated from a single-sized protein product, corresponding to full-length YidC-GFP, as checked for production from some BCD variants and Lemo21(DE3). (b) Western blot performed with anti-his-tag antibody to visualize both inclusion body and well-folded YidC-GFP-his (c) Western blot performed with anti-IbpB[21,22] to visualize inclusion body binding protein B for YidC-GFP expression.

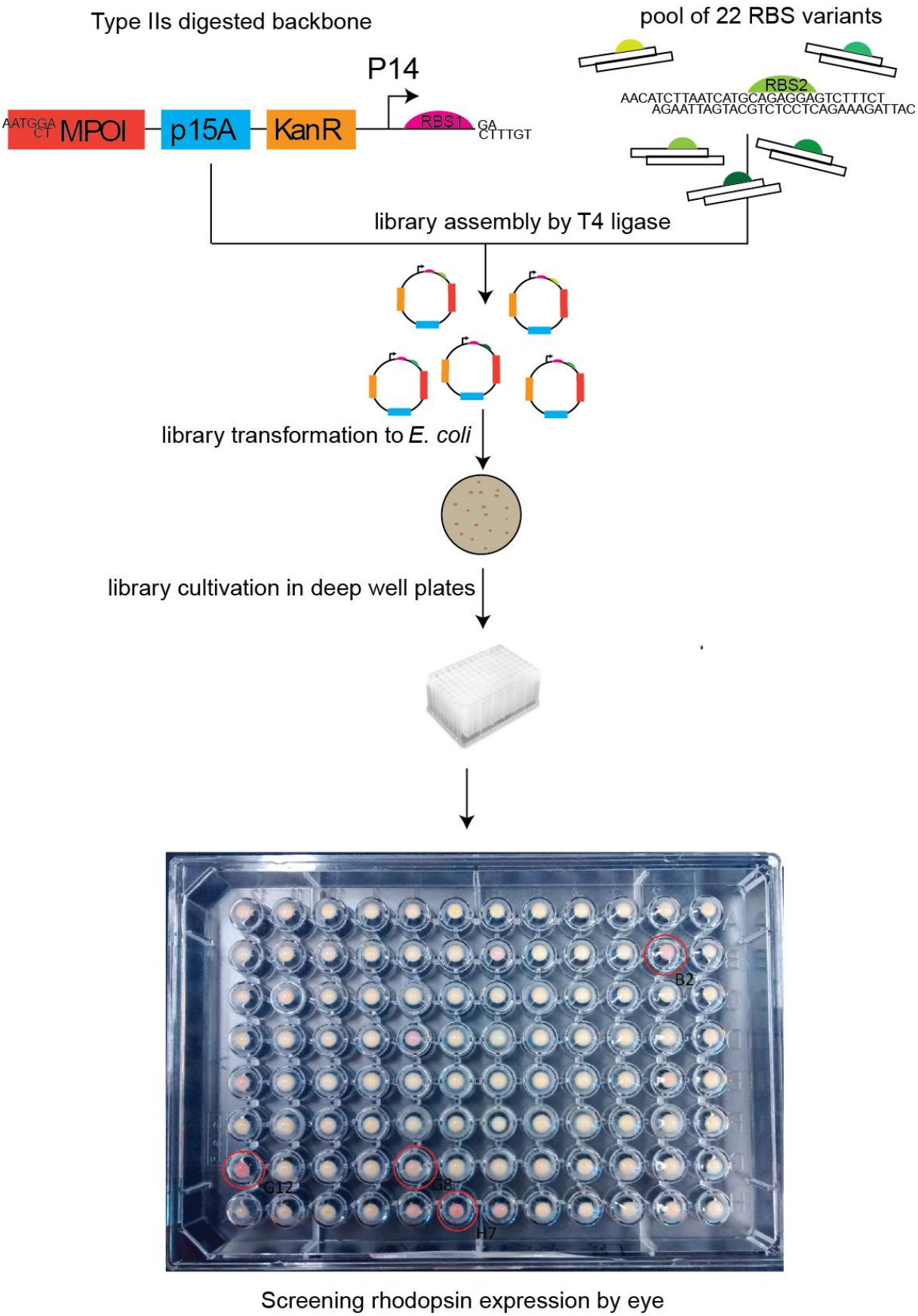
(c) (d) The fluorescent signal from AraH-GFP cultures comes predominantly from full size AraH-GFP, and only a very minor fraction from a small product, probably loose GFP, as confirmed for two BCD variants and production from the pET-opt-AraH-GFP vector. (e) Western blot performed with anti-his-tag antibody to AraH-GFP-his (f) Western blot performed with anti-IbpB[21,22] to visualize inclusion body binding protein B for AraH-GFP expression.
In-gel fluorescence and Western blots were performed on samples obtained from representative cultures, cultivated for 22 hours at 30ºC (or 25ºC for pET-opt-AraH-GFP).



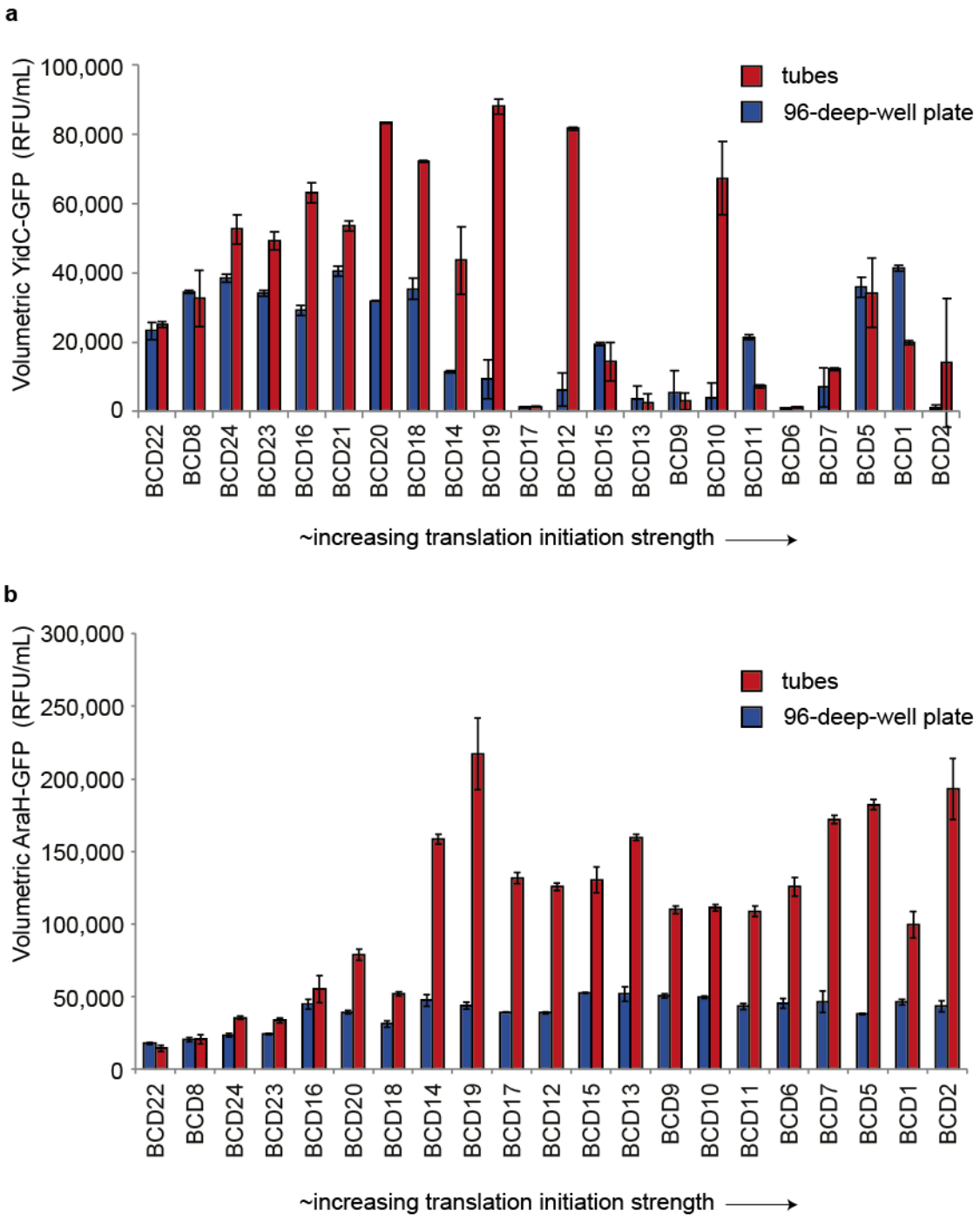
Supplementary Figure S2. Comparing production at 25ºC versus 30 ºC. Production of YidC-GFP (a) and AraH-GFP (b) from the BCD variants was compared at 25 °C and 30°C after cultivation in 96-deep-well plates for 22h. Whole-cell fluorescence for pET-opt-AraH-GFP was also measured per volume (c) and normalized per OD_{600} (c) at 25ºC for 5 hours (as in original publication of optimized vector[2]) and at 30ºC for 5 hours and at 25ºC and after 22h of induction.



Supplementary Figure S3. Volumetric production of YidC-GFP (a) and AraH-GFP (b) during 72h stability experiment.. *E. coli* BL21(DE3) harbouring BCD vectors were re-inoculated 1:50 into fresh LB kanamycin medium every 24 hours. Volumetric production data come from 4 biological replicate cultures, except for BCD2-YidC-GFP, where only 2 pre-cultures were available that still had an initial high production level.



Supplementary Figure S4. Workflow for pooled cloning and visual screening for high-producing rhodopsin clones. The plate with cell pellets depicted contains the BCD-TR library from which B2, G12 and H7 (all encircled red) were selected for sequence analysis and tube cultivation.



Supplementary Figure S5. Comparing cultivation of BCD strains in tubes versus 96-deep-well plates. All BCD variants for YidC-GFP (a) and AraH-GFP (b) were cultivated in 50 mL tubes (10 mL medium) and 96-deep-well plates (0.5 mL medium). Volumetric, membrane-integrated production was measured and normalized using the same method; this demonstrated different performance dependent on the cultivation conditions. All cultures were grown at 30ºC and data for tubes are from at least 3 biological replicates and for plates from 2 biological replicates.

Supplementary Data 1. Gene sequences codon adapted rhodopsin genes.

Codon-optimized GR +his

ATGCTGATGACCGTTTTTAGCAGCGCACCGGAACTGGCACTGCTGGGTAGCACCTTTGCACAGGTTGATCCGAGCA
ATCTGAGCGTTAGCGATAGCCTGACCTATGGTCAGTTTAATCTGGTGTATAACGCATTTAGCTTTGCCATTGCAGCA
ATGTTTGCAAGCGCACTGTTTTTTTTCAGCGCACAGGCACTGGTTGGTCAGCGTTATCGTCTGGCCCTGCTGGTGA
GCGCAATTGTTGTTAGCATTGCAGGCTATCATTATTTCCGCATTTTCAATAGCTGGGATGCAGCATATGTTCTGGAAA
ATGGTGTTTATAGTCTGACCAGCGAGAAATTCAATGATGCCTATCGTTATGTTGATTGGCTGCTGACCGTTCCGCTG
CTGCTGGTTGAAACCGTTGCAGTTCTGACCCTGCCTGCAAAAGAAGCACGTCCTCTGCTGATCAAACTGACCGTTG
CAAGCGTTCTGATGATTGCAACCGGCTATCCGGGTGAAATTAGTGATGATATTACCACCCGTATTATTTGGGGCACC
GTTAGCACCATTCCGTTTGCATATATTCTGTATGTTCTGTGGGTTGAACTGAGCCGTAGCCTGGTTCGTCAGCCTGC
CGCAGTGCAGACCCTGGTGCGTAATATGCGTTGGTTACTGCTGCTGAGCTGGGGTGTTTATCCGATTGCATATCTG
CTGCCGATGCTGGGTGTGAGCGGCACCAGCGCAGCAGTTGGTGTTCAGGTTGGTTATACCATTGCAGATGTTCTG
GCCAAACCTGTTTTTGGTCTGCTGGTTTTTGCAATTGCCCTGGTTAAAACCAAAGCAGATCAAGAAAGCAGCGAACC
GCATGCAGCAATTGGTGCAGCAGCAAATAAAAGCGGTGGTAGCCTGATTAGCCACCACCACCACCACCACTAA

Codon-harmonized TR + his

ATGCGGATGTTACCCGAACTGAGCTTTGGAGAATATTGGTTAGTCTTTAACATGCTGAGCCTGACCATTGCGGGCAT
GTTAGCGGCGTTTGTCTTTTTTCTGTTAGCTCGGAGCTATGTGGCGCCGCGTTATCATATTGCGCTGTATCTGAGCG
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CAGTCTTAATGTTAGCGCTTGGCTATGTGGGAGAAGTGAACACTGAACCGGGACCGCGGACCTTATGGGGCGCGT
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ATGCGTTAGGTACCTGGCTGCCGGGAGGCGCTGCGCAGGAAGTGGCGATTCAGATAGGTTATAGCCTTGCTGATT
TAATTGCGAAACCGATTTATGGTTTATTAGTCTTTGCGATTGCGCGCGCGAAAAGCCTGGAAGAAGGTTTTGGTGTG
GAAGCTAAAGCGGCGTTAGAGCACCACCACCACCACCACTAA

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