Supplementary information for:

Simple and robust *in vivo* engineering of plasmid DNA at any copy number in *Escherichia coli*

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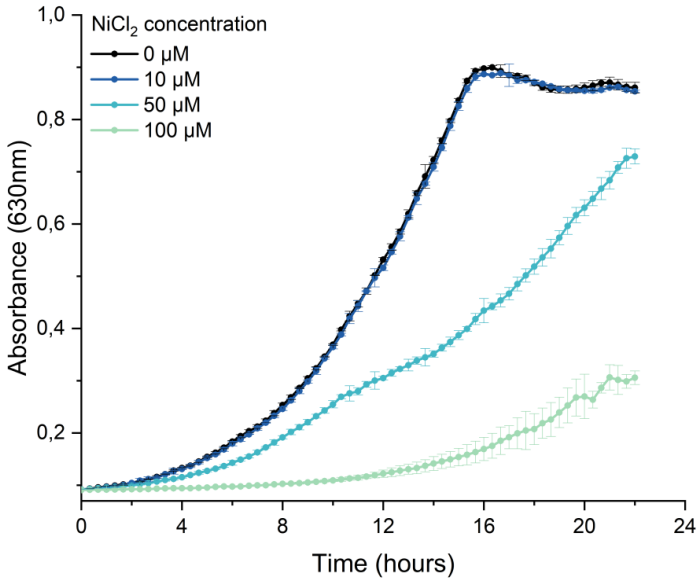
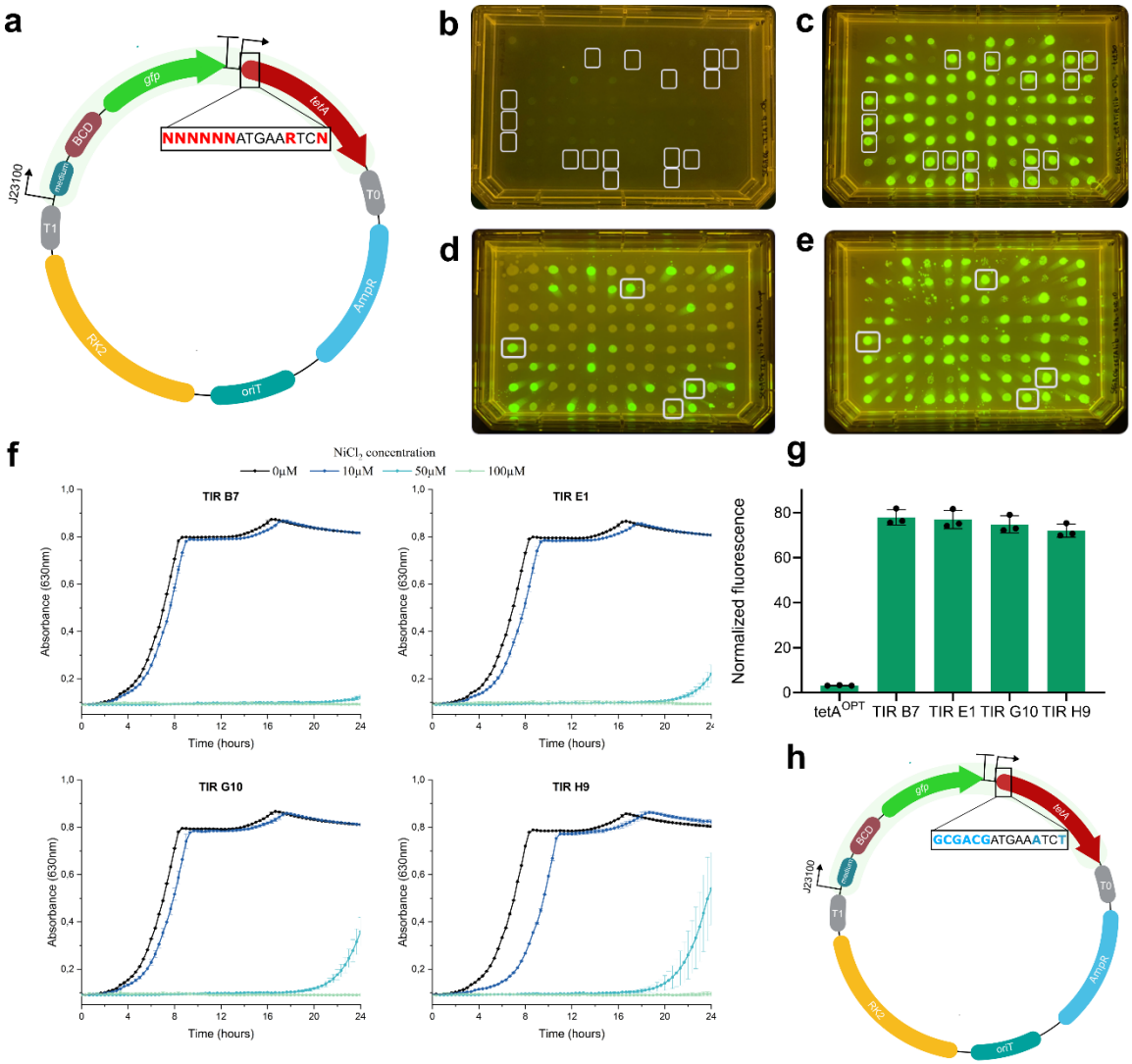
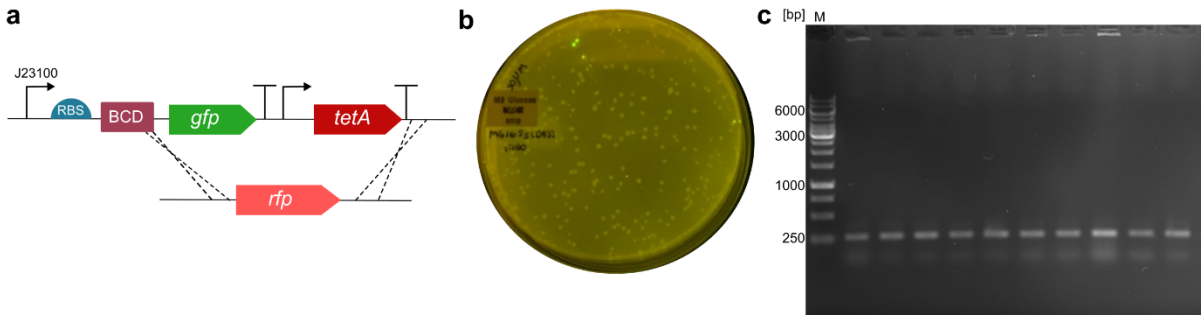


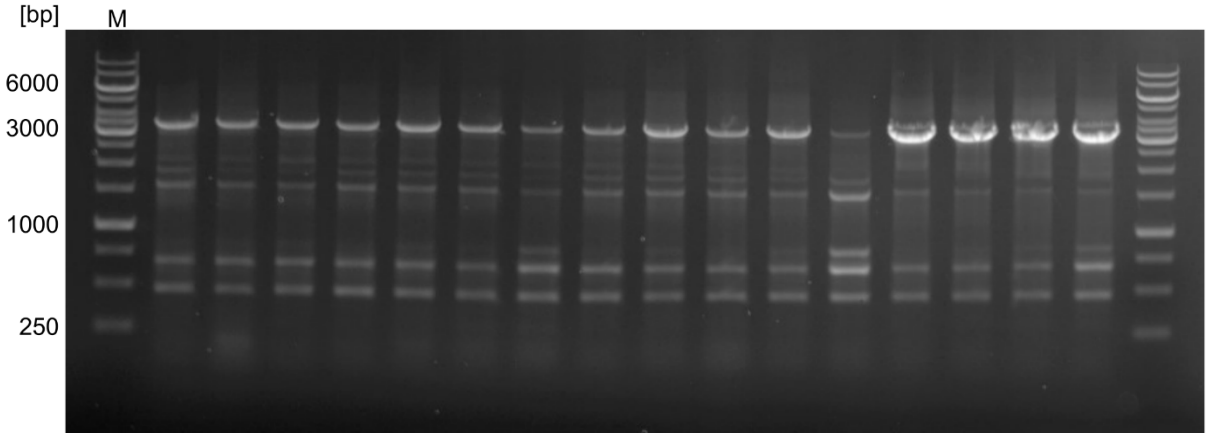
Supplementary Figure 1: Growth of *E. coli* K12-MG1655 (DE3) with pSEVA521 in the presence of different concentrations of $NiCl_2$.



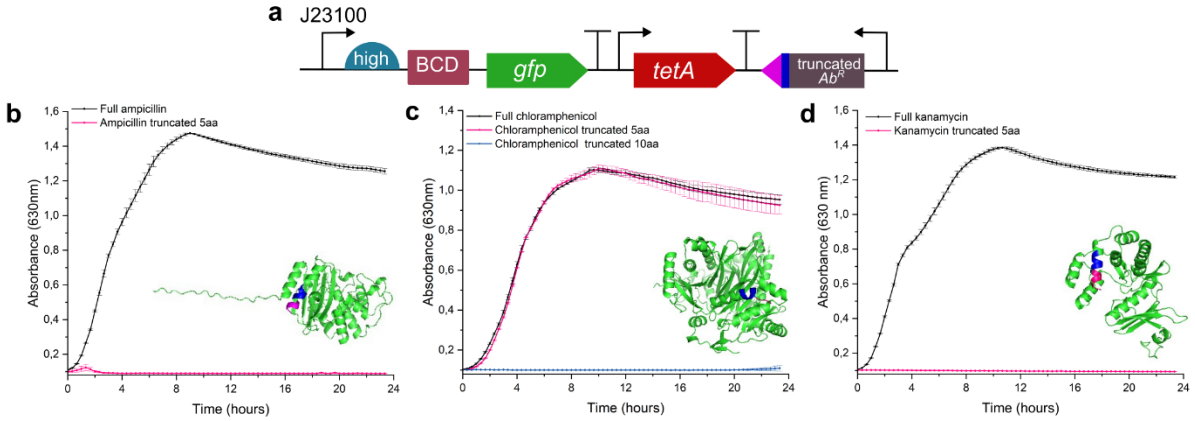
Supplementary Figure 2: *tetA* translation initiation region (TIR) optimization for stable maintenance in a RK2 plasmid. a) Illustration of the plasmid showing the double selection cassette together with the RK2 origin of replication. oriT is the origin of transference that allows for conjugative mobilization of plasmids into organisms in which no alternative transformation protocols are available. **b)** 94 TIR variants plated on M9 glucose supplemented with 50μM of $NiCl_2$. 16 variants highlighted with white squares show no growth. **c)** 94 TIR variants plated on M9 glucose supplemented with tetracycline. **d)** After 48 hours of liquid growth in LB media, the cultures were spotted on plates with ampicillin or **e)** tetracycline and four variants selected for further analysis based on fluorescence (plasmid stability). **f)** The four variants were further characterized with regards to sensitivity to $NiCl_2$ and **g)** fluorescence in liquid culture. **h)** Illustration of the TIR B7 variant that was selected for the final selection cassette.



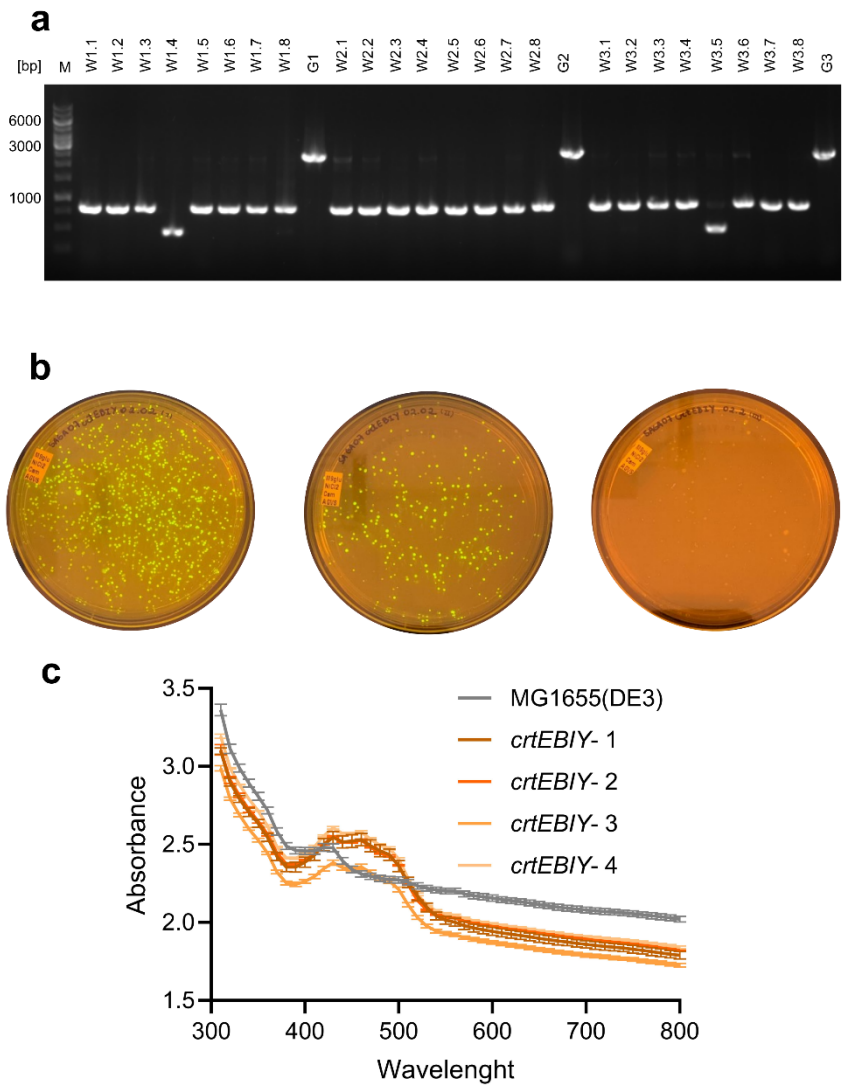
Supplementary Figure 3: Recombination of DNA fragments into the double selection cassette. a) Illustration of the genetic parts in the double selection cassette and in a DNA fragment encoding *rfp* to be integrated in the cassette. **b)** Representative plate showing the loss of fluorescence in the majority of colonies resulting from the attempted recombineering. **c)** PCR screening of 10 non-green colonies from the recombineering shows a 250 bp PCR product indicating absence of both the selection cassette and the *rfp* fragment.



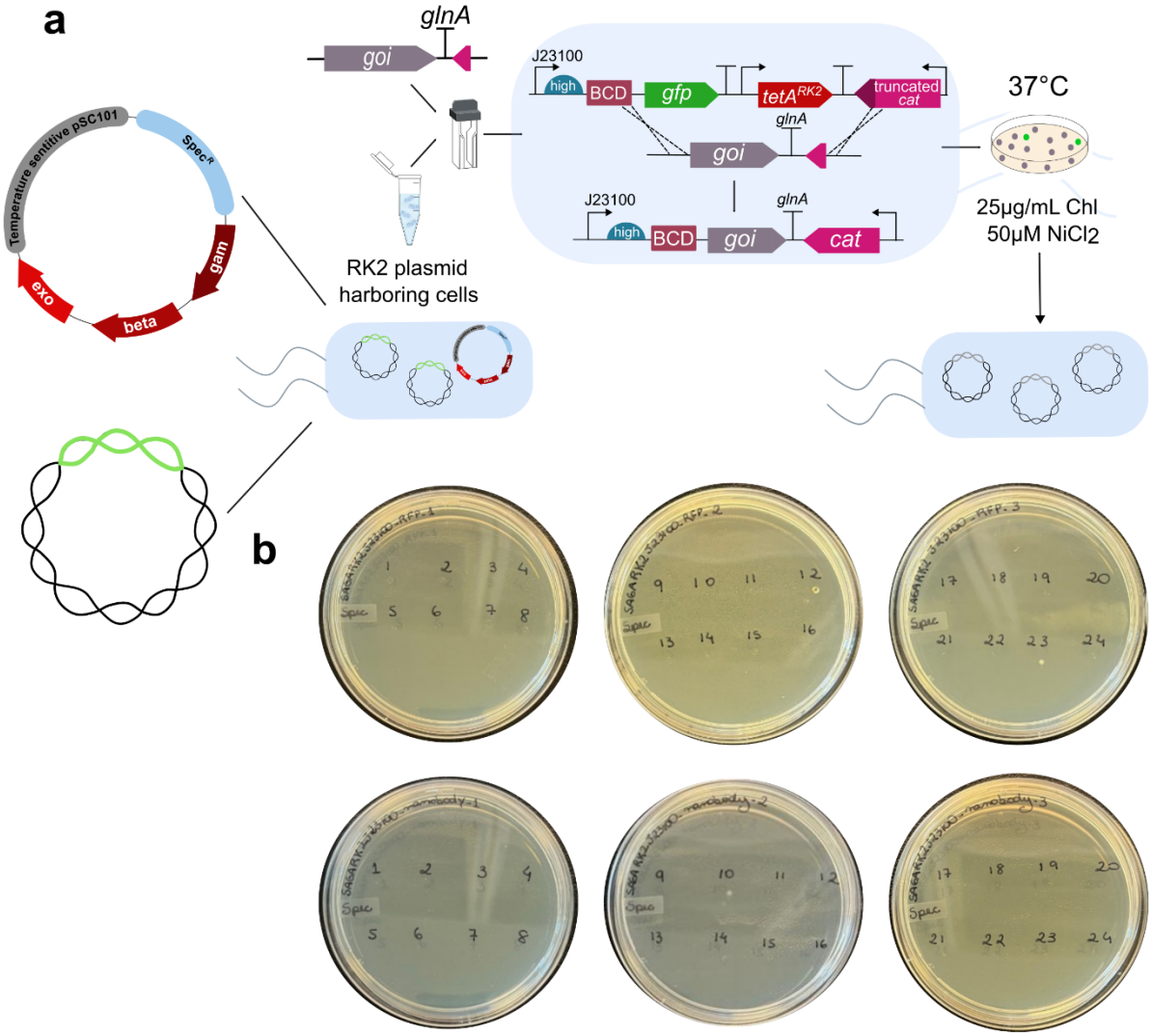
Supplementary Figure 4: PCR screening of colonies when attempting to complement a truncated promoter driving expression of an antibiotic resistance marker. The gel shows 16 individual colonies evaluated and shows a mix of differently sized PCR products: the original *gfp* 3439 bp, the *rfp* integrated 2126 bp and the version where the cassette was looped out 1569 bp, indicating inaccurate recombineering.



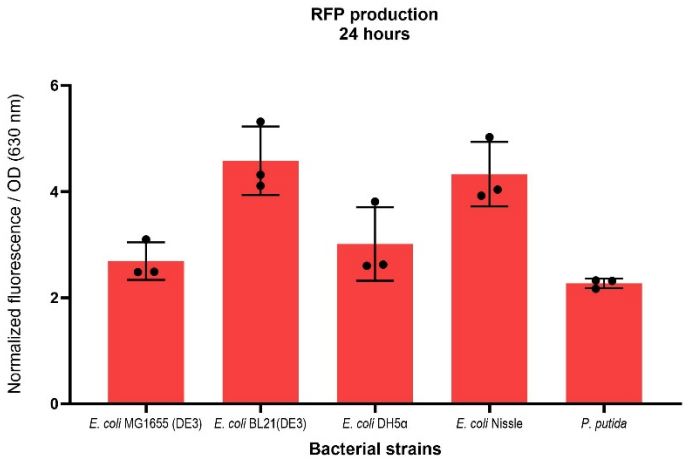
Supplementary Figure 5: Illustration of C-terminally truncated antibiotic resistance markers and analysis of the growth of truncated variants. a) Schematic representation of the system with antibiotic inversion. **b)** Growth in liquid culture with the full ampicillin resistance marker (black line) or with the five amino acid truncation (pink line) was evaluated in LB supplemented with 100 µg/mL ampicillin. **c)** Growth in liquid culture with the full chloramphenicol resistance marker (black line) or with the five (pink line) or 10-amino acid (blue line) truncation was evaluated in LB supplemented with 50 µg/mL chloramphenicol. **d)** Growth in liquid culture with the full kanamycin resistance marker (black line) or with the five amino acid truncation (pink line) was evaluated in LB supplemented with 50 µg/mL kanamycin. Structural analysis: based on the amino acid sequences for the antibiotic resistance markers, their structural model was generated using the web-based software SWISS-MODEL45. Ampicillin, based on beta-lactamase TEM (PDB: 1ZG4); Chloramphenicol, based on chloramphenicol acetyltransferase (PDB: 3U9B); and kanamycin based on aminoglycoside 3'-phosphotransferase AphA1-IAB (PDB: 4FEV). The figures of structural analyses were generated using PyMOL software (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC, New York, NY, USA).The C-terminal five amino acids are colored in pink, and the next five amino acids colored in blue



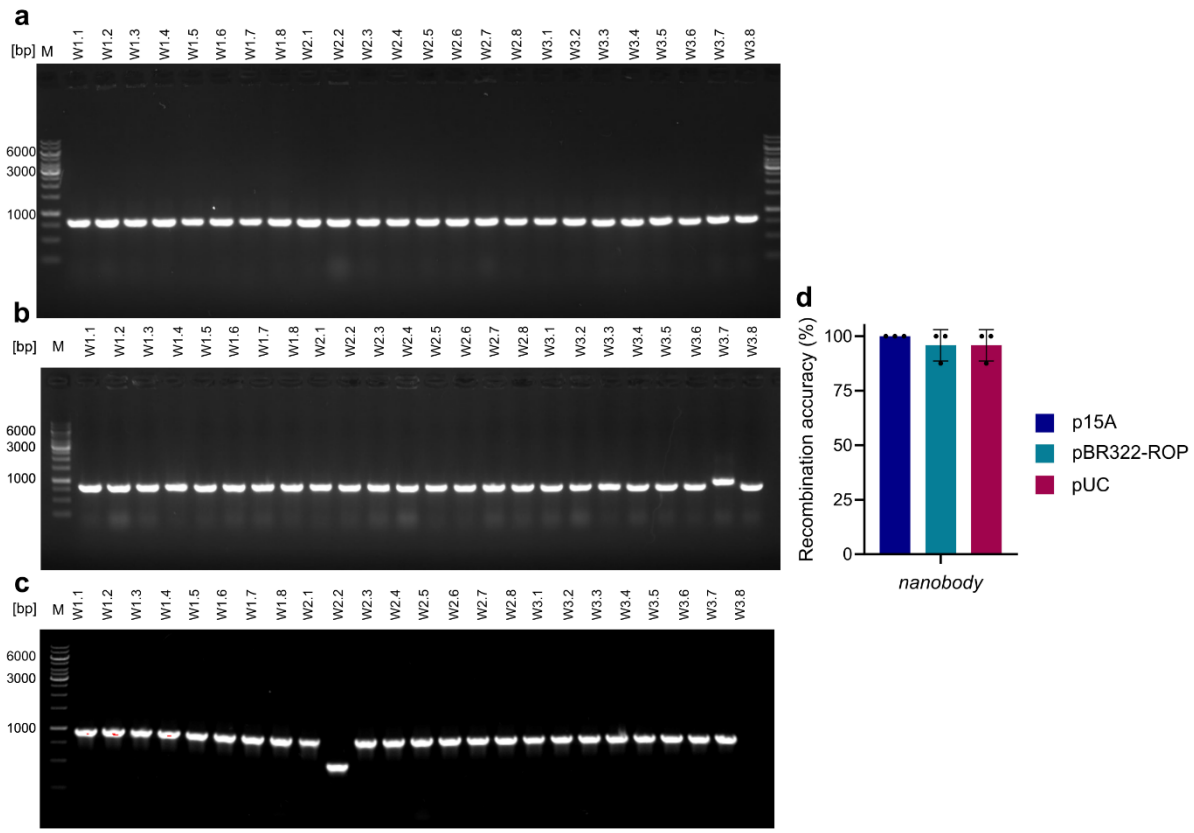
Supplementary Figure 6: Recombination of DNA fragments into the triple selection cassette. a) PCR screening of eight white colonies and one green colony from triplicate recombineering attempts with an antibody fragment. The expected size for the correct recombination was 826 bp, whereas the undisturbed triple selection cassette will lead to a 2510 bp PCR product. **b)** Triplicate plates showing large variation upon the attempted recombination of a large *crtEBIY* DNA fragment. **c)** The absorbance spectrum of four different positive colonies was evaluated as well, with the MG1655(DE3) as a control, all in triplicate. β-carotene has maximum absorbances at wavelengths around 450 nm and 470 nm, and we can observe a peak in this range on the clone variants, showing that the pathway is active.



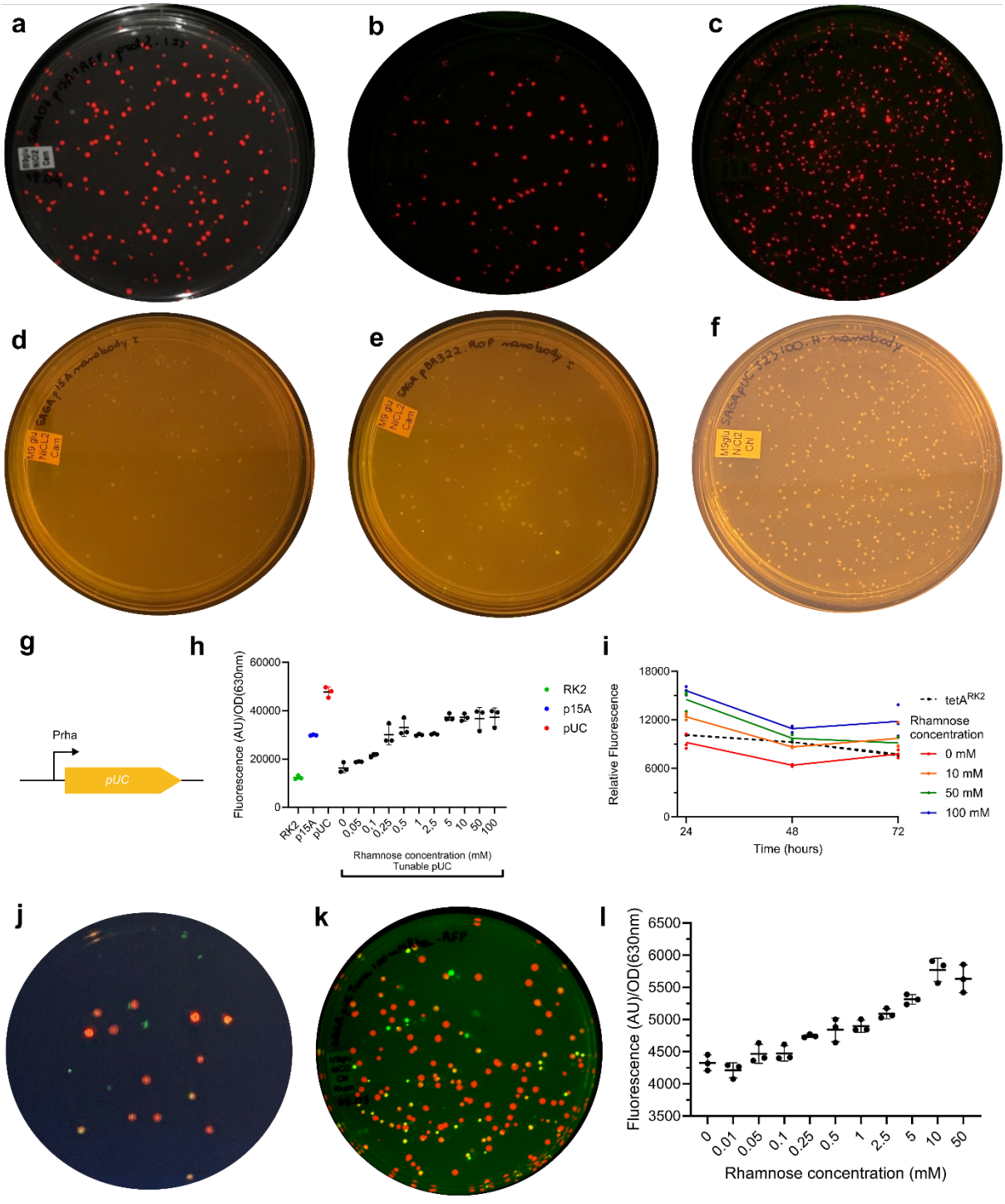
Supplementary Figure 7: Workflow of lambda-red auxiliary plasmid pSIM19 curing in the cloning process. a) Lambda-red genes *gam*, *beta,* and *exo* are expressed from an auxiliary plasmid pSIM19 hosting a temperature-sensitive origin of replication. Growth at 37 °C leads to loss of this plasmid. **b) In a test for pSIM19 loss, only two out of** twenty-four colonies from the *rfp* and *nanobody* recombination were able to grow in the presence of spectinomycin (the antibiotic marker present on pSIM19).



Supplementary Figure 8: Quantification of red fluorescence from RK2-*rfp* plasmids transferred to different hosts.

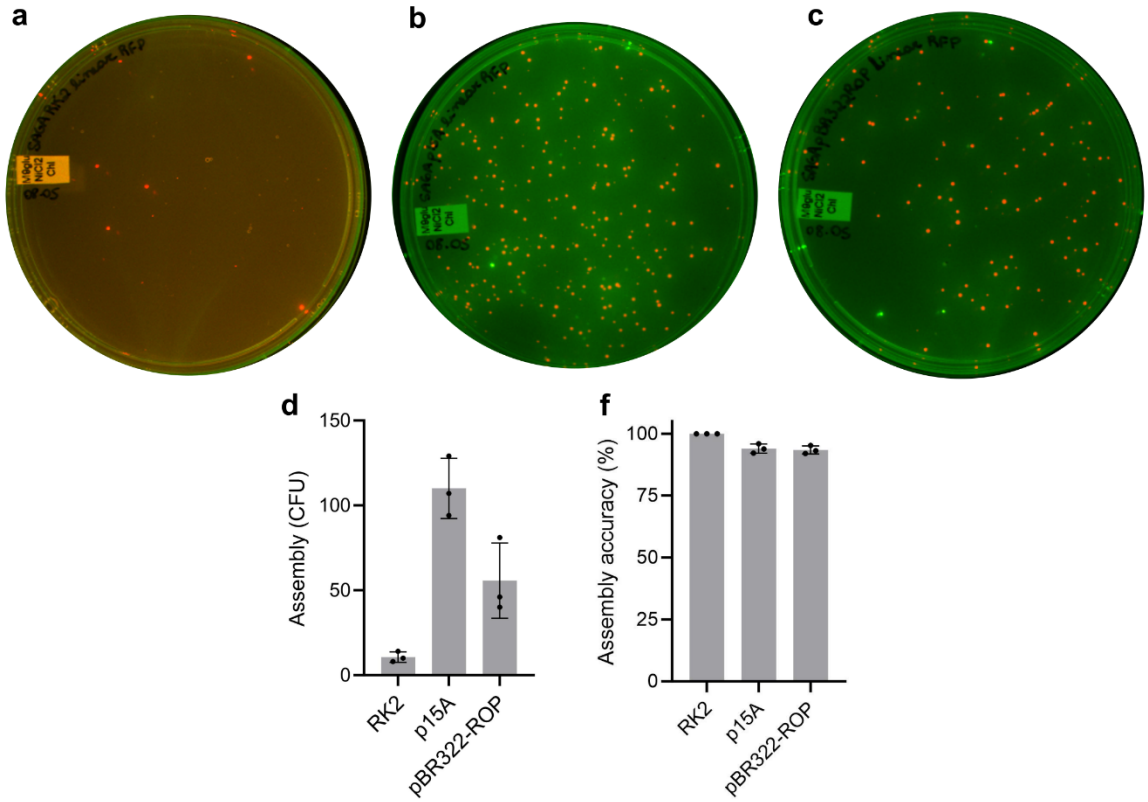


Supplementary Figure 9: PCR screening of colonies when recombineering the antibody fragment into higher copy number plasmids. Eight white colonies were selected from triplicate experiments with **a)** the p15A plasmid **b)** the pBR322-ROP plasmid and **c)** pUC plasmid. The expected size for the correct recombination is 826 bp, and for the unmodified triple selection cassette 2510 bp. **d)** Overall accuracy of the system was measured by integrating nanobody and verifying through PCR the size of the inserted elements; for the medium copy number, the accuracy was 100%, and for the high and super high copy number, the measured accuracy was 95 ± 5 %

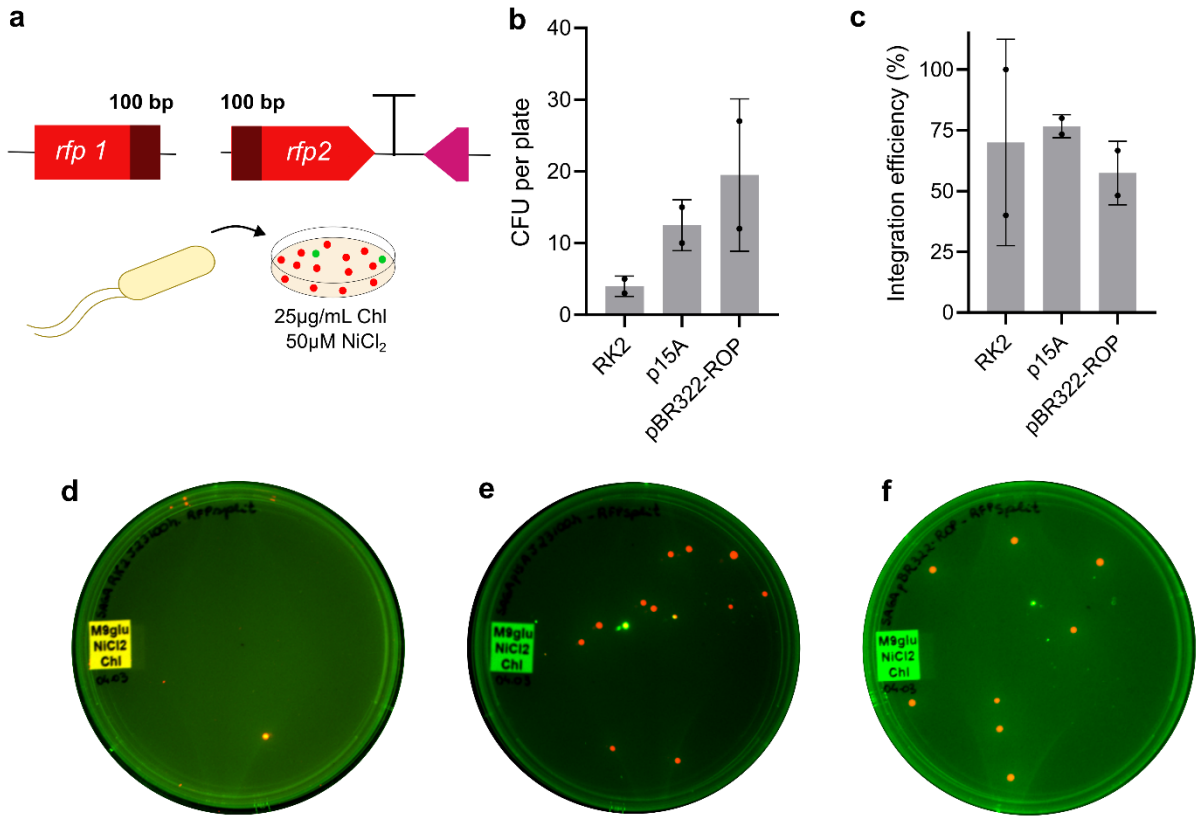


Supplementary Figure 10: Expansion of the triple-selection cassette for medium and high copy number plasmids. Representative plates from *rfp* DNA fragment recombineering into plasmids with **a)** p15A, **b)** pBR322-ROP, **c)** pUC origins of replication and nanobody fragment recombineering into plasmids with **d)** p15A, **e)** pBR322-ROP and **f)** pUC **g)** Illustration of the rhamnose-controlled copy number of pUC^{TUNE}. **h)** Fluorescence from pUC^{TUNE} with a constitutively expressed *gfp* with different rhamnose levels compared to the same expression cassette present in plasmids with the RK2, p15a, and pUC origins of replication. **i)** Stability of pUC^{TUNE} with the

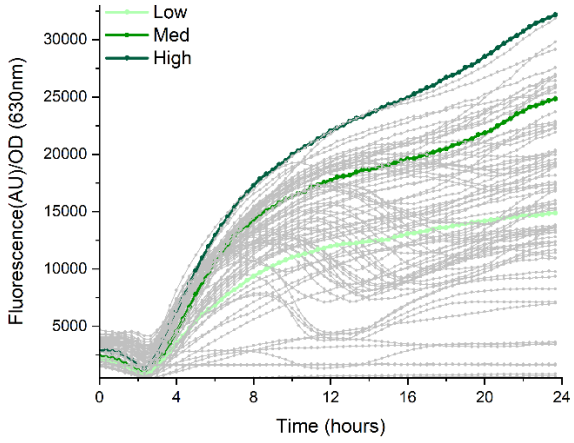
tetA$^{pBR322\text{-}ROP}$ was evaluated by measuring the fluorescence over time with different rhamnose concentrations and without antibiotic in the media. The RK2 version was included as a positive (stable) control. The fluorescence was relative to the wildtype MG1655(DE3) to ensure reproducibility between data collected on different days. Representative plates of *rfp* fragment recombineering into pUC^{TUNE} using **j)** no rhamnose and **k)** 100mM of rhamnose, and **l)** Evaluation of copy number tunability by measuring RFP fluorescence with different rhamnose concentrations form one of the clones isolated from the *rfp* pUC^{TUNE} recombineering.



Supplementary Figure 11: Recombineering between the triple selection cloning cassette fragment and linearized plasmids. Representative plates showing recombineering with the plasmid a) RK2, b) p15A and c) pBR322-ROP. d) The assembly efficiency in CFU was low on the low copy plasmid but increased for medium and high copy plasmids. F) Assembly accuracy was higher than 90% in all cases.



Supplementary Figure 12: Multiple fragment incorporation. a) To evaluate the cloning system with multiple fragment incorporation, we split *rfp* into two pieces with 100bp of homology between them. **b)** CFU observed for the different copy numbers. **c)** Recombination efficiency observed for the different copy numbers. Representative plates for **d)** RK2, **e)** p15A and **f)** pBR322-ROP.



Supplementary Figure 13: TIR library evaluation at the pBR322-ROP PT7^{CONS} histidine cluster plasmid. A TIR library was generated and the resulting variants were evaluated in liquid culture based on fluorescence. The green lines show the selected variants for low, medium and high expression.

***tetA* optimized TIR sequences**

- Low copy number plasmid

*ccaattattgaaggccgctaacgcggccttttttgtttctggtctccc*AAAAATTTATTTGCTTATTAATCATCCGGCTCGTATA
ATGTGTGGAGGGCCCAAGTTCACTTAAAAAGGAGAGCGACG**ATG**AAATCTAACAATGCGCTCATC
GTCATCCTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGGTTATGCCGGTACTGCCGGGC
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CCGCCTCGGCGAGCACATGGAACGGGTTGGCATGGATTGTAGGCGCCGCCCTATACCTTGTCTGCC
TCCCCGCGTTGCGTCGCGGTGCATGGAGCCGGGCCACCTCGACCTGAGACAATTGTC*cagataaaaaaaa
tccttagctttcgctaaggatgatttct*

Italic lower case: terminators

Underlined: P3 promoter

Bold: Start codon

Red: Translation initiation region

- Medium copy number plasmid

*ccaattattgaaggccgctaacgcggccttttttgtttctggtctccc*AAAAATTTATTTGCTTATTAATCATCCGGCTCGTATA
ATGTGTGGAGGGCCCAAGTTCACTTAAAAAGGAGA
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CCTCGACCTGAGACAATTGTC*cagataaaaaaaatccttagctttcgctaaggatgatttct*

Italic lower case: terminators

Underlined: P3 promoter

Bold: Start codon

Red: Translation initiation region

- High copy number plasmid

*ccaattattgaaggccgctaacgcggccttttttgtttctggtctccc*AAAAATTTATTTGCTTATTAATCATCCGGCTCGTATA
ATGTGTGGAGGGCCCAAGTTCACTTAAAAAGGAGA
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CCTCGACCTGAGACAATTGTC*cagataaaaaaaatccttagctttcgctaaggatgatttct*

Italic lower case: terminators

Underlined: P3 promoter

Bold: Start codon

Red: Translation initiation region

BCD-*gfp* sequence

atgaaagcaattttcgtactgaaacatcttaatcatgctaaggaggttttctaatgAGCAAAGGAGAAGAACTTTTCACTGGAGTT
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GAAGGTGATGCTACATACGGAAAACTCACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTT
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ATGGCATGGATGAGCTCTACAAATGA

Bold lower case: BCD sequence

Upper case: *gfp* sequence

***crtEBIY* sequence**

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GTACAGCCTGCCGCTCTCGCCGACCAGATTGTTAATTGAAGACACGCACTATATTGATAATGCGAC
ATTAGATCCTGAATGCGCGCGGCAAAATATTTGCGACTATGCCGCGCAACAGGGTTGGCAGCTTCA
GACACTGCTGCGAGAAGAACAGGGCGCCTTACCCATTACTCTGTCGGGCAATGCCGACGCATTCTG
GCAGCAGCGCCCCCTGGCCTGTAGTGGATTACGTGCCGGTCTGTTCCATCCTACCACCGGCTATTC
ACTGCCGCTGGCGGTTGCCGTGGCCGACCGCCTGAGTGCACTTGATGTCTTTACGTCGGCCTCAAT
TCACCATGCCATTACGCATTTTGCCCGCGAGCGCTGGCAGCAGCAGGGCTTTTTCCGCATGCTGAA
TCGCATGCTGTTTTTAGCCGGACCCGCCGATTCACGCTGGCGGGTTATGCAGCGTTTTTATGGTTTA
CCTGAAGATTTAATTGCCCGTTTTTATGCGGGAAAACTCACGCTGACCGATCGGCTACGTATTCTG
AGCGGCAAGCCGCCTGTTCCGGTATTAGCAGCATTGCAAGCCATTATGACGACTCATCGTTAA

***nanobody* sequence**

ATGGCTCAGGTCCAACTGGTCGAATCAGGCGGCGCTCTGGTCCAACCGGGCGGCTCTCTGCGTCTG
TCGTGTGCTGCTTCAGGCTTCCCGGTTAACCGTTATAGCATGCGTTGGTACCGTCAGGCACCGGGT
AAAGAACGTGAATGGGTCGCGGGCATGAGCTCTGCCGGTGATCGTAGTTCCTATGAAGACTCAGT
GAAGGGTCGCTTTACCATTTCGCGTGATGACGCACGCAACACGGTGTACCTGCAAATGAATAGTCT
GAAACCGGAAGATACCGCTGTTTATTACTGTAATGTTAATGTCGGTTTTGAATACTGGGGTCAGGG
CACGCAGGTCACGGTTAGCAGCAAGCTTGCGGCCCATCATCATCACCACCACCACCACTGA

***rfp* sequence**

ATGGTTTCCAAGGGCGAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCA
CATGGAGGGCTCCGTGAACGGCCACGAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACG
AGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAGGGTGGCCCCCTGCCCTTCGCCTGGGACATC
CTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCACCCCGCCGACATCCCCGACTAC
TTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGT
GGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGCG
GCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCC
GAGCGGATGTACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGG
ACGGCGGCCACTACGACGCTGAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCC
GGCGCCTACAACGTCAACATCAAGTTGGACATCACCTCCCACAACGAGGACTACACCATCGTGGA
ACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGCATGGACGAGCTGTACAAGTAA

***galK* sequence**

ATGAGTCTGAAAGAAAAAACACAATCTCTGTTTGCCAACGCATTTGGCTACCCTGCCACTCACACC
ATTCAGGCGCCTGGCCGCGTGAATTTGATTGGTGAACACACCGACTACAACGACGGTTTCGTTCTG
CCCTGCGCGATTGATTATCAAACCGTGATCAGTTGTGCACCACGCGATGACCGTAAAGTTCGCGTG
ATGGCAGCCGATTATGAAAATCAGCTCGACGAGTTTTCCCTCGATGCGCCCATTGTCGCACATGAA
AACTATCAATGGGCTAACTACGTTCGTGGCGTGGTGAAACATCTGCAACTGCGTAACAACAGCTTC
GGCGGCGTGGACATGGTGATCAGCGGCAATGTGCCGCAGGGTGCCGGGTTAAGTTCTTCCGCTTCA
CTGGAAGTCGCGGTCGGAACCGTATTGCAGCAGCTTTATCATCTGCCGCTGGACGGCGCACAAATC
GCGCTTAACGGTCAGGAAGCAGAAAACCAGTTTGTAGGCTGTAACTGCGGGATCATGGATCAGCT

AATTTCCGCGCTCGGCAAGAAAGATCATGCCTTGCTGATCGATTGCCGCTCACTGGGGACCAAAGC
AGTTTCCATGCCCAAAGGTGTGGCTGTCGTCATCATCAACAGTAACTTCAAACGTACCCTGGTTGG
CAGCGAATACAACACCCGTCGTGAACAGTGCGAAACCGGTGCGCGTTTCTTCCAGCAGCCAGCCC
TGCGTGATGTCACCATTGAAGAGTTCAACGCTGTTGCGCATGAACTGGACCCGATCGTGGCAAAAC
GCGTGCGTCATATACTGACTGAAAACGCCCGCACCGTTGAAGCTGCCAGCGCGCTGGAGCAAGGC
GACCTGAAACGTATGGGCGAGTTGATGGCGGAGTCTCATGCCTCTATGCGCGATGATTTCGAAATC
ACCGTGCCGCAAATTGACACTCTGGTAGAAATCGTCAAAGCTGTGATTGGCGACAAAGGTGGCGT
ACGCATGACCGGCGGCGGATTTGGCGGCTGTATCGTCGCGCTGATCCCGGAAGAGCTGGTGCCTG
CCGTACAGCAAGCTGTCGCTGAACAATATGAAGCAAAAACAGGTATTAAAGAGACTCTTTACGTT
TGTAAACCATCACAAGGAGCAGGACAGTGCTGA

pUC^{TUNE} sequence

GGCCGGCCccgtagaaaagatcaaaggatcttcttgaCACCACAATTCAGCAAATTGTGAACATCATCACG
TTCATCTTTCCCTGGTTGCCAATGGCCCATTTTCCTGTCAGTAACGAGAAGGTCGCGAATT
CAGGCGCTTTTTAGACTGG**TGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTT**
TGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCG
CAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAAC
TCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC
AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAA
GGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGA
ACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCT
TCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGA
GAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGG
GTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGA
GCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGG
CCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATT
ACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGA
GTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCG
CGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGG
CAGTGAGCGCAACGCaattaatgtgagttagctcactcattaggcaGGCGCGCC

Blue: FseI restriction enzyme site

Pink: Rhamnose promoter

Black bold: pUC origin of replication

Green: AscI restriction enzyme site

Histidine cluster-optimized TIR sequences

- Low expression level

tttgtttaactttaagaaggagaCCCGTT**ATG**GGTAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGC
CGCGCGGCAGCCATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGC

Lower case: RBS

Bold: Start codon

Red: Translation initiation region - low

Blue: 6x Histidine

Green: Thrombin protease recognition site (TPS)

Orange: T7-Tag

- Medium expression level

tttgtttaactttaagaaggagaATCCGG**ATG**GGTAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGC
CGCGCGGCAGCCATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGC

Lower case: RBS

Bold: Start codon

Red: Translation initiation region – high

Blue: 6x Histidine

Green: Thrombin protease recognition site (TPS)

Orange: T7-Tag

- High expression level

tttgtttaactttaagaaggagaGCTGTA**ATG**GGAAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGC
CGCGCGGCAGCCATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGC

Lower case: RBS

Bold: Start codon

Red: Translation initiation region – high

Blue: 6x Histidine

Green: Thrombin protease recognition site (TPS)

Orange: T7-Tag

Triple terminator (T2) sequence

TCGACCTAGCATAACCCCGCGGGGCCTCTTCGGGGGTCTCGCGGGGTTTTTTGCTGAAAGAAGCTT
CAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGCCAAAG
CCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCC
TCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAT

Blue: TUUGG terminator

Green: rrnB T1 terminator

Orange:T7 terminator

Supplementary Table 1 - Oligonucleotides used in this study

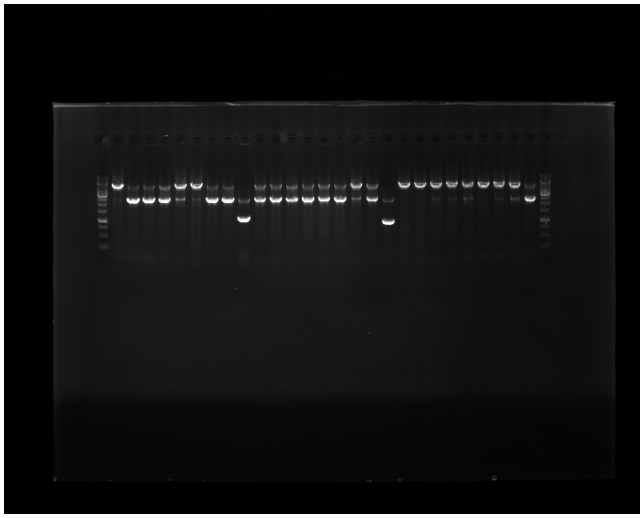
Number	Sequence	Description
01	GGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTTT AATTAAGCGTTGCGCTTGACG	SEGA landing pad with J23100 promoter on SEVA 121 fwd
02	TGGAGTTCTGAGGTCATTACTGGATCTATCAACAGGAGTCCAA GACTAGTGACAATTGTCTCAGGTCGAGG	SEGA landing pad on SEVA 121 rev

03	AAGTTCACTUAAAAAGGAGANNNNNNATGAARTCNAACAATG CGCTCATCGTCATC	*tetA* TIR library
04	AGTGAACTUGGGCCCTCCAC	*tetA* TIR library
05	GCCGGCACCTGTCCTACGAGTTGC	*tetA* sequencing rev
06	ACTCACACAATGTATACATCACG	*tetA* sequencing fwd
07	AGACCCTTGUCCTTTTCCGCTGCAT	SEVA bb amplification for antibiotic truncation – OriT
08	ACTGGATTCUCACCAATAAAAAACGCCC	SEVA bb amplification for antibiotic truncation – T0
09	ACAAGGGTCUAGTAGCCCGCCTAATGAGC	amp truncation rev
10	AGAATCCAGUTAGCTCGCGCCAATTTCC	5 aa amp truncation fwd
11	ATTTTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT AATGGTTTCCAAGGGCGAGGAGGA	BCD RFP integration - fwd
12	AGGCGACGATGGATGAACGTAACCGTCAGATTGCGGAAATTG GCGCGAGCCTGATTAAACATTGGTAATTCCTGACTTAAGCGGC GCTGGTTATCCATCGGAGCCATCCGTATTGTGTTTACTTGTAC AGCTCGTCCA	RFP integration *amp* restoration - rev
13	ACAAGGGUCTGTGTCTCAAAATCTCTGATGT	*kan* truncation rev
14	AGAATCCAGUTACATCAGATGAAATTGCAGTTTG	5 aa kan truncation fwd
15	AATATGGCATTGATAATCCGGATATGAACAAACTGCAATTTCA TCTGATGCTGGATGAATTTTTCTAATTCCTGACTTAAGCGGCG CTGGTTATCCATCGGAGCCATCCGTATTGTGTTTACTTGTACA GCTCGTCCA	RFP integration *kan* restoration - rev
16	ACAAGGGTCUGGCGAAAATGAGACGTTG	*cat* truncation rev
17	AGAATCCAGUTACTCATCGCAGTACTGTTG	5 aa *cat* truncation fwd
18	AGAATCCAGUTATTGTAATTCATTAAGCATTCTGCC	10 aa *cat* truncation fwd
19	ATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTA ATGAATTACAACAGTACTGCGATGAGTGGCAGGGCGGG GCGTAATTCCTGACTTAAGCGGCGCTGGTTATCCATCGG AGCCATCCGTATTGTGTTTACTTGTACAGCTCGTCCA	RFP integration *cat* restoration - rev
20	ATTTTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT AATGACGGTCTGCGCAAAAAAACA	BCD *crtEBIY* Integration
21	ATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGA ATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATT CCTGACTTAAGCGGCGCTGGTTATCCATCGGAGCCATCCGTAT TGTGTTTAACGATGAGTCGTCATAA	*crtEBIY* Integration *cat* restoration - rev
22	ATTTTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT AATGGCTCAGGTCCAAC	BCD Nanobody Integration
23	ATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGA ATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATT CCTGACTTAAGCGGCGCTGGTTATCCATCGGAGCCATCCGTAT TGTGTTCAGTGGTGGTGGTGGTGATGATG	Nanobody Integration *cat* restoration - rev
24	GCTCAGTCCTAGGTACAGTGCTAGC	Landing pad screening oligo fwd
25	GGTGAAAACCTGGCCTATTTCCCTAAAG	Landing pad screening oligo rev
26	AACCCTGCUTCGGGGTCATTATAGCGATTT	Binding in OriT for ori amplification fwd
27	AGGCATCAAAUAAAACGAAAGGCTCAGTCG	Binding in T1 for ori amplification rev
28	ATTTGATGCCUTTAATTAAGCGTTGCGCTTGAC	Binding in landing pad for back bone amplification fwd
29	AGCAGGGTUATGCAGCGGAAAAGGACA	Binding in OriT for backbone amplification rev
30	TGTATCCAACGGCGTCAGCC	Screening ori region fwd
31	GCATGGAGCCGGGCC	Screening ori region rev
32	AGTCCTAGGUACAGTGCTAGCTACTAGAGATTCGTAATGAAA TTCTTTTTAAG	Amplification of SEVA with GFP_fwd
33	ACCTAGGACUGAGCTAGCCGTCAATTAATTAAAGGCATCAAA TAAAAC	Amplification of SEVA with GFP_rev

34	ATCTTTCCCUGGTTGCCAATGGCCCATTTTCCTGTCAGTAACG AGAAGGTCGCGAATTCAGGCGCTTTTTAGACTGGTGCAAACA AAAAAACCAC	Amplification of Rhamn-pUC_fwd
35	AGGGAAAGAUGAACGTGATGATGTTCACAATTTGCTGAATTG TGGTGTCAAGAAGATCCTTTGATCTTTTCTACGGGGCCGGCCT AC	Amplification of Rhamn-pUC_rev
36	ATTAAGCGUTGCGCTCACTGCC	Amplification of lac promoters fwd
37	ATTAAGCGUTGCGCTTAATCTTTCTGC	Amplification of PrhaBAD promoter fwd
38	AGTTCTTCUCCTTTGCTCATTAGAAAACCTC	Different promoter insertion from SEGA rev
39	ATGATGATGAUGATGGCTGCTGCCCATGGTATATCTCCTTCTT AAAGTTAAACAAAATTATTTCTAGAGGGGAATTGTTATCCGCT CACAATTC	Amplification of the histidine tag cluster fwd
40	ATCATCATCAUCACAGCAGCGGCCTGGTGCCGCGCGGCAGCC ATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGCATGA GCAAAGGAGAAGAACTTTTC	Amplification of the histidine tag cluster rev
41	GAAGAACUTTTCACTGGAGTTGTGCC	Plasmid backbone amplification for histidine cluster fwd
42	ACGCTTAAUTAAAGGCATCAAATAAAACGAAAGG	Plasmid backbone amplification for histidine cluster rev
43	AATACGACUCACTATAGGGAGAGGAATTGTGAGCGGATAAC	T7CONS sequence restoration - fwd
44	AGTCGTATUAATTTCGCGGGATCG	T7CONS sequence restoration - rev
45	GCCATCTGATCGTTGGCAACCAGC	Screening for the histidine cluster
46	AATTTATGCUGCTAACAAAGCCCGAAA	pET28 backbone amplification - fwd
47	ATTTGCTGUCCACCAGTCATGCTAG	pET28 backbone amplification - rev
48	ACAGCAAAUGGGTCGCATGAGCAAAGGAGAAGAAC	Insertion of GFP on pET28 - fwd
49	AGCATAAATUTTTGGGAGACCAGAAACAA	Insertion of GFP on pET28- rev
50	TGGCAGCAGCCAACTCAGCTTC	Screening for GFP in pET28 - fwd
51	GGGAATTGTGAGCGGATAAC	Screening for GFP in pET28 - rev
52	ATCATCATCAUCATCACAGCAGCGG	TIR library of the histidine cluster - fwd
53	ATGATGATGAUGGCTRCTNCCCATNNNNNNTCTCCTTCTTAAA GTTAAACAAAATT	TIR library of the histidine cluster - rev
54	ATCTGTTGUTTGTCGCCAAAGCCCGAAAGGAAGCTGAGTTGG CTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGC CTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACT ATATCCGGATAAAAATTTATTTGCTTATTAATCATCC	T2 on plasmid - fwd
55	ACAACAGAUAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTT TCGTTTTATTTGAAGCTTCTTTCAGCAAAAAACCCCGCGAGAC CCCCGAAGAGGCCCCGCGGGGTTATGCTAGGTCGATCATTTGT AGAGCTCATCC	T2 on plasmid -rev
56	GGTGATGTCGGCGATATAGG	Screening for T2 insert - rev
57	GTACAGGAACGCACTATATCTTTC	Screening for T2 insert - rev
58	ATTTTCGTACUGAAACATCTTAATCATGCTAAGGAG	BCD binding -fwd
59	AGTACGAAAAUNGCYTTCATNNNNNNTTTCTCCTCTTTAATCT CTAGTAGC	BCD TIR library- rev
60	ATTTTCGTACTGAAACATCTTAATCATGCTAAGGAGGTTTTCT AATGAGTCTGAAAGAAAAAACACAATCTC	*galK* Integration *cat* restoration - fwd
61	ATGCCGTTTGTGATGGCTTCCATGTCGGCAGAATGCTTAATGA ATTACAACAGTACTGCGATGAGTGGCAGGGCGGGGCGTAATT	*galK* Integration *cat* restoration - rev

	CCTGACTTAAGCGGCGCTGGTTATCCATCGGAGCCATCCGTAT TGTGTTCAGCACTGTCCTGCTC

Uncropped gel images:



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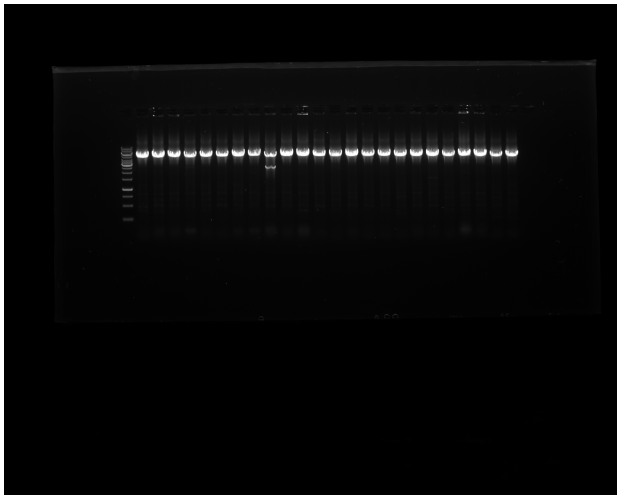

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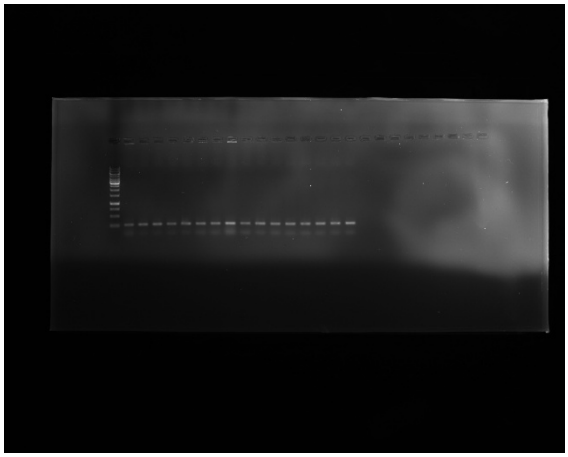
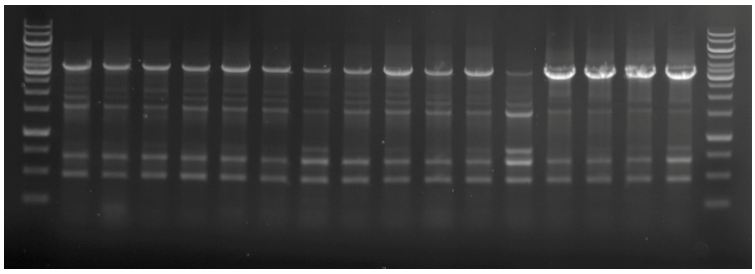


Uncropped gel Supplementary Figure 3c



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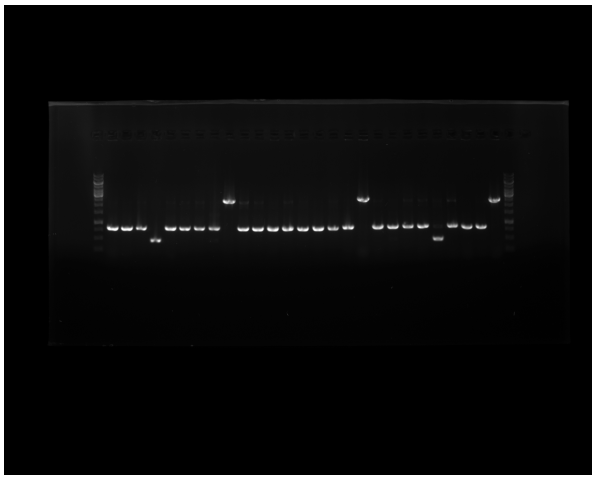
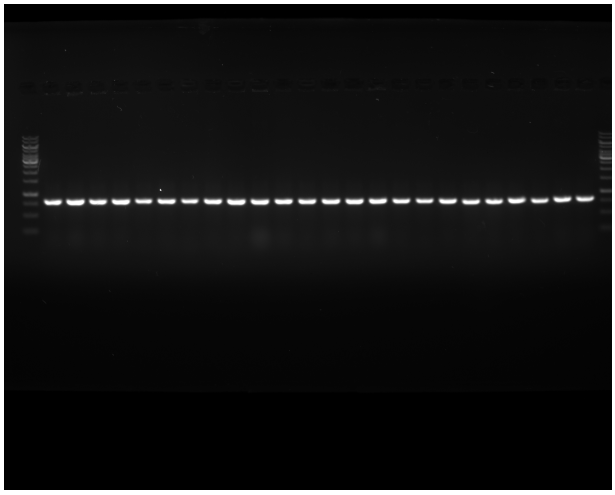


Uncropped gel Supplementary Figure 6a



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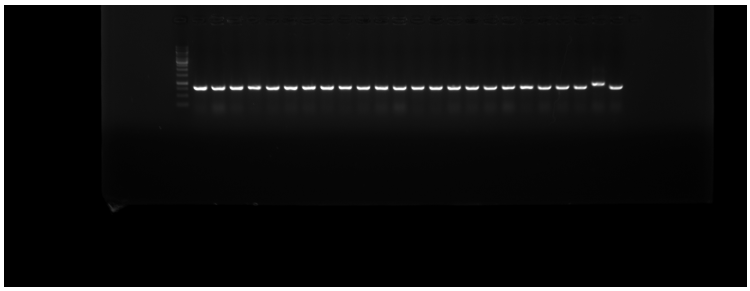

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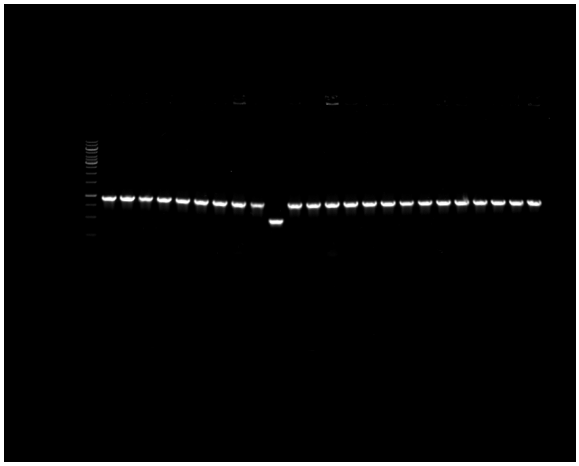

Uncropped gel Supplementary Figure 9c