nature portfolio

Peer Review File

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

Corresponding Author: Dr Morten Nørholm

This manuscript has been previously submitted to another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Brief summary and recommendation
In this study, the authors developed a methodology for in vivo engineering of plasmids applicable to any copy number. Specifically, they constructed a triple-selection cassette comprising a GFP expression module (distinguishable by colors) and two selection modules (tetA: responsive to tetracycline and NiCl2, and cat: responsive to chloramphenicol). This three-component cluster facilitates the selection and characterization of recombinant plasmid DNA when combined with Lambda-Red homologous recombination technology, exhibiting high efficiency and accuracy. Additionally, they extended the system's application to screen sequence libraries. The results demonstrate the predictability, availability, and reliability of the proposed design.
The article builds on the SEGA genome engineering methodology established by the authors in prior work, introducing a significant advancement in plasmid engineering in vivo. It offers valuable insights for researchers in synthetic biology. However, the manuscript contains several major and minor issues that require attention. Besides, one key concern must be addressed to enhance the method's reliability and reproducibility.
In summary, I recommend that the manuscript be considered for publication after major revisions, provided the authors fully address the following concerns.

Key concern
1. According to the Methods section, enzymes for Lambda-Red recombination are expressed from a plasmid (pSIM19), which facilitates the production of recombinant DNA in vivo. However, it is unclear how pure constructs can be obtained from E. coli that harboring two plasmids (pSIM19 and the target construct) after manipulation. The manuscript does not provide a detailed explanation of this process. If the authors have conducted experiments addressing this, they should include the detailed results in the revised manuscript. Otherwise, they should perform experiments to clarify the workflow. Additional results should be presented and include: a schematic diagram of the workflow, experimental results, and calculations of efficiency and accuracy.

Major concerns
1. (Lines 36-38) The Introduction section discusses DNA manipulation methods, including in vitro approaches. However, the description of their limitations (e.g., time-consuming, challenging to automate, expensive, and fragile) seems inappropriate. While highlighting the novelty and significance of this study is essential, the current phrasing may unfairly criticize widely recognized methods. I recommend revising this section to simplify the critique of existing methods and instead emphasize the novelty and gap-filling contributions of this work.
2. The authors employed different TIRs (translation initiation regions) to modify protein expression levels (e.g., tetA in Figures 1 and 3). Why did they not use and screen commonly reported "promoter-ribosome binding site" combinations (e.g., J23100-B0034 for strong expression or J23109-B0033 for weak expression)? The necessity of TIR screening over using well-characterized parts should be explained.
3. (Lines 230-231) The authors report the in vivo recombineering of two co-transformed linear DNA fragments but only provide plate images in Supplementary Figure 10. Detailed calculations and statistical analyses should be included to support the claims.

Minor concerns
1. (Lines 60-61) Provide a brief explanation of why tetA is sensitive to NiCl2, as this may not be familiar to all readers.
2. (Lines 73-74) Clarify the reason of the instability in the double selection cassette (e.g., mutation or cell toxicity).
3. Ensure consistent formatting of value-unit pairs. For example, “0μM” in Figure 1e lacks a space, whereas “100 μM” in line 383 includes one. Review and correct such inconsistencies throughout the manuscript.
4. Remove undesired black borders from several figures, including Figures 1e, 1f, 3f, 5b-5d, 5k-5m, and Supplementary Figures 5b-5d, 8c, 9g.
5. Standardize formatting for uppercase/lowercase usage (e.g., “hours” in Figure 1e and “Hours” in Figure 1f).
6. Provide details on statistical analyses, including error bar meanings and the number of biological replicates for each figure with numerical data (e.g., Figures 1e and 1f).
7. (Line 146) Briefly explain the function of the small antibody-encoding fragment.
8. (Line 176) Add a legend entry for Figure 2j.
9. (Lines 192 and 196) Ensure consistency in describing the copy number of pBR322-ROP. Clarify whether pET series plasmids are medium- or high-copy-number systems. To my knowledge, the pET series plasmids contain the same ori as pBR322-ROP.
10. (Figure 4) Correct spelling errors for “Fluorescence” in Figures 4c-4f and 4h. Check for similar typos throughout the manuscript.
11. (Lines 312-313) Provide references supporting the toxic effects of the galK gene.
12. Correct subscript formatting issues (e.g., “CaCI2” in line 383 and “H2O” in line 386).
13. (Line 423) Revise “1,5mL” to “1.5 mL”.
14. (Supplementary Figures) Many Supplementary Figures (e.g., Supplementary Figure 2f) have low resolution. Provide higher-resolution versions.
15. (Supplementary Figure 2a) What is the function of oriT in this study? Please explain this.
16. (Supplementary Figure 2) Correct the second “e)” in the Supplementary Figure 2 legend to “g)”.
17. (Supplementary Figure 4) Add an explanation of the samples in different lanes.
18. (Supplementary Figure 5) Check whether “Illustration of N-terminally truncated” should be “Illustration of C-terminally truncated”.
19. It is recommended to include a list of DNA sequences used in the study (e.g., GFP, tetA, nanobody, crtEBIY) to help readers better understand and follow the work. This information could be provided in the Supplementary Information.

Reviewer #2

(Remarks to the Author)
Sepulchro et al., in the manuscript titled “Simple and robust in vivo engineering of plasmid DNA at any copy number,” describe an in vivo recombineering approach for modifying plasmid DNA in Escherichia coli, overcoming common challenges associated with in vitro manipulation. The study presents a triple-selection system enabling robust recombineering at varying plasmid copy numbers. By integrating positive selection, counterselection, and fluorescence-based screening, the authors offer a streamlined methodology for plasmid modification, which could be widely applicable in synthetic biology and biotechnology.

I recommend the manuscript be published; however, some additional requirements need to be addressed:

In the introduction, the authors mention in vivo cloning methods using overlapping fragments, such as Gibson Assembly, as “incremental improvements,” while these methods completely transformed how we assemble DNA. That should be corrected.

In the introduction, the authors did not properly cite newer publications. For example, how much improvement do the current methods provide over this study?
Kostylev M, et al. “Cloning Should Be Simple: Escherichia coli DH5α-Mediated Assembly of Multiple DNA Fragments with Short End Homologies.” PLoS One. 2015 Sep 8;10(9):e0137466. doi: 10.1371/journal.pone.0137466. PMID: 26348330; PMCID: PMC4562628.

How about multiple fragment incorporation? Did the authors try that? At the very least, there needs to be a paragraph discussing what was done and whether it is predicted to work with their system.

The authors should provide a more comprehensive discussion of prior work related to in vivo recombineering for plasmid modification. While the challenges of plasmid copy number and multimer formation are mentioned, previous approaches to addressing these issues should be reviewed in greater detail to contextualize the novelty of the present study. It would be beneficial for the authors to discuss the potential drawbacks of the approach, particularly in comparison to other DNA assembly techniques, such as Gibson Assembly or CRISPR-based methods.

It would be worth describing the bicistronic design in this manuscript's introduction.

The authors state that a large DNA fragment, which is actually only 4542 bp, can be incorporated. With the advancement of synthetic biology, such fragments are no longer considered large. They also required extensive purification of clones by restreaking to obtain the correct clones. Overall, the authors need to add a paragraph describing the limitations of their technology.

Reviewer #3

(Remarks to the Author)
In this paper, the authors report on an in vivo plasmid DNA construction method using recombinase in bacterial cells. This method is claimed to have achieved high efficiency and accuracy by using a triple selection system that uses negative selection based on NiCl2 sensitivity by TetA, positive selection by reconstructing a chloramphenicol-resistant gene, and GFP fluorescence as an indicator. Typically, designed plasmid constructs are prepared in vitro using expensive enzymes. The in vivo plasmid construction proposed by the authors could provide an inexpensive and rapid method. Thus, this paper may be of interest to many molecular biologists. However, several concerns are raised in this paper.

Major points:
(1) Stability: It was suggested that the expression level of tetA should be carefully adjusted to match the copy number of the plasmid. On the other hand, in pUCtune, where the copy number is variable, the authors report that the plasmid is “stable” with a single TIR optimized for the pBR322-ROP (line 217-218). Is pUCtune stable at all copy numbers (i.e., at all rhamnose concentrations)? The experimental procedures and results should be presented in detail. It is also necessary to discuss these seemingly contradictory observations.
(2) Fidelity: Plasmid recombination is judged to have high ACCURACY based on the size of the PCR product. However, point mutations and short indels have not been evaluated. If the authors propose this method as a generic platform for plasmid construction, evaluation by sequencing is necessary.
(3) Homogenicity: In the low copy number pRK2, heterologous plasmids due to different recombination events were found within a single colony. On the other hand, surprisingly, a high degree of homogeneity is observed in p15A and pBR322-ROP with high copy numbers. With high copy plasmids, multiple plasmids can be recombined differently, resulting in a heterogeneous plasmid population within a single bacterial cell. For example, if a plasmid with a functional chloramphenicol gene derived from correct recombination arises, a plasmid with only a nonfunctional chloramphenicol gene that has simply lost its selection cassette will survive in the same bacterial cell. The authors observation probably is due to differences in the transformation procedure. Possible mechanisms should be discussed in detail.

Minor points:

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“a piece of DNA”
rfp?

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“The graph shows fluorescence relative to the wild-type strain without plasmid.”
Why not simply evaluate fluorescence intensity minus background?

Figure 2d
The magnification of the photo is too low to see the individual colonies clearly.

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What was the phenotype? Was the synthesis of β-carotene confirmed?

Figure 3f
Is the integration efficiency of nanobody in pUC and pUCtune 0 or ND?

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Version 1:

Reviewer comments:

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I sincerely appreciate the authors for fully addressing my concerns. The quality of the revised manuscript has been significantly improved. I recommend that this revised version be accepted for publication. Congratulations to the authors on their excellent work!

Reviewer #2

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The authors of the manuscript titled 'Simple and robust in vivo engineering of plasmid DNA at any copy number' describe an in vivo recombineering approach for modifying plasmid DNA in Escherichia coli, overcoming common challenges associated with in vitro manipulation. They have addressed all my concerns, and I therefore recommend that the manuscript be published.

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R: We thank the reviewer for raising this point. We have now performed a stability assessment over time with different rhamnose concentrations and additionally performed the cloning with 100 mM of rhamnose – a high copy number situation. The description of these experiments is included in the revised version of the manuscript (Lines 250 - 256) and the data in the revised version of the supplementary information Figure 10.

(my 2nd round comment)
Supplementary Fig. 10i do not clearly show the stability of the plasmid; please present the data as in Figure 1f (horizontal axis: time, vertical axis: fluorescence intensity).
Positive and negative control experiments are needed to evaluate stability. The authors should show the results of other plasmids whose stability or instability is clear.
In Supp Fig 10, fluorescence is shown as a relative value only in "i". Please define what the fluorescence is normalized to.
Data points overlap and are not legible. Please improve them.

Detailed Response to the Reviewers´ and Editor´s comments

We would like that thank the three reviewers for their very kind and detailed comments. We are confident that the manuscript has improved considerably due to this process. Detailed responses are marked with an R below and highlighted in green font. Furthermore, a version of the revised manuscript with track-changes have been uploaded together with the clean version.

Reviewer #1 (Remarks to the Author):

Brief summary and recommendation

In this study, the authors developed a methodology for in vivo engineering of plasmids applicable to any copy number. Specifically, they constructed a triple-selection cassette comprising a GFP expression module (distinguishable by colors) and two selection modules (tetA: responsive to tetracycline and NiCl2, and cat: responsive to chloramphenicol). This three-component cluster facilitates the selection and characterization of recombinant plasmid DNA when combined with Lambda-Red homologous recombination technology, exhibiting high efficiency and accuracy. Additionally, they extended the system's application to screen sequence libraries. The results demonstrate the predictability, availability, and reliability of the proposed design.

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<u>Key concern:</u>

1. According to the Methods section, enzymes for Lambda-Red recombination are expressed from a plasmid (pSIM19), which facilitates the production of recombinant DNA in vivo. However, it is unclear how pure constructs can be obtained from E. coli that harboring two plasmids (pSIM19 and the target construct) after manipulation. The manuscript does not provide a detailed explanation of this process. If the authors have conducted experiments addressing this, they should include the detailed results in the revised manuscript. Otherwise, they should perform experiments to clarify the workflow. Additional results should be presented and include: a schematic diagram of the workflow, experimental results, and calculations of efficiency and accuracy.

R: We thank the reviewer for pointing out the lack of such an important explanation in our manuscript. The auxiliary plasmid responsible for expressing lambda-red enzymes is under control of a temperature-sensitive version of the pSC101origin of replication, which is cured at 37 °C. We have now provided a workflow and performed experiments that evaluate the

curing efficiency. This workflow and explanation is now presented in the main manuscript (Lines 178-182) and in the supplementary information Figure 7.

Major concerns:

1. (Lines 36-38) The Introduction section discusses DNA manipulation methods, including in vitro approaches. However, the description of their limitations (e.g., time-consuming, challenging to automate, expensive, and fragile) seems inappropriate. While highlighting the novelty and significance of this study is essential, the current phrasing may unfairly criticize widely recognized methods. I recommend revising this section to simplify the critique of existing methods and instead emphasize the novelty and gap-filling contributions of this work.

R: Thank you for this advice! We have revised the section accordingly

2. The authors employed different TIRs (translation initiation regions) to modify protein expression levels (e.g., tetA in Figures 1 and 3). Why did they not use and screen commonly reported "promoter-ribosome binding site" combinations (e.g., J23100-B0034 for strong expression or J23109-B0033 for weak expression)? The necessity of TIR screening over using well-characterized parts should be explained.

R: We previously successfully optimized the expression of *tetA* from the genome and therefore chose the same approach. We have now added this information to the section.

3. (Lines 230-231) The authors report the in vivo recombineering of two co-transformed linear DNA fragments but only provide plate images in Supplementary Figure 10. Detailed calculations and statistical analyses should be included to support the claims.

R: The requested calculations and analyses were done and added to the supplementary data (previous Supplementary Figure 10 now Supplementary Figure 11)

Minor concerns

1. (Lines 60-61) Provide a brief explanation of why tetA is sensitive to NiCl2, as this may not be familiar to all readers.

R: A more detailed explanation on TetA counterselection and sensitivity towards $NiCl_2$ was added to the revised version of the manuscript (to our knowledge it is not known with certainty why *tetA* expression makes cells nickel-sensitive, but the given reference suggests the explanation that nickel is transported directly by the protein).

2. (Lines 73-74) Clarify the reason of the instability in the double selection cassette (e.g., mutation or cell toxicity).

R: We clarified this point by exchanging *excessive burden* with *negative fitness effect*. Further, we have now detailed that it led to total or partial deletion of the cassette.

3. Ensure consistent formatting of value-unit pairs. For example, "0µM" in Figure 1e lacks a space, whereas "100 µM" in line 383 includes one. Review and correct such inconsistencies throughout the manuscript.

R: Corrections were made throughout the manuscript as requested by the reviewer.

4. Remove undesired black borders from several figures, including Figures 1e, 1f, 3f, 5b-5d, 5k-5m, and Supplementary Figures 5b-5d, 8c, 9g.

R: The undesired black borders seem to be a result of the online pdf conversion. We have tried to re-generate the figures with a different background to avoid this.

5. Standardize formatting for uppercase/lowercase usage (e.g., "hours" in Figure 1e and "Hours" in Figure 1f).

R: Figure 1 was modified accordingly.

6. Provide details on statistical analyses, including error bar meanings and the number of biological replicates for each figure with numerical data (e.g., Figures 1e and 1f).

R: The requested details were added to the relevant figure legends. Graph 1f was modified so that all individual data are displayed.

7. (Line 146) Briefly explain the function of the small antibody-encoding fragment.

R: A more detailed explanation was added to the revised version of the manuscript.

8. (Line 176) Add a legend entry for Figure 2j.

R: The missing legend entry was added to the revised version of the manuscript.

9. (Lines 192 and 196) Ensure consistency in describing the copy number of pBR322-ROP. Clarify whether pET series plasmids are medium- or high-copy-number systems. To my knowledge, the pET series plasmids contain the same ori as pBR322-ROP.

R: Thank you for pointing this out. It is true that the pET plasmids contain pBR322-ROP and that this is usually considered a medium copy plasmid. For this reason, and for consistency throughout the manuscript, we have now changed the description of the plasmids to low (RK2), low-medium (p15a), medium (pBR322-ROP) and high (pUC).

10. (Figure 4) Correct spelling errors for "Fluorescence" in Figures 4c-4f and 4h. Check for similar typos throughout the manuscript.

R: We apologize for spelling mistakes throughout the manuscript. An in-depth correction was made to correct all typos in the revised version of the manuscript.

11. (Lines 312-313) Provide references supporting the toxic effects of the galK gene.

R: This statement was based on our own empirical observations when trying to clone *galk*, which is why used the phrase "we have previously observed".

12. Correct subscript formatting issues (e.g., "CaCl2" in line 383 and "H2O" in line 386).

R: This was corrected in the revised version of the manuscript.

13. (Line 423) Revise "1,5mL" to "1.5 mL".

R: This was corrected in the revised version of the manuscript.

14. (Supplementary Figures) Many Supplementary Figures (e.g., Supplementary Figure 2f) have low resolution. Provide higher-resolution versions.

R: Figure resolutions were improved to the extent possible in the revised version of Supplementary Information.

15. (Supplementary Figure 2a) What is the function of oriT in this study? Please explain this.

R: oriT is the origin of transference that allows for conjugative mobilization of plasmids into organisms in which no alternative transformation protocols are available. We added this information to the legend.

16. (Supplementary Figure 2) Correct the second "e)" in the Supplementary Figure 2 legend to "g)".

R: This was corrected in the revised version of the Supplementary information.

17. (Supplementary Figure 4) Add an explanation of the samples in different lanes.

R: A more detailed explanation was added to the revised version of the Supplementary information.

18. (Supplementary Figure 5) Check whether "Illustration of N-terminally truncated" should be "Illustration of C-terminally truncated".

R: This was corrected in the revised version of Supplementary information, and now it states, "Illustration of C-terminally truncated."

19. It is recommended to include a list of DNA sequences used in the study (e.g., GFP, tetA,

nanobody, crtEBIY) to help readers better understand and follow the work. This information could be provided in the Supplementary Information.

R: Following the reviewer's recommendation, all sequences were added to the revised version of Supplementary information.

Reviewer #2 (Remarks to the Author):

Sepulchro et al., in the manuscript titled "Simple and robust in vivo engineering of plasmid DNA at any copy number," describe an in vivo recombineering approach for modifying plasmid DNA in Escherichia coli, overcoming common challenges associated with in vitro manipulation. The study presents a triple-selection system enabling robust recombineering at varying plasmid copy numbers. By integrating positive selection, counterselection, and fluorescence-based screening, the authors offer a streamlined methodology for plasmid modification, which could be widely applicable in synthetic biology and biotechnology.

I recommend the manuscript be published; however, some additional requirements need to be addressed:

In the introduction, the authors mention in vivo cloning methods using overlapping fragments, such as Gibson Assembly, as "incremental improvements," while these methods completely transformed how we assemble DNA. That should be corrected.

R:We have edited this section in response to the comments from both reviewer #1 and #2.

In the introduction, the authors did not properly cite newer publications. For example, how much improvement do the current methods provide over this study?
Kostylev M, et al. "Cloning Should Be Simple: Escherichia coli DH5α-Mediated Assembly of Multiple DNA Fragments with Short End Homologies." PLoS One. 2015 Sep 8;10(9):e0137466. doi: 10.1371/journal.pone.0137466. PMID: 26348330; PMCID: PMC4562628.

R: Thank you for this comment. It is not possible to mention or cite all methods developed for DNA assembly, but we agree that the mentioned method is particularly relevant due to the similarity to our approach. We have added a small paragraph on this in the discussion with relevant references.

How about multiple fragment incorporation? Did the authors try that? At the very least, there needs to be a paragraph discussing what was done and whether it is predicted to work with their system.

R: Thank you for this suggestion. We designed and performed a new experiment where we split *rfp* in two fragments so we could assess the incorporation of multiple fragments. The

description of this can be found in the revised version of the main manuscript (Lines 267 - 271) and the supporting data on the revised version of the Supplementary Information Figure 12.

The authors should provide a more comprehensive discussion of prior work related to in vivo recombineering for plasmid modification. While the challenges of plasmid copy number and multimer formation are mentioned, previous approaches to addressing these issues should be reviewed in greater detail to contextualize the novelty of the present study. It would be beneficial for the authors to discuss the potential drawbacks of the approach, particularly in comparison to other DNA assembly techniques, such as Gibson Assembly or CRISPR-based methods

.

R: Thank you for this suggestion. In the introduction, we have added some additional background on prior attempts to in vivo modify plasmids and as described in response to reviewer comments above, we have also added a sentence to explain when in vivo recombination might be preferred over in vitro methods.

It would be worth describing the bicistronic design in this manuscript's introduction.

R: We added a description of the BCD to the introduction.

The authors state that a large DNA fragment, which is actually only 4542 bp, can be incorporated. With the advancement of synthetic biology, such fragments are no longer considered large. They also required extensive purification of clones by restreaking to obtain the correct clones. Overall, the authors need to add a paragraph describing the limitations of their technology.

R: We modified the description of this experiment and added a sentence in the discussion describing this limitation.

Reviewer #3 (Remarks to the Author):

In this paper, the authors report on an in vivo plasmid DNA construction method using recombinase in bacterial cells. This method is claimed to have achieved high efficiency and accuracy by using a triple selection system that uses negative selection based on NiCl2 sensitivity by TetA, positive selection by reconstructing a chloramphenicol-resistant gene, and GFP fluorescence as an indicator. Typically, designed plasmid constructs are prepared in vitro using expensive enzymes. The in vivo plasmid construction proposed by the authors could provide an inexpensive and rapid method. Thus, this paper may be of interest to many molecular biologists. However, several concerns are raised in this paper.

Major points:

(1) Stability: It was suggested that the expression level of tetA should be carefully adjusted to match the copy number of the plasmid. On the other hand, in pUCtune, where the copy number is variable, the authors report that the plasmid is "stable" with a single TIR optimized for the pBR322-ROP (line 217-218). Is pUCtune stable at all copy numbers (i.e., at all rhamnose concentrations)? The experimental procedures and results should be presented in detail. It is also necessary to discuss these seemingly contradictory observations.

R: We thank the reviewer for raising this point. We have now performed a stability assessment over time with different rhamnose concentrations and additionally performed the cloning with 100 mM of rhamnose – a high copy number situation. The description of these experiments is included in the revised version of the manuscript (Lines 250 - 256) and the data in the revised version of the supplementary information Figure 10.

(2) Fidelity: Plasmid recombination is judged to have high ACCURACY based on the size of the PCR product. However, point mutations and short indels have not been evaluated. If the authors propose this method as a generic platform for plasmid construction, evaluation by sequencing is necessary.

R: Thank for raising another good point! We sequenced 48 clones and the description of this can be found in the revised version of the manuscript (Lines 150- 153, Lines 161-163 and Lines 400 - 404).

(3) Homogenicity: In the low copy number pRK2, heterologous plasmids due to different recombination events were found within a single colony. On the other hand, surprisingly, a high degree of homogeneity is observed in p15A and pBR322-ROP with high copy numbers. With high copy plasmids, multiple plasmids can be recombined differently, resulting in a heterogeneous plasmid population within a single bacterial cell. For example, if a plasmid with a functional chloramphenicol gene derived from correct recombination arises, a plasmid with only a nonfunctional chloramphenicol gene that has simply lost its selection cassette will survive in the same bacterial cell. The authors observation probably is due to differences in the transformation procedure. Possible mechanisms should be discussed in detail.

R: Thank for this suggestion. We have now expanded on this topic in the discussion.

Minor points:

Line 106
"a piece of DNA"
rfp?

R: "A piece of DNA" was replaced by *rfp* in the revised version of the manuscript.

line 102-103
"The graph shows fluorescence relative to the wild-type strain without plasmid."
Why not simply evaluate fluorescence intensity minus background?

R: As data was collected over many days, this normalization was necessary to make up for the differences in measurements that the equipment can present over longer time scales.

Figure 2d
The magnification of the photo is too low to see the individual colonies clearly.
R: Magnification squares were made bigger in the revised version of the manuscript.

Line 146
"a small (351 bp) antibody-encoding fragment"
Nonobody?

R: Following this note as well as the one raised by Reviewer 1, further clarification on the nanobody segment was given.

Line 154-155
"analyzing non-green colonies by diagnostic PCR"
What was the phenotype? Was the synthesis of β-carotene confirmed?

R: Experiments aiming at verifying the β-carotene synthesis were performed using screening of the absorbance at different wavelengths, and the result can be found in the supplementary Figure 6.

Figure 3f
Is the integration efficiency of nanobody in pUC and pUCtune 0 or ND?

R: In the initial version of this manuscript, we did not have the data. In the revised version, however, we performed nanobody integration on pUC, and the data was added to the revised version of the manuscript. The new data can be found in Figure 3 of the revised manuscript.

Detailed Response to the Reviewers´ and Editor´s comments

Reviewer #1 (Remarks to the Author):

I sincerely appreciate the authors for fully addressing my concerns. The quality of the revised manuscript has been significantly improved. I recommend that this revised version be accepted for publication. Congratulations to the authors on their excellent work!

R: Thank you very much!

Reviewer #2 (Remarks to the Author):

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R: We thank the reviewer for raising this point. We have now performed a stability assessment over time with different rhamnose concentrations and additionally performed the cloning with 100 mM of rhamnose – a high copy number situation. The description of these experiments is included in the revised version of the manuscript (Lines 250 - 256) and the data in the revised version of the supplementary information Figure 10.

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Supplementary Fig. 10i do not clearly show the stability of the plasmid; please present the data as in Figure 1f (horizontal axis: time, vertical axis: fluorescence intensity).
Positive and negative control experiments are needed to evaluate stability. The authors should show the results of other plasmids whose stability or instability is clear.
In Supp Fig 10, fluorescence is shown as a relative value only in "i". Please define what the

fluorescence is normalized to.
Data points overlap and are not legible. Please improve them.

R: We apologize for not addressing this point fully in the first revision. We have now revised the figure so that the data is plotted as in Figure 1f, added a stable (positive) control, and explained the normalization in the legend. We would argue that it is not possible to meaningfully include an unstable (negative) control because upon transformation of such constructs fluorescent clones simply do not occur. In addition to the revised figure, we have described the observations in more detail on page 10 in the manuscript: *"In the absence of rhamnose, the fluorescence from pUC^{TUNE} indicates a copy number that is close to that of the low-copy RK2 plasmid, but the plasmid nevertheless seems stable with the TIR selected for the medium copy number plasmid. This may indicate that this tetA TIR is stable in plasmids with a copy number higher than RK2."*