

Peer Review File

A Frugal CRISPR Kit for Equitable and Accessible Education in Gene Editing and Synthetic Biology



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Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

Summary of results

The authors of this manuscript have developed a kit for conducting CRISPRi in K-12 education classrooms. The experimental design involves using CRISPRi to block transcription of introduced chromoproteins in a cell free system, allowing for easy visualization of color readouts. The authors first established the baseline performance of their CRISPRi system in a typical 'high-tech' laboratory setting, and then tested the performance in a 'low-tech' set-up. Finally, the authors tested how the kit would perform in the hands of high school students. The overall approach is sound and the data from each of their experiments suggests that this kit will be an exciting and feasible way for students in high school classrooms to both learn about and implement CRISPRi techniques.

The authors have presented both the kit as an education tool as well as a study of the kit performance. Below I have divided my comments based on both.

Review of the CRISPRi kit as an education tool:

Strengths: The kit is presented as low-cost and easy to implement in a classroom setting, and the use of chromoprotein expression is new – the focus on an easily visualizable effect is a strength. The videos showcase how to use the kit and the supplement includes notes for teachers, and the authors have developed a novel tool for quantifying chromoprotein expression.

Weaknesses: The authors have developed a unique kit that leverages CRISPRi. CRISPR kits themselves are not novel, there are kits from the Innovative Genomics Institute (<https://store.lab-aids.com/kits-and-modules/the-power-of-crispr-base-unit-and-consumable-package>; currently \$1642 for 5 packages) and BioRad (<https://www.bio-rad.com/en-us/product/out-blue-crispr-genotyping-extension-kits?ID=Q0JGD4E0801Y>; currently \$262 per kit), these include resources for classroom use including NGSS connections and powerpoints for teachers. Kits have previously been developed for remote instruction during the COVID-19 pandemic (McDonnell et al., 2022). When compared to what has already been published or is available, two weaknesses of the presentation of this kit as a tool are a need for clarity of cost, and strength of the in the instructions and resources for teachers.

Suggestions:

1. Clarity of cost: Throughout the manuscript the authors frequently have commented on how the kit is a frugal set-up compared to experiments with CRISPR in a high tech setting. While it's clear this is the case, the authors have not included a total cost for the kit itself – a current cost for the reagents included will help emphasize that the kit is feasible for high school classrooms.
2. Protocol: While the authors have included resources for use, there is room for improvement.
 - a. The manual for teachers is missing instructions for teachers for how to store the kits until use.
 - b. The manual for teachers is missing information for how to dispose of the tubes, loops, and liquids. It will help to include confirmation that EHS has verified that bleach and typical trash disposal (if bleach is sufficient) will be enough.
 - c. The manual for teachers would benefit from specific instructions. For example, in the methods the authors say that an arms length works for images – depending on the age of the students conducting the experiments there may be wide variation in arms length, which could result in variation in pixel resolution. I would encourage the authors to specify that consistency in distance for the images will be one way of reducing this variation.
 - d. It would be helpful if the authors provided recommendations based on the high school students'

implementation of the kits. Do they recommend one kit per class, one kit per pair of students, one kit per student?

e. Since students will be setting up several reactions with the reagents, a worksheet or table that they can use to check off which reagents have been added might minimize risk of contamination or missing addition of reagents. This would be a good addition to Supplemental Note 2.

3. Curriculum

a. K-12 standards: I appreciate that the authors included core concepts related to the CRISPR kit. It would strengthen the manuscript and may encourage use from additional teachers if the authors include a list of high school biology standards that can be supported by using the kit. For example, the next-generation science standards may be a good place to start.

b. Building off of which standards are supported by the kit, it would be beneficial for teachers to have recommendations for how to integrate the lab in with their curriculum. Specifically, what background knowledge students should have in order to maximize their understanding in lab. There are resources such as Harvard's EdX LabXchange that might be a good reference to include in curriculum for preparing students to conduct CRISPRi experiments.

c. It would be helpful for other educators to know the level of biology course that tested the kit – was this in a general high school biology course, AP biology course, etc?

Review of the CRISPRi kit performance between in bench lab and classroom settings:

Strengths: The approach of first testing feasibility of the kit, then comparing bench and low-cost settings, and finally including kit results when in the hands of high school students is sound.

Weaknesses:

Data and methodology: One concern I have about the data and methodology is the small sample size for each of the experiments. Throughout the manuscript the authors relied on a sample size of 3 for each condition. Since the experimental set-up was in vitro, I wonder why they did not aim for an N of ~10 for each condition, which would have allowed them to conduct statistical tests between conditions. With the current data there are clear trends and differences identified, but this is a large limitation that could have been addressed during their experimental design.

Analytical approach:

The researchers have mixed biological and technical replicates in their figures and conclusions throughout the paper.

In Figure 3B the authors present the normalized fluorescence for each condition. There are six datapoints in each of these conditions, but these six represent only 3 biological replicates with 2 technical replicates each, and they are presenting the mean – was the mean calculated from the 3 biological replicates or from the six samples? Clarifying the mean is from the biological replicates (e.g. the mean value of the two technical replicates for each sample) will help if this is the case.

I would encourage the authors to think about what information should be conveyed from each graph. I believe the focus of Figure 3B is to showcase the fold of repression, and not show the consistency of repeated measurements of a sample. I think the most helpful information to keep in this figure would be the data from biological replicates. I do see value of presenting technical replicates to showcase consistency of the approaches for measuring individual samples - If presenting the six replicates, I strongly suggest that the authors color code which technical replicates were associated with each biological replicates. The authors could present a table showcasing the values of each of the technical replicates per sample, to highlight the consistency of measurements.

Second, the manuscript is currently lacking statistical tests. In figure 2D the authors present means fluorescence values of three biological replicates. This would benefit from an ANOVA with post-hoc tests. The fold differences are striking and convincing on their own, but it should be feasible to add in these tests.

Suggestions:

1. Throughout: While I acknowledge that the authors may not have the resources to increase their sample size, I encourage the authors to separate biological and technical replicates, and use the ones that are most appropriate for their questions. For example, in figure 3B the authors should present only the biological replicates.
2. Clarity of what data is presented:
 - a. Supplemental Figure 1A: the caption states there are results from three biological replicates presented, but there are 6 dots. Are the additional dots technical replicates?
 - b. Figure 4D: same comment
 - c. Supplemental Figure 2B: It appears technical replicates are being presented in figure 2B. I don't think these should be included, since presenting repeated measurements of the same sample being included in a correlation is misleading. The correlation should only be on biological replicates, and I think it will be more appropriate to compare for 1 individual sample the mean of its technical replicates from plate reader data with the mean of its technical replicates for the smart phone data.
 - d. Figure 4F: the caption states this is biological replicates only and is based on data from 4c and 4d with 3 biological replicates, but since Figure 4D appears to include more than 3 replicates this is a bit confusing. I advise checking that this is only biological replicates.
 - e. Figure 5C: there is data from 8 experiments here but more than 8 dots per condition, similar comment as before – either present means of technical replicates or make it clear which are technical replicates for each sample. As I mentioned earlier in the reviews, the authors could present a table showcasing the values of each of the technical replicates per sample, to highlight the consistency of measurements.

Other minor concerns

1. Figure 2A: I believe the authors should check the directionality of their PAM sequences – the figure shows a PAM site on the bottom strand “GGN” and another PAM site on the top strand “GGN.” One of these should be NGG. This figure would benefit from 5' and 3' directionality.
2. This is a small point, but since the high school students tested the materials and their data is included in the manuscript I would suggest to the authors that they include the students in their acknowledgments.

Reviewer #2:

Remarks to the Author:

Key results

In this work by Collins et al., CRISPR kits were designed for K-12 use. These CRISPR kits utilize a cell free system (CFS), dCas9, and chromoproteins, which are proteins with pigmented prosthetic groups that can be visualized without specialized equipment. The sgRNAs provided in the kit each target a single chromoprotein (eForRed, fwYellow, and aeBlue), and a color change readout can be observed by eye or determined through plate reader measurements. Importantly, the authors also developed an algorithm named CRISPECTRA that enables smartphone-based quantification of results, eliminating the requirement for a plate reader. High school students were able to successfully use the frugal CRISPR kit.

Data, methodology, and validity

In general, the data presented here support the conclusions and claims of the authors. Data obtained from CRISPR experiments were analyzed in one of three ways: 1) fluorescence measurements from a plate reader, 2) visual inspection of reaction tubes or droplets on parafilm, and 3) quantification of smartphone images using the CRISPECTRA algorithm. The authors present convincing evidence that smartphone quantifications are comparable to plate reader measurements (SFig. 2B and Fig. 4F) and adequately address the variability in smartphone quantification by high school students (Fig. 5C).

Concerns related to data interpretation and conclusions:

Visual inspection and interpretation is clear and straightforward in single-color CRISPR experiments (Figs. 2F and 2G). However, it is less clear in dual-color CRISPR experiments in Figs. 3C, 4D, and 5B. Two suggestions: 1) either label the images directly with the expected color (ex: orange) or color-code the labeling text as appropriate (ex: R + Y written in orange text), 2) improve the overall quality of the images used in these panels. This observation does not significantly affect the findings in the manuscript, but does emphasize the importance of the quantification tool CRISPEctra for those who are visually impaired or have difficulty interpreting color as such (see below).

In panel 6E, addition of sgYellow leads to a general knockdown/decrease in chromoprotein color that is not addressed. This problem does not occur in Figure 2, and on pg. 8 the authors note "We also confirmed that the sgRNAs acted orthogonally and only repressed their cognate targets." Please address this discrepancy.

Significance

Compared to previous literature (for example, Huang et al. 2018), the significant advancements described here are 1) the design of CRISPR experiments that can be performed in a K-12 setting without standard laboratory equipment such as incubators or pipettes, and 2) the creation of a smartphone-based algorithm for quantification of visual color-change readouts. These advancements emphasize the authors' commitment to providing accessible science tools for students of all socioeconomic backgrounds. This manuscript can represent a significant contribution to the field of K-12 educational resources, but relies on distribution, affordability, and accessibility of the designed CRISPR kits. The website (crisprkit.org) demonstrates accessibility of K-12 protocols and ability to contact the manuscript authors for kits. The manuscript may not provide a significant contribution to the field of synthetic biology (see below).

Concerns related to significance:

Cell-free systems have been used previously in K-12 settings (Huang et al. 2018), which is noted by the authors. In addition, the BioBits™ kits can be designed with multiple readout types including detection of fragrances and hydrogels, allowing these kits to address several types of biological questions which are more broadly interesting. While the authors' implementation of CRISPR kits in a K-12 setting is noteworthy and important for equitable access to this biological tool, the biological questions that can currently be addressed with the kit are quite limited in scope.

The discussion could be strengthened by highlighting other biological questions that can be addressed by students using this system. The question currently posed, "Which genes are in the tube?" may be suitable for middle school but does not represent an opportunity for advanced high schoolers to consider meaningful biological questions using this kit.

In Figure 1a and its corresponding title legend, the authors emphasize the biosafety and biohazard concerns of using CRISPR. However, CRISPR is routinely performed in microorganisms such as *S. cerevisiae* and does not require specialized BSL2 facilities or tissue culture equipment. While I appreciate that CFS eliminates any requirement for cellular or biohazard disposal, I believe that some organisms would be safe to work with in high school settings and that the concerns here are overstated.

The CFS system used here is commercially available and was not designed by the authors, while the sgRNA and chromoprotein plasmids were. This distinction is important for the consideration of the manuscript's significance to the synthetic biology field, which may be limited. While the use of a commercial mastermix is clear from the methods section, it could be explicitly stated in the results section.

A concern above (related to interpretation of results) relies on the ability to use the CRISPEctra analysis algorithm which is not easily accessible (or accessible at all?) from the website. After watching Movie S2, I located the website (<https://myckitresults.com/>). CRISPEctra may not be user-friendly unless its use is accompanied by direct instruction and/or the video is linked/available. I would recommend 1) modifications to the website to include detailed

instructions on each page corresponding to each step, and/or 2) a link to the video on the first page.

While the authors state that the price of the frugal CRISPR kit may eventually reach <\$1/kit, there is no discussion of the current cost. This should be explicitly stated in the sentence "We manufactured the frugal CRISPR kits in bulk and distributed them..." on pg. 12. In the discussion, the cost could be compared to other kits used at the K-12 level such as BioBits™ kits which range from \$85-\$225.

Panel 6F shows a schematic of chromoprotein colors that are not yet available for the CRISPR kits. Possible expansions of the kit may be more appropriate for the discussion, with Fig. 6F being moved to the supplementary material.

Response to Reviewer Comments

Reviewer #1:

The authors of this manuscript have developed a kit for conducting CRISPRi in K-12 education classrooms. The experimental design involves using CRISPRi to block transcription of introduced chromoproteins in a cell free system, allowing for easy visualization of color readouts. The authors first established the baseline performance of their CRISPRi system in a typical 'high-tech' laboratory setting, and then tested the performance in a 'low-tech' set-up. Finally, the authors tested how the kit would perform in the hands of high school students. The overall approach is sound and the data from each of their experiments suggests that this kit will be an exciting and feasible way for students in high school classrooms to both learn about and implement CRISPRi techniques. The authors have presented both the kit as an education tool as well as a study of the kit performance. Below I have divided my comments based on both.

Response: Thank you for recognizing our work.

Review of the CRISPRi kit as an education tool:

Strengths: The kit is presented as low-cost and easy to implement in a classroom setting, and the use of chromoprotein expression is new – the focus on an easily visualizable effect is a strength. The videos showcase how to use the kit and the supplement includes notes for teachers, and the authors have developed a novel tool for quantifying chromoprotein expression.

Response: Thank you for recognizing the strengths of the work.

Weaknesses: The authors have developed a unique kit that leverages CRISPRi. CRISPR kits themselves are not novel, there are kits from the Innovative Genomics Institute (<https://store.lab-aids.com/kits-and-modules/the-power-of-crispr-base-unit-and-consumable-package>; currently \$1642 for 5 packages) and BioRad (<https://www.bio-rad.com/en-us/product/out-blue-crispr-genotyping-extension-kits?ID=Q0JGD4E08O1Y>; currently \$262 per kit), these include resources for classroom use including NGSS connections and powerpoints for teachers. Kits have previously been developed for remote instruction during the COVID-19 pandemic (McDonnell et al., 2022). When compared to what has already been published or is available, two weaknesses of the presentation of this kit as a tool are a need for clarity of cost, and strength of the in the instructions and resources for teachers.

Response: Thank you for mentioning the previous kits for classroom teaching and for suggesting improvements to strengthen our CRISPRkit. In response to these suggestions, we have implemented a much cheaper version of CRISPRkit by utilizing in-house prepared cell free reagents. These in-house reagents have significantly reduced the overall cost of CRISPRkit, as they were previously the major expense. We have provided a detailed cost breakdown of the reagents used in the current CRISPRkit, which is approximately \$2.01 per kit (**Table S4**). This cost includes both reagents (\$1.13 per kit) and plastic consumables (\$0.88 per kit).

In comparison, the previously reported BioBits™ Bright kit costs an estimated \$105 per kit (excluding plastic consumables, <https://www.minipcr.com/product-category/biobits/>). The lower cost of our CRISPRkit makes it a more accessible resource for classroom teaching related to CRISPR, especially in low-resource settings. Additionally, we have included more comprehensive instructions and resources for teachers to facilitate the integration of the kits into their curricula (**Supplementary Note 5**, see details below).

Suggestions:

1. Clarity of cost: Throughout the manuscript the authors frequently have commented on how the kit is a frugal set-up compared to experiments with CRISPR in a high tech setting. While it's clear this is the case, the authors have not included a total cost for the kit itself – a current cost for the reagents included will help emphasize that the kit is feasible for high school classrooms.

Response: In this revision, we have developed a more affordable version of CRISPRkit by utilizing in-house cell free system (i.e., in vitro transcription-translation reagents). These in-house reagents have significantly reduced the cost of the kit, as they were previously the major expense. We have provided a detailed cost sheet for our current CRISPRkit, which is approximately \$2.01 per kit (**Supplementary Table 4**). For a classroom of 40 students, with groups of 2 students performing one CRISPRkit experiment, the total cost is \$40.20. This low cost makes our kit a more affordable and accessible resource for classroom teaching, especially in low-resource settings.

2. Protocol: While the authors have included resources for use, there is room for improvement.

a. The manual for teachers is missing instructions for teachers for how to store the kits until use.

Response: We have added this information to **Supplementary Note 5: CRISPRkit Instructions for Teachers**. Briefly, we recommend that teachers store all reagents in a -20°C freezer for long-term storage, or in a 4°C fridge for short-term storage of 1-2 days. If the reagents are stored in a -20°C freezer, they should be thawed before classroom use.

b. The manual for teachers is missing information for how to dispose of the tubes, loops, and liquids. It will help to include confirmation that EHS has verified that bleach and typical trash disposal (if bleach is sufficient) will be enough.

Response: We have included information in **Supplementary Note 5** on how to dispose of the tubes, loops, and liquids, along with relevant safety information. According to the NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (Version April 2024) Section III-F-2 (Page 24, https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf), reagents that are not in organisms, cells, or viruses and have not been modified to penetrate cellular membranes can be disposed of as regular trash. Because the CRISPRkit is not used in organisms or cells and thus can be disposed of as regular trash (reference: <https://ehs.berkeley.edu/sites/default/files/76recombdna.pdf>).

c. The manual for teachers would benefit from specific instructions. For example, in the methods the authors say that an arms length works for images – depending on the age of the students conducting the experiments there may be wide variation in arms length, which could result in variation in pixel resolution. I would encourage the authors to specify that consistency in distance for the images will be one way of reducing this variation.

Response: We have included this information in **Supplementary Note 5: CRISPRkit Instructions for Teachers**. We recommend that all images be taken with the smartphone (or camera) placed 6 inches away from the imaging tubes to ensure optimal pixel resolution for downstream image analysis. This consistency in distance will help reduce variation in image quality.

d. It would be helpful if the authors provided recommendations based on the high school

students' implementation of the kits. Do they recommend one kit per class, one kit per pair of students, one kit per student?

Response: Thank you for the suggestion. Based on our testing in two high schools, we recommend 2-3 students per kit. We have found that a collaborative effort between 2-3 students enhances the learning experience. These recommendations have been added to **Supplementary Note 5**: CRISPRkit Instructions for Teachers and our website (also see [link](#)).

e. Since students will be setting up several reactions with the reagents, a worksheet or table that they can use to check off which reagents have been added might minimize risk of contamination or missing addition of reagents. This would be a good addition to Supplemental Note 2.

Response: Thank you for the suggestion. We have added a worksheet to help students track reagent addition, minimizing the risk of contamination or omission. This worksheet is now available on our website (see [link](#)) and has been included in **Supplementary Note 4**.

3. Curriculum

a. K-12 standards: I appreciate that the authors included core concepts related to the CRISPR kit. It would strengthen the manuscript and may encourage use from additional teachers if the authors include a list of high school biology standards that can be supported by using the kit. For example, the next-generation science standards may be a good place to start.

Response: As the kits are intended for high school students, we align our scientific standards with those outlined in the AP Biology curriculum, which includes six key areas: concept explanation, visual representations, questioning and methodology, representing and describing data, statistical tests and data analysis, and argumentation. CRISPRkit meets all these standards. Additionally, we have referenced to the Next Generation Science Standards (<https://www.nextgenscience.org>) to guide the development of CRISPRkit. We have included a detailed document explaining how the kit supports these biological standards in the **Supplementary Note 5** as well as on our website ([here](#)).

b. Building off of which standards are supported by the kit, it would be beneficial for teachers to have recommendations for how to integrate the lab in with their curriculum. Specifically, what background knowledge students should have in order to maximize their understanding in lab. There are resources such as Harvard's EdX LabXchange that might be a good reference to include in curriculum for preparing students to conduct CRISPRi experiments.

Response: In **Supplementary Note 5**, section 'How can the experiment be integrated into the curriculum?', we have provided detailed recommendations for integrating the lab into high school curricula. This section includes insights on the necessary background knowledge students should have to maximize their understanding and engagement in the lab. These recommendations are based on our testing with two high schools. Additionally, we suggest utilizing resources such as Harvard's EdX LabXchange (<https://www.labxchange.org>) to help prepare students for conducting CRISPRi experiments.

c. It would be helpful for other educators to know the level of biology course that tested the kit – was this in a general high school biology course, AP biology course, etc?

Response: While high school students of all levels should be able to conduct the experiment, for optimal understanding of the concepts and results, we recommend implementing this kit in an

AP Biology class or a biotechnology class. This information is included in **Supplementary Note 5**.

Review of the CRISPRi kit performance between in bench lab and classroom settings:
Strengths: The approach of first testing feasibility of the kit, then comparing bench and low-cost settings, and finally including kit results when in the hands of high school students is sound.

Response: Thank you for recognizing the strengths.

Weaknesses:

Data and methodology: One concern I have about the data and methodology is the small sample size for each of the experiments. Throughout the manuscript the authors relied on a sample size of 3 for each condition. Since the experimental set-up was in vitro, I wonder why they did not aim for an N of ~10 for each condition, which would have allowed them to conduct statistical tests between conditions. With the current data there are clear trends and differences identified, but this is a large limitation that could have been addressed during their experimental design.

Response: Thank you for making this important suggestion. To address the sample size issue, we have expanded the number of biological replicates for several key experiments. Specifically:

1. We have performed new experiments using the in-house cell-free system reagents for the dual-color experiment with N=10 biological replicates (**Figs. 5b, 5c**).
2. We have conducted additional tests on the dual-color experiment in an AP Biology class with N = 15 (approximately 40 students with groups of 2 or 3). This data is included in the new **Fig. 6d**. The two figures together demonstrate that our low-tech kit works satisfactorily both in the hands of a biological research professional and in the hands of high-school students.
3. We have added statistical tests in the figures. We have ensured that all experiments were conducted with at least N=3, which is sufficient to carry out the statistical analysis.

For example, we attach Fig 5b-5c with 10 biological replicates:



Figure 5 | Testing the frugal CRISPRkit using in-house cell-free system. b-c. Experimental data showing dual-color CRISPRi reactions using in-house CFS, measured by a fluorescent plate reader (b) or smartphone images and analyzed by CRISPECTRA (c). From left to right, each group of bars (red – eforRed, yellow – fwYellow) show eforRed single-color positive control, fwYellow single-color positive control, eforRed + fwYellow dual-color positive control, dual-color with eforRed-targeting sgRNA (sgRed), dual-color with fwYellow-targeting sgRNA (sgYellow), dual-color with both eforRed- and fwYellow-targeting sgRNAs, and negative control (water only). For b-c, ten biological replicates are plotted for each group. The bars represent the mean of biological replicates, and the error bars represent the standard deviation. Two one-way ANOVA were performed; * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001; n.s., not significant.

Data from new classroom testing in **Fig. 6d** with more students:

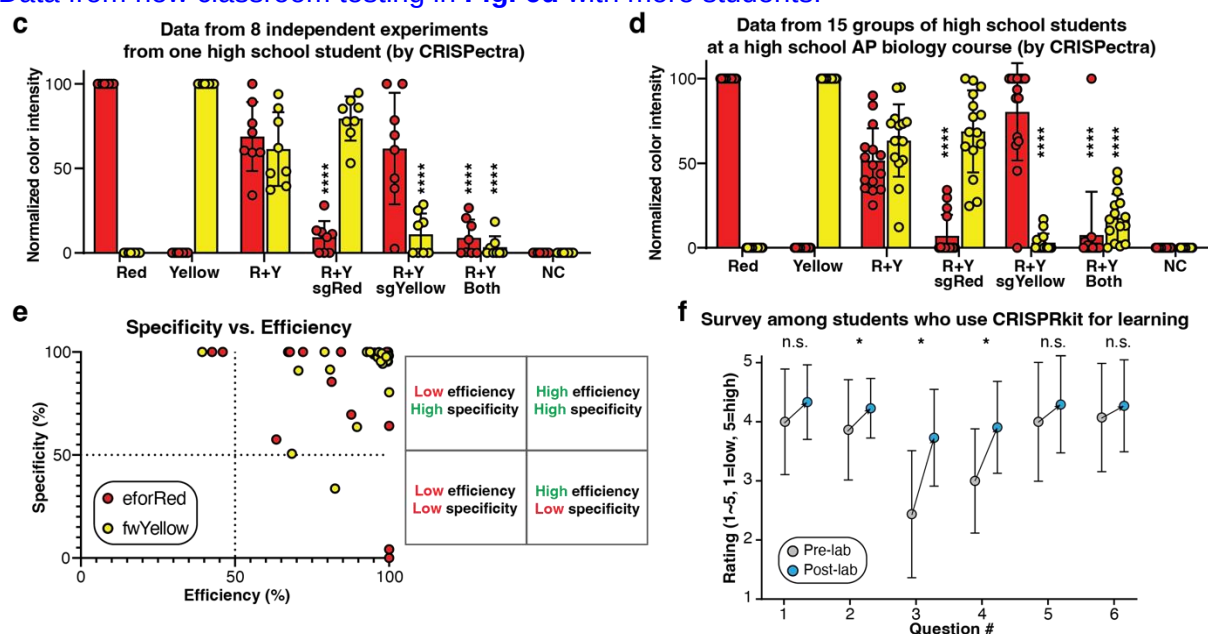


Figure 6 | Testing the frugal dual-color CRISPRkit by high school students in a classroom setting. b. Illustration of the components of the frugal CRISPRkit. A, Reagent tubes (5), containing the chromoprotein plasmid for eforRed, fwYellow, dCas9 protein, and sgRNA targeting eforRed or fwYellow. B, Reaction tubes (7, preloaded with the cell-free system). C, Imaging tubes (7), for the phone image purpose. D, Inoculation loops (17 loops for each kit, and 3 spare ones). E, User manual and protocol. c. Experimental data from 8 independent CRISPRkit experiments performed by one high school student in the frugal setting. d. Experimental data from 15 independent CRISPRkit experiments performed by 15 groups of high school students in the classroom setting. For c-d, the bars represent the mean of biological replicates, and the error bars represent the standard deviation. Two one-way ANOVA were performed for each figure; * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001; n.s., not significant. e. 2D scatter plot showing the calculated efficiency and specificity for each reaction for eforRed (red) and fwYellow (yellow). The top right quadrant shows reactions with high efficiency and high specificity. Individual dots represent biological replicates. Data points with both specificity and efficiency of 100% were jittered by <5% to facilitate visualization of many overlapping data points. f. Pre- and post-lab survey data among students who used CRISPRkit. Each dot represents the mean of ratings and the error bars represent the standard deviation. Multiple Student's t-test were performed with the Holm-Sidak correction; * < 0.05; n.s., not significant.

Analytical approach:

The researchers have mixed biological and technical replicates in their figures and conclusions throughout the paper.

Response: We have fixed this issue by calculating the mean value across technical replicates for each biological replicate, and then displaying this mean as a single data point. Where technical replicates were measured, the number of technical replicates for each biological replicate is indicated in the figure legends (Figs. 2c, 2d, 3b, 3d, 4b, 4c, 4d, 5a, 5b, 5c, 6c, 6d, 7d, 7e, 8d, 8e, Supplementary Figs. 3a, 4a, 6b, 7b, 13b, 13c).

In Figure 3b the authors present the normalized fluorescence for each condition. There are six datapoints in each of these conditions, but these six represent only 3 biological replicates with 2 technical replicates each, and they are presenting the mean – was the mean calculated from the 3 biological replicates or from the six samples? Clarifying the mean is from the biological

replicates (e.g. the mean value of the two technical replicates for each sample) will help if this is the case.

Response: As indicated above, in the revised figure, each data point displayed in the new **Figure 3b** is now the mean of technical replicate measurements for each biological replicate. We've also made similar changes to all data figures throughout the manuscript.

I would encourage the authors to think about what information should be conveyed from each graph. I believe the focus of Figure 3B is to showcase the fold of repression, and not show the consistency of repeated measurements of a sample. I think the most helpful information to keep in this figure would be the data from biological replicates. I do see value of presenting technical replicates to showcase consistency of the approaches for measuring individual samples - If presenting the six replicates, I strongly suggest that the authors color code which technical replicates were associated with each biological replicates. The authors could present a table showcasing the values of each of the technical replicates per sample, to highlight the consistency of measurements.

Response: Thank you for this suggestion and we completely agree. In the new **Figure 3b**, we now present each individual data point as a biological replicate by calculating the mean of technical replicates. We've also added this information to the figure legend. We've also included all raw values of technical measurements for biological replicates in the **Supplementary Data**, with each tab in that file organized for each individual figure.

Second, the manuscript is currently lacking statistical tests. In figure 2D the authors present means fluorescence values of three biological replicates. This would benefit from an ANOVA with post-hoc tests. The fold differences are striking and convincing on their own, but it should be feasible to add in these tests.

Response: Thank you for the suggestion. Statistical tests have now been added throughout the manuscript. For example, we've conducted an ANOVA with post-hoc tests and added statistical significance for relevant comparisons in **Figure 2d** and other figures. We've also added statistical information to each figure legend.

Suggestions:

1. Throughout: While I acknowledge that the authors may not have the resources to increase their sample size, I encourage the authors to separate biological and technical replicates, and use the ones that are most appropriate for their questions. For example, in figure 3B the authors should present only the biological replicates.

Response: Thank you. Each data point displayed in **Figure 3b** is now a single biological replicate value which is the mean of technical replicate measurements. We've also added this information to the figure legend. We've also made similar changes to all data figures throughout the manuscript.

2. Clarity of what data is presented:

a. Supplemental Figure 1A: the caption states there are results from three biological replicates presented, but there are 6 dots. Are the additional dots technical replicates?

Response: Each data point shown in **Supplementary Figure 3a** (previously Supplementary Figure 1a) is now a biological replicate value which is the mean of technical replicates. We've also added this information to the figure legend.

b. Figure 4D: same comment

Response: Each data point shown in the new **Figure 4d** is now a biological replicate value which is the mean of technical replicates. We've also added this information to the figure legend.

c. Supplemental Figure 2B: It appears technical replicates are being presented in figure 2B. I don't think these should be included, since presenting repeated measurements of the same sample being included in a correlation is misleading. The correlation should only be on biological replicates, and I think it will be more appropriate to compare for 1 individual sample the mean of its technical replicates from plate reader data with the mean of its technical replicates for the smart phone data.

Response: Each data point shown in **Supplementary Figure 4b** (previously Supplementary Figure 2b) and **Supplementary Figure 3b** (previously Supplementary Figure 1b) is now a biological replicate value which is the mean of technical replicates. We've also added this information to the figure legend.

d. Figure 4F: the caption states this is biological replicates only and is based on data from 4c and 4d with 3 biological replicates, but since Figure 4D appears to include more than 3 replicates this is a bit confusing. I advise checking that this is only biological replicates.

Response: We've corrected these issues. For example, in the new **Figure 4d**, each data point shown is now a biological replicate value which is the mean of technical replicates. We've also added this information to the figure legend.

e. Figure 5C: there is data from 8 experiments here but more than 8 dots per condition, similar comment as before – either present means of technical replicates or make it clear which are technical replicates for each sample. As I mentioned earlier in the reviews, the authors could present a table showcasing the values of each of the technical replicates per sample, to highlight the consistency of measurements.

Response: In the new **Supplementary Figure 6b** (previous Figure 5c), each data point shown is now an independent experiment. We've also added this information to the figure legend. We've also included all raw values of technical measurements for biological replicates in the **Supplementary Data**, with each tab in that file organized for each individual figure.

Summary: Thank you for the suggestions regarding biological vs. technical replicates. We've now presented data points presenting biological replicates, indicated in the figure legends, and included the raw data for technical replicates in the **Supplementary Data**.

Other minor concerns

1. Figure 2A: I believe the authors should check the directionality of their PAM sequences – the figure shows a PAM site on the bottom strand “GGN” and another PAM site on the top strand “GGN.” One of these should be NGG. This figure would benefit from 5' and 3' directionality.

Response: Thank you for the suggestion. We've corrected this mistake.

2. This is a small point, but since the high school students tested the materials and their data is included in the manuscript I would suggest to the authors that they include the students in their acknowledgments.

Response: We've acknowledged the teachers and their high schools involved in testing CRISPRkit in Acknowledgements.

Reviewer #2:

Key results

In this work by Collins et al., CRISPR kits were designed for K-12 use. These CRISPR kits utilize a cell free system (CFS), dCas9, and chromoproteins, which are proteins with pigmented prosthetic groups that can be visualized without specialized equipment. The sgRNAs provided in the kit each target a single chromoprotein (eForRed, fwYellow, and aeBlue), and a color change readout can be observed by eye or determined through plate reader measurements. Importantly, the authors also developed an algorithm named CRISpectra that enables smartphone-based quantification of results, eliminating the requirement for a plate reader. High school students were able to successfully use the frugal CRISPR kit.

Data, methodology, and validity

In general, the data presented here support the conclusions and claims of the authors. Data obtained from CRISPR experiments were analyzed in one of three ways: 1) fluorescence measurements from a plate reader, 2) visual inspection of reaction tubes or droplets on parafilm, and 3) quantification of smartphone images using the CRISpectra algorithm. The authors present convincing evidence that smartphone quantifications are comparable to plate reader measurements (SFig. 2B and Fig. 4F) and adequately address the variability in smartphone quantification by high school students (Fig. 5C).

Concerns related to data interpretation and conclusions:

Visual inspection and interpretation is clear and straightforward in single-color CRISPR experiments (Figs. 2F and 2G). However, it is less clear in dual-color CRISPR experiments in Figs. 3C, 4D, and 5B. Two suggestions: 1) either label the images directly with the expected color (ex: orange) or color-code the labeling text as appropriate (ex: R + Y written in orange text), 2) improve the overall quality of the images used in these panels. This observation does not significantly affect the findings in the manuscript, but does emphasize the importance of the quantification tool CRISpectra for those who are visually impaired or have difficulty interpreting color as such (see below).

Response: Thank you for the suggestion. We have color-coded the text for all the relevant figures in their respective colors for easy visualization (**Figs. 3c, 4d, Supplementary Figs. 6a, 7a**). We have also included detailed information regarding what components are added to each reaction for better understanding of visual output in the new **Supplementary Figure 2** (for chromoprotein experiments) and **Supplementary Figure 12** (for the new melanin production experiment). For the quality of images, we tried to present the raw images to give users information about what they might observe when using CRISPRkit.

In panel 6E, addition of sgYellow leads to a general knockdown/decrease in chromoprotein color that is not addressed. This problem does not occur in Figure 2, and on pg. 8 the authors note “We also confirmed that the sgRNAs acted orthogonally and only repressed their cognate targets.” Please address this discrepancy.

Response: The previous Figure 6e contained only one biological replicate. In this revised manuscript, we have repeated the ‘guess-and-test’ experiment with a greater number of biological (N=3) and technical (N=2) replicates. This data is now included in the new **Figure 7d**, with the addition of sgYellow no longer leading to a decrease in other chromoprotein colors, as quantified by the CRISpectra method. In addition, no change is visible to the naked eye, as can be seen in **Figure 7c**.

Significance

Compared to previous literature (for example, Huang et al. 2018), the significant advancements described here are 1) the design of CRISPR experiments that can be performed in a K-12 setting without standard laboratory equipment such as incubators or pipettes, and 2) the creation of a smartphone-based algorithm for quantification of visual color-change readouts. These advancements emphasize the authors' commitment to providing accessible science tools for students of all socioeconomic backgrounds. This manuscript can represent a significant contribution to the field of K-12 educational resources, but relies on distribution, affordability, and accessibility of the designed CRISPR kits. The website (crisprkit.org) demonstrates accessibility of K-12 protocols and ability to contact the manuscript authors for kits. The manuscript may not provide a significant contribution to the field of synthetic biology (see below).

Response: Thank you for recognizing the utility of CRISPRkit for K-12 education. Indeed, in the past several months, we've received requests from over 30 high schools regarding the adoption of CRISPRkit in their classrooms. Meanwhile, we agree that the first version of the manuscript utilizing chromoprotein systems follows a more standard CRISPRi protocol, although our designs of the CRISPRi sgRNAs to repress expression of individual chromoproteins such as eforRed, fwYellow, and aeBlue are new.

To further strengthen the utility of the CRISPRkit for synthetic biology uses, in this revision, we've added a new exciting experiment showing that the CRISPRkit can be used for controlling a metabolic pathway related to melanin production (**Figure 8a-e, Supplementary Figs. 12 and 13a-d**). We designed CRISPR sgRNAs to repress the expression of a tyrosinase (*melC*) gene derived from bacteria (*Priestia megaterium*), which modulates multiple steps of conversion of tyrosine to melanin. Then we showed that CRISPRkit can generate robust *melC* knockdown and visible melanin outcomes in the cell-free system. To our knowledge, this is the first application of a cell-free system to this melanin production pathway in vitro. Furthermore, we've expanded the CRISPRkit to Cas-based gene editing (**Fig. 2c**) and a fluorescent protein (**Fig. 7e**). Therefore, we hope the reviewer will agree that the revised manuscript presents useful data to the synthetic biology community.

We attach the melanin results for your reference (Fig. 8; additional data are in Supplementary Fig. 12 and 13):

Figure 8

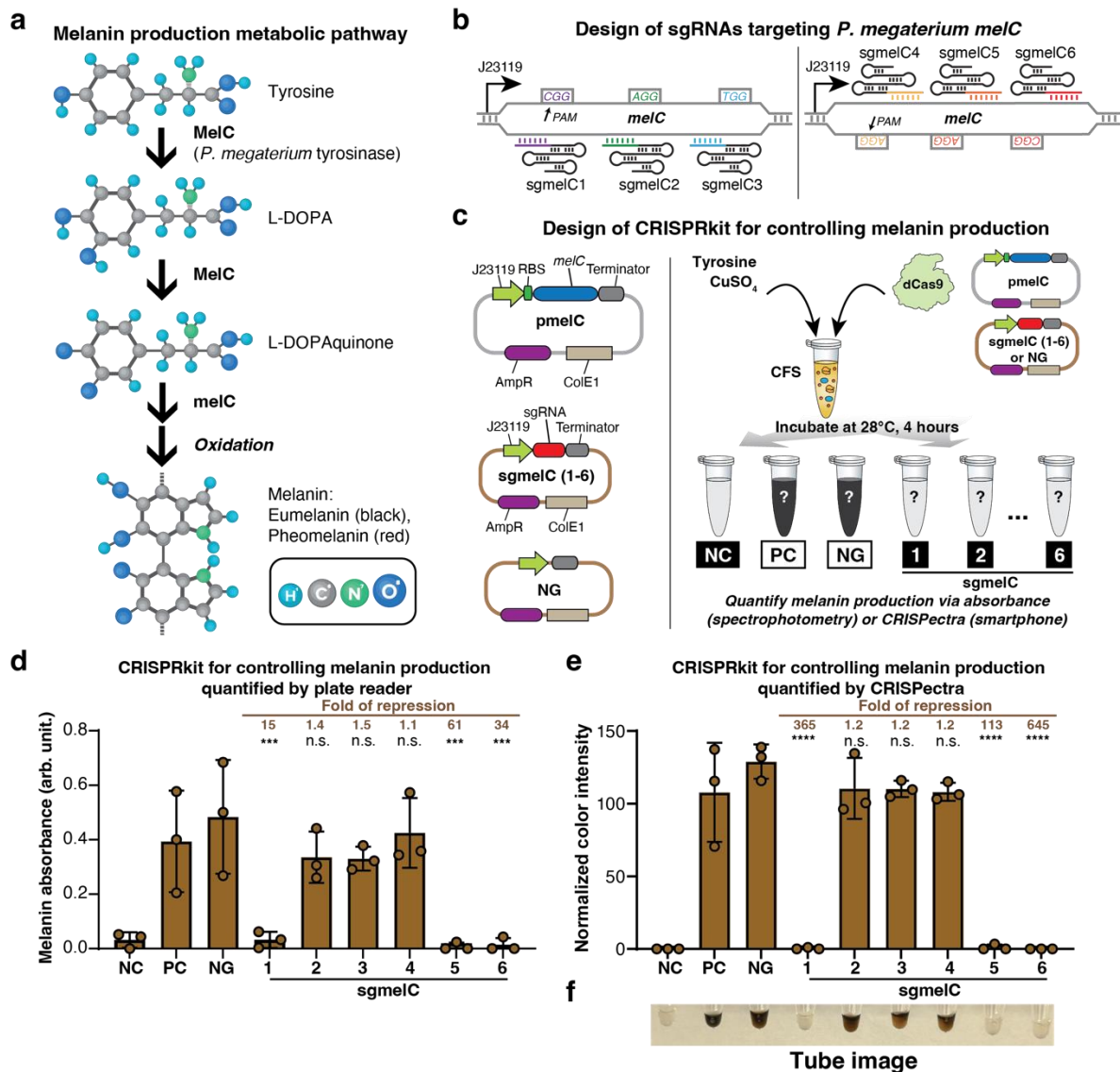


Figure 8 | CRISPRkit experiment for controlling melanin production. a. schematic of the melanin production metabolic pathway. The *melC* tyrosinase converts tyrosine (substrate) to L-DOPA and L-Dopaquinone, which subsequently converts to melanin consisting of eumelanin or pheomelanin. b. Design of sgRNAs that target the *melC* coding region on the template strand (left) or the non-template strand (right). The PAM for each sgRNA is shown. c. Experimental setup for the high-tech single-color experiment using the CRISPRkit with different sgRNAs. After incubation at 28°C for 4 hours, we quantify the absorbance using a fluorescence plate reader or smartphone images and CRISPECTRA. d-e. Experimental data showing CRISPRkit reactions for controlling melanin production, measured by a fluorescent plate reader (d) or smartphone images and analyzed by CRISPECTRA (e). Three biological replicates are plotted for each group. The bars represent the mean of biological replicates, and the error bars represent the standard deviation. A one-way ANOVA was performed for each dataset; * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001; n.s., not significant. f. Raw tube images showing one group of CRISPRkit reactions for controlling melanin production.

Concerns related to significance:

Cell-free systems have been used previously in K-12 settings (Huang et al. 2018), which is

noted by the authors. In addition, the BioBits™ kits can be designed with multiple readout types including detection of fragrances and hydrogels, allowing these kits to address several types of biological questions which are more broadly interesting. While the authors' implementation of CRISPR kits in a K-12 setting is noteworthy and important for equitable access to this biological tool, the biological questions that can currently be addressed with the kit are quite limited in scope.

Response: We thank the reviewer for this critique. We believe the cell-free system (CFS) combined with CRISPR has numerous applications that can generate interesting readouts. As mentioned above as an example, our lab has long been interested in using CFS + CRISPR to modulate metabolic pathways. In this revision, as described above, we've added a new experiment using the CRISPRkit for controlling a melanin production pathway (**Figure 8**; see above). By designing CRISPR guides to repress the expression of a bacterial tyrosinase (*melC*) gene that modulates multiple steps of conversion of tyrosine to melanin, we showed that CRISPRkit can generate robust gene knockdown outcomes on the melanin pathway. Beyond melanin production, we believe that CRISPRkit can inspire more users to design their own content and applications to generate other interesting readouts (such as production of visible or tangible biopolymers or biomaterials).

The discussion could be strengthened by highlighting other biological questions that can be addressed by students using this system. The question currently posed, "Which genes are in the tube?" may be suitable for middle school but does not represent an opportunity for advanced high schoolers to consider meaningful biological questions using this kit.

Response: We have revised the text in the Discussion (Page 11), which is included below:

"Furthermore, we demonstrated that CRISPRkit offers an expandable platform for users to add their own content. Besides chromoproteins, we showed that CRISPRkit can work robustly with fluorescent proteins and metabolic pathways such as melanin production. For example, by designing sgRNAs that specifically repress transcription of *melC* tyrosinase, we can use CRISPRkit to modulate enzyme expression and characterize melanin production using CRISPR Spectra. We expect the frugal CRISPRkit could be further expanded to include additional biotechnology content in the future, such as production of biomaterials and sequestration of CO₂."

The concept behind CRISPRi is probably too advanced for middle school students. We have also consulted with high school teachers regarding the design of the question in **Figure 7b** 'Which genes are in the tube?', and they think it is an explorative question to inspire high school students to learn more about mechanisms of CRISPRi and how to design the experiment themselves to test it.

In Figure 1a and its corresponding title legend, the authors emphasize the biosafety and biohazard concerns of using CRISPR. However, CRISPR is routinely performed in microorganisms such as *S. cerevisiae* and does not require specialized BSL2 facilities or tissue culture equipment. While I appreciate that CFS eliminates any requirement for cellular or biohazard disposal, I believe that some organisms would be safe to work with in high school settings and that the concerns here are overstated.

Response: One major motivation for us to develop CRISPRkit is accessibility, in addition to safety. We agree that routine CRISPR experiments in *E. coli* and *S. cerevisiae* do not need BSL2 facilities and it is meaningful to encourage students to conduct CRISPR experiments in

living organisms if the high schools can afford to set up such experiments. However, after discussing with many teachers, we've come to recognize that setting up experiments in living organisms is not an easy feat for many teachers, because it requires significant effort and resources for the teachers to prepare or purchase competent cells, reagents for transformation, media such as transformation reagents or sterilized culture media, and consumables including LB agar plates. What prevents many high schools from offering CRISPR experiments is not just biosafety level requirements, but the accessibility including cost, efforts, equipment, teacher's training, and the large student body (e.g., a local high school has 60 students in their AP biology classroom). Because of these practical limitations, we believe that CRISPRkit is a useful contribution to education. We are not claiming that CRISPRkit can replace educational CRISPR experiments in organisms but can greatly increase accessibility, and we have changed our statement in the introduction (**Page 3**):

"Performing CRISPR experiments in living organisms, such as in microbes or mammalian cells, in a classroom setting presents multiple challenges. These include the high cost of equipment and reagents, biosafety requirements for handling living cells and biohazards, and lengthy, complex protocols. These obstacles are often insurmountable for most high schools, leaving students with limited hands-on experience with CRISPR technology."

The CFS system used here is commercially available and was not designed by the authors, while the sgRNA and chromoprotein plasmids were. This distinction is important for the consideration of the manuscript's significance to the synthetic biology field, which may be limited. While the use of a commercial mastermix is clear from the methods section, it could be explicitly stated in the results section.

Response: In this revised manuscript, we utilized an in-house developed CFS, distinguished from the commercial CFS in the first submission. In the new **Figure 5a-c**, we show that our in-house CFS has comparable performance to the commercial CFS. We further used the in-house CFS reagents for conducting the melanin production experiment and the sfGFP repression experiment. The use of in-house CFS reagents greatly reduced the cost of the CRISPRkit. We've clarified this in the main text and **Methods** about which reagents were used in different parts of the manuscript related to particular experiments.

A concern above (related to interpretation of results) relies on the ability to use the CRISpectra analysis algorithm which is not easily accessible (or accessible at all?) from the website. After watching Movie S2, I located the website (<https://myckitresults.com/>). CRISpectra may not be user-friendly unless its use is accompanied by direct instruction and/or the video is linked/available. I would recommend 1) modifications to the website to include detailed instructions on each page corresponding to each step, and/or 2) a link to the video on the first page.

Response: We've created a new **Supplementary Table 6** to include all permanent links online related to the resources and algorithms for the CRISPRkit and CRISpectra. The CRISpectra analysis GUI can be accessed here: (ckitdual.com). The link to the GUI can also be readily accessed from the main website (<https://www.crisprkit.org/>). The website is also modified and more information is incorporated to demonstrate how the algorithm works as well as how to use it for data analysis. Videos on how to conduct a CRISPRkit experiment and how to analyze results using CRISpectra are also published on our YouTube channel (<https://www.youtube.com/@CRISPRkit>).

While the authors state that the price of the frugal CRISPR kit may eventually reach <\$1/kit,

there is no discussion of the current cost. This should be explicitly stated in the sentence “We manufactured the frugal CRISPR kits in bulk and distributed them...” on pg. 12. In the discussion, the cost could be compared to other kits used at the K-12 level such as BioBits™ kits which range from \$85-\$225.

Response: We have included a new **Supplementary Table 4**, which includes a cost breakdown of our CRISPRkit in terms of biological reagent and plastic consumable components. According to our calculations, the reagents associated with each CRISPRkit experiment is \$1.13, and the plastic consumable components are \$0.88. Since we do not need additional instruments (other than a smartphone), the combined cost, \$2.01 per kit is much lower than the BioBits™ kits (\$149.96, <https://pubs.acs.org/doi/10.1021/acssynbio.8b00381>), which also did not include the cost of plastic consumables in their cost analysis. We've added discussion of the cost in the manuscript (**Page 11**, Discussion).

Panel 6F shows a schematic of chromoprotein colors that are not yet available for the CRISPR kits. Possible expansions of the kit may be more appropriate for the discussion, with Fig. 6F being moved to the supplementary material.

Response: Thank you for this suggestion. The former Figure 6f has been moved to the Supplementary Figures and is now **Supplementary Figure 10**.

Reviewers' Comments:

Reviewer #1:

Remarks to the Author:

The revised manuscript is much improved. Below I have divided my comments into three sections: the aspects focused on CRISPRi as an education tool, the data and analysis, and the new sections added in this version.

Education Tool:

In this revised manuscript the authors have included new supplemental materials that sufficiently addressed my comments and suggestions regarding the CRISPRi kit as an educational tool. Supplemental Table 4 provides a detailed cost breakdown, the new worksheet (Supplementary Note 4) will provide clarity and organization for the students working through the protocol, and the new manual for teachers (Supplementary Note 5) appropriately addresses learning goals, waste disposal, storage of reagents, and provides specificity in instructions. Additionally, I appreciate the inclusion of data showcasing testing from a class of high school students (Figure 6).

Revision of analyses:

The authors have expanded their biological sample size (Figure 5), sufficiently addressed the issue of biological vs technical replicates in the prior analyses, and have added statistical tests that support their conclusions. These new analyses address my prior concerns regarding sample size and analyses.

New data:

The authors have included a new anonymous survey (Supplementary Table 5).

- Major concern: Since the authors are including responses from students, the data in Figure 6F is human subjects research with students who were under 18. The authors need to include a statement clarifying whether the IRB deemed the research exempt.
- Minor concern: The survey question design details are currently not included in the methods – were these items derived from previously validated instruments, or did the authors develop and validate the items in-house before implementation?
- Suggestion: Analysis for 6F: The R package “Likert” may be useful for presenting an overview of the range of student responses. If the authors would like to continue to present means, since the data are paired the authors could present the change in responses.

The authors have included a new section: application of the CRISPRi kit to control melanin production.

- Suggestion: Additional details here would be helpful regarding the types of experiments the authors recommend the students do with the 6 gRNAs – is the suggestion that students predict how combinations of gRNAs will impact expression? It may help to have a worksheet for this protocol as well for students, with recommended discussion questions.
- Suggestion: The selection of melanin synthesis as a focus for the CRISPRi kit could allow for instructors to link the material to other AP Biology topics. HHMI has developed a classroom unit <https://www.biointeractive.org/classroom-resources/biology-skin-color> that could be referenced as a recommended resource.

Other:

- Supplementary figure 9 has typo's – correct to “Knockdown” gene

Reviewer #2:

Remarks to the Author:

The authors have sufficiently addressed all reviewer comments. Particularly impressive are the new instructional resources included in the supplement, the new application of the CRISPRkit

(using CRISPR to knock down melanin production), testing of in-house CFS reagents, and the clarification on biological/technical replicates and statistical analysis for many of the figures. Nice work!

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- Major concern: Since the authors are including responses from students, the data in Figure 6F is human subjects research with students who were under 18. The authors need to include a statement clarifying whether the IRB deemed the research exempt.

[Response: Thank you for pointing this out. The design of our survey mirrored a previous study using in vitro cell free system for education, which was declared 'not human subject research' \(reference 36\). In our verbal conversation with the Stanford IRB committee, they commented that this survey is likely classified as 'program evaluation' and 'not human subject research'. The IRB committee is currently in the process of making a formal determination. However, we expect this process to take at least 3 months to complete. We also note that in the first round of revision, none of the reviewers asked us to perform a survey using the CRISPRkit. We added this data mainly to highlight user experiences. But we believe this survey data is not important to the core claims of the overall manuscript. The effectiveness and performance of the CRISPRkit is well supported by the experimental data presented in the manuscript. Therefore, in light of the less important role of the survey data and the long IRB determination process, we have decided to remove the survey data \(Figure 6f\) from the manuscript.](#)

• Minor concern: The survey question design details are currently not included in the methods – were these items derived from previously validated instruments, or did the authors develop and validate the items in-house before implementation?

Response: As described above, we removed the survey data. Thus, this question is no longer applicable.

• Suggestion: Analysis for 6F: The R package “Likert” may be useful for presenting an overview of the range of student responses. If the authors would like to continue to present means, since the data are paired the authors could present the change in responses.

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Response: As suggested by this reviewer, we have added a new protocol and worksheet (**Supplementary Note 6**) with a protocol and table to instruct students to test how individual or combinatorial sgRNAs can repress melanin production using CRISPRkit. We also listed a few exemplar discussion questions in the worksheet:

- Based on your experiment, which sgRNA(s) represses melanin the best?
- If your experiment failed (output does not match intended result for some tubes), can you think of reasons why this happened?
- Does having two sgRNAs repress melanin better than a single sgRNA?
- Can you design an experiment to further enhance repressive effects beyond using two sgRNAs? How would you test your hypothesis?

• Suggestion: The selection of melanin synthesis as a focus for the CRISPRi kit could allow for instructors to link the material to other AP Biology topics. HHMI has developed a classroom unit <https://www.biointeractive.org/classroom-resources/biology-skin-color> that could be referenced as a recommended resource.

Response: Thank you for pointing out this useful resource. We have now included a link to this HHMI classroom unit related to the biology of skin color in our **Supplementary Note 6**.

Other:

• Supplementary figure 9 has typo's – correct to “Knockdown” gene

Response: Thank you. We've corrected the typo.

Reviewer #2 (Remarks to the Author):

The authors have sufficiently addressed all reviewer comments. Particularly impressive are the new instructional resources included in the supplement, the new application of the CRISPRkit

(using CRISPR to knock down melanin production), testing of in-house CFS reagents, and the clarification on biological/technical replicates and statistical analysis for many of the figures. Nice work!

Response: We thank the reviewer for providing constructive comments and recognizing the revised manuscript.