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Validating CRISPR Prime Editing for Targeted NR2B Inhibition in Ischemic Stroke Using an *E. coli* GFP Reporter System

Keywords: CRISPR prime editing, NR2B (GRIN2B), ischemic stroke, GFP reporter system, *Escherichia coli*, gene editing verification

1. Introduction

Ischemic stroke remains one the leading causes of mortality and disability worldwide, with around 7.8 million new cases and 3.6 million deaths in 2021 alone (Feigin et al., 2025). While reperfusion therapies such as mechanical thrombectomy restore blood flow, they do not directly address neuronal damage that follows the initial ischemic insult, leaving many recovered patients with permanent side effects (Dammavalam et al., 2024). This has motivated years of study into neuroprotective strategies that target the cellular mechanisms of injury rather than blood flow alone.

A central driver of this secondary damage is glutamate excitotoxicity. During ischemic stroke, energy failure causes excessive glutamate release and reuptake dysfunction, leading to sustained overactivation of N-methyl-D-aspartate receptors (NMDARs) on postsynaptic neurons (Yu et al., 2023). Critically, not all NMDAR subtypes contribute equally to this damage. For instance, the GluN2B (NR2B) subunit that strongly associated with pro-death signalling, whereas GluN2A containing receptors tend to promote neuronal survival. This variance functional identifies NR2B as an appealing for specific therapeutic target, in contrast to pan-NMDAR antagonists, which have repeatedly failed in clinical trials. Possibly, that because they inhibit both the harmful GluN2B pathway and the protective GluN2A pathway simultaneously (Wu et al., 2023).

Achieving NR2B inhibition pharmacologically has proven difficult, motivating interest in precision genome editing tools that could mutate a single target gene with minimal off-target dislocation. CRISPR prime editing is one such tool since it uses impaired Cas9 fused to an engineered reverse transcriptase, guided by a prime editing guide RNA (pegRNA) without generating double-strand breaks or requiring a donor template (Anzalone et al., 2019). Because prime editing can make precise insertions, such as premature stop codons that shorten a protein and abolish its function, it provides a promising approach to targeted, functional inactivation of NR2B (GRIN2B) as a treatment for ischemic stroke.

Before such an approach can be tested in a mammalian or clinical context, it is important to prove that prime editing can reliably install a loss of function edit and that this edit can be verified. Prior work has demonstrated this principle in *Escherichia coli* by using prime editing to insert premature stop codons into the gene encoding green fluorescent protein (GFP), with loss of fluorescence functioning as an easily scored proxy for successful gene disruption (Tong et al., 2021a). The present study adapts this bacterial GFP based design as a first step toward the longer-term goal of NR2B-targeted prime editing. Using *E. coli* expressing GFP as a substitute target, we designed a pegRNA to insert a premature stop codon into the GFP coding sequence, hypothesizing that successful editing would silence GFP expression and be visually detectable as loss of fluorescence. Showing that this method works in a simple, well-studied system like *E. coli* gives us a reliable way to test and screen for successful edits that can subsequently be extended to NR2B silencing in ischemic stroke models.

2. Methods

2.1 Stains, Plasmid, Media, and Growth Condition

Escherichia coli Top10 cells and the plasmids used in this study were adapted from the CRISPR-Prime Editing toolkit for *E. coli* (Tong et al., 2021b). Cultures were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (Fisher Scientific). Appropriate antibiotics were supplemented at the following working concentrations: spectinomycin (50 μg/mL), ampicillin (100 μg/mL), and chloramphenicol (25 μg/mL), with anhydrotetracycline (0–1 μg/mL) used to induce expression of the Cas9n–reverse transcriptase fusion. Two percent agar was used for agar plates. Antibiotics were purchased from Sigma-Aldrich (USA); enzymes and cloning reagents were purchased from New England Biolabs (USA); and plasmid purification kits were purchased from Qiagen (Germany).

2.2 pegRNA Design and Plasmid Construction

A pegRNA targeting the GFP+ coding sequence was designed to insert a premature stop codon at the fifth codon position, following the standard prime editing guide RNA architecture (20-nucleotide protospacer, gRNA scaffold, reverse transcriptase template, and primer binding site, each ~13 nucleotides). The pegRNA cassette was synthesized as a gBlock and assembled into the empty pPEgRNA backbone by Gibson Assembly (NEB Gibson Assembly Master Mix, E2611S) using NEBuilder compatible overhangs. Plasmid backbones were amplified by PCR using Q5 High-Fidelity 2X Master Mix (NEB, M0492S).

2.3 Transformation and Clone Selection

Assembled plasmid DNA was introduced into chemically competent Top10 cells by heat-shock transformation, cells were equilibrated on ice with the Gibson Assembly product for 5 minutes, heat-shocked at 42°C for 30 seconds, returned to ice, recovered in SOC medium at 37°C for 1 hour, and plated on LB agar containing the appropriate antibiotic. Individual colonies were picked for downstream screening.

2.4 Plasmid Isolation and PCR Verification

Plasmid DNA was isolated from overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, 27104). PCR products were

purified using the QIAquick PCR Purification Kit (Qiagen, 28104) and resolved on agarose gels (Sigma, A6013) stained with SYBR Safe (Thermo Fisher, S33102) in 1X TAE buffer, alongside a 1 Kb Plus DNA Ladder (Thermo Fisher, 10787018).

2.5 Fluorescence and Growth Measurements

Bacterial growth (OD_{600}) and GFP fluorescence intensity were measured using a SPARK multimode microplate reader in a 96-well plate format. Wells containing LB medium only (no bacteria) served as the blank control. Colonies were additionally imaged under UV illumination using a gel documentation system (SGC-W5000 Plus, Servicebio) to visually assess fluorescence.

2.6 Fluorescence Image Analysis

Fluorescence images were quantified using Fiji (ImageJ). Regions of interest were manually drawn around individual colonies, and fluorescence intensity was extracted along a line profile and exported to Microsoft Excel for analysis.

2.7 Editing Confirmation by Sanger Sequencing

Positive clones identified by loss of GFP fluorescence were verified at the DNA level by colony PCR followed by Sanger sequencing across the target region, confirming the intended stop codon insertion at the fifth codon of the GFP coding sequence.

3. Results

3.1 Bacterial Growth and Fluorescence Intensity

OD_{600} was monitored across three timepoints (10:45 AM, 1:30 PM, and 4:48 PM) to assess bacterial growth for the Stop Codon construct relative to the CRISPR plasmid only and pegRNA plasmid only controls (Fig. 2a). All three conditions showed comparable growth trajectories, reaching similar final OD_{600} values (0.14–0.16) by the last timepoint. Subsequently, the Stop Codon construct showed a decline in GFP fluorescence across the three measured timepoints, from 47,344 a.u. at 10:45 AM to 37,891 a.u. at 1:30 PM and 3,417 a.u. at 4:48 PM (Fig. 2b). Both negative controls, CRISPR plasmid only and pegRNA plasmid only, remained at consistently low fluorescence throughout the day (7,879–18,018 a.u.). By the final timepoint, all three samples had converged to a similar, low fluorescence range (3,417–3,631 a.u.).

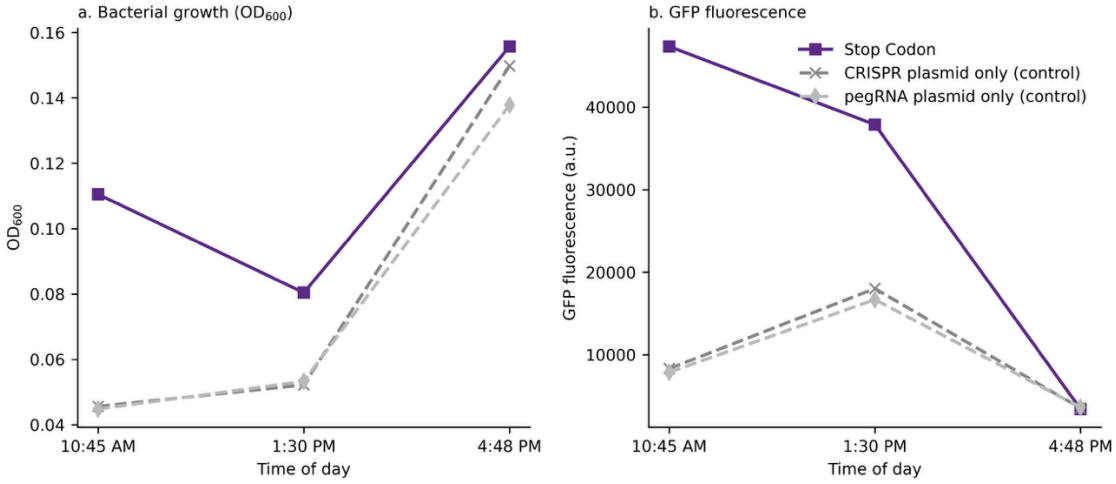


Figure 2: Bacterial growth and GFP fluorescence for the Stop Codon construct and plasmid only controls. (a) OD_{600} growth curves and (b) GFP fluorescence over three time points for the Stop Codon construct compared to CRISPR plasmid only and pegRNA plasmid only controls.

Figure 3 represents plate images and corresponding fluorescence quantification for two bacterial colony samples grown under different selection conditions. The agar plate for Experiment Set A (Fig. 3a) displayed colonies at moderate density that were individually distinguishable across the surface, whereas the plate for Sample C_f (Fig. 3b) showed markedly higher colony density, with several regions approaching confluence. Background corrected fluorescence intensity (RawIntDen) was then measured for six individually selected colonies from each plate. In Experiment Set A (Fig. 3c), values ranged from 19,747 to 40,270 across Colonies 1–6, yielding a mean of 26,313, with Colony 2 showing the highest fluorescence and Colony 5 the lowest, in Sample C_f (Fig. 3d), values ranged from 3,128 to 12,542, yielding a mean of 7,294, with Colony 4 showing the highest fluorescence and Colony 5 again the lowest. Fig. 3c, d are plotted on different y-axis scales (0–50,000 and 0–16,000, respectively), and the mean fluorescence value in Fig. 3c is approximately 3.6 times that in Fig. 3d.

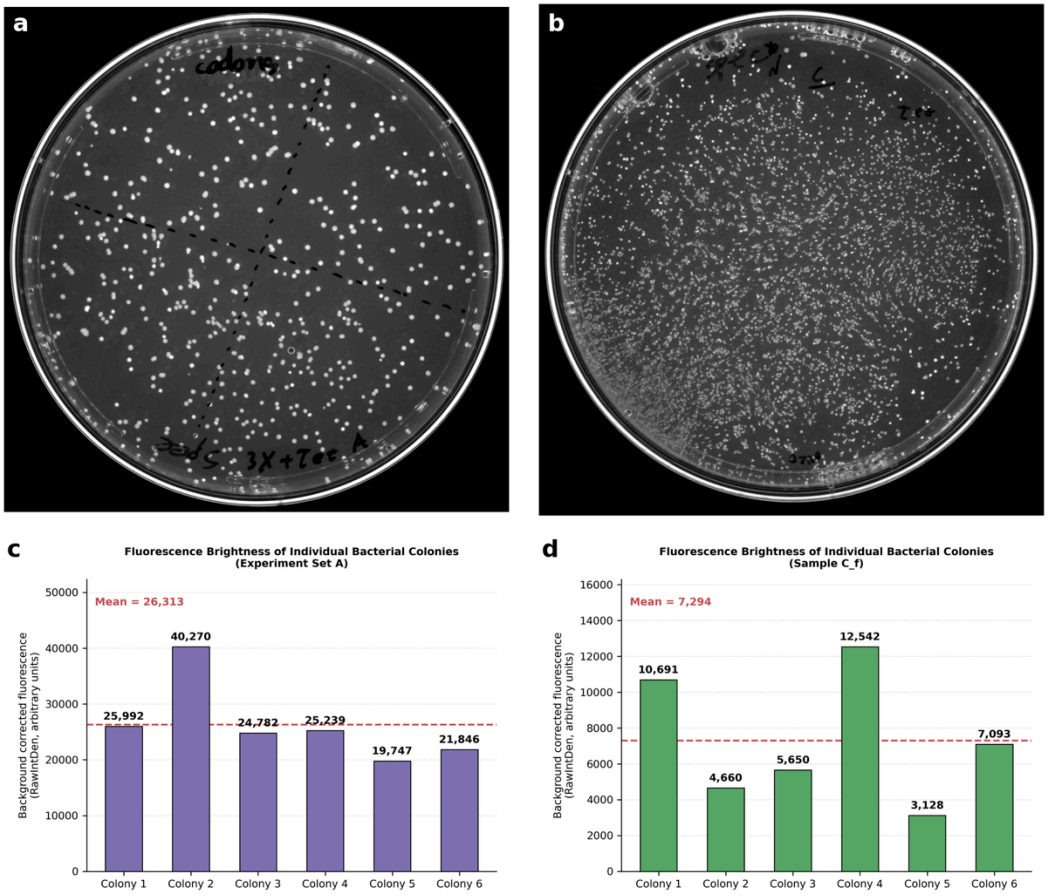


Figure 3: Colony fluorescence in Codon construct plates under TET induction. (a) Representative plate image of Codon construct colonies, TET-induced (Experiment Set A). (b) Representative plate image of Codon construct colonies, TET-induced (sample C_f). (c) Quantified fluorescence brightness of individual colonies from Experiment Set A (n = 6). (d) Quantified fluorescence brightness of individual colonies from sample C_f (n = 6).

3.2 Sequencing Confirmation

Figure 4 presents Sanger sequencing chromatograms and quality scores at the codon 5 target site for three Stop Codon colonies. The unedited GFP reference (no PEgRNA control, Fig. 4a) reads GAA at codon 5. The three test colonies, C_t_1 (well B08, forward read, Fig. 4b), C_f (well H04, reverse read, Fig. 4c), and C_d (well D05, reverse read, Fig. 4d), read GAA, TTC, and TTC at codon 5, respectively (TTC being the reverse complement of GAA on the reverse strand), matching the sequence in Figure 4a. None of the three colonies shows the TAG sequence. In addition, average Sanger sequencing quality scores across the 21 bp window centered on codon 5 (Fig. 4e) were 57.4 for C_t_1, 54.2 for C_f, and 59.4 for C_d, compared with 59.0 for the unedited control (dashed red line). C_t_1, C_f, and C_d were sequenced from bulk liquid culture, whereas

Tong et al. (2021b) sequenced isolated single colonies.

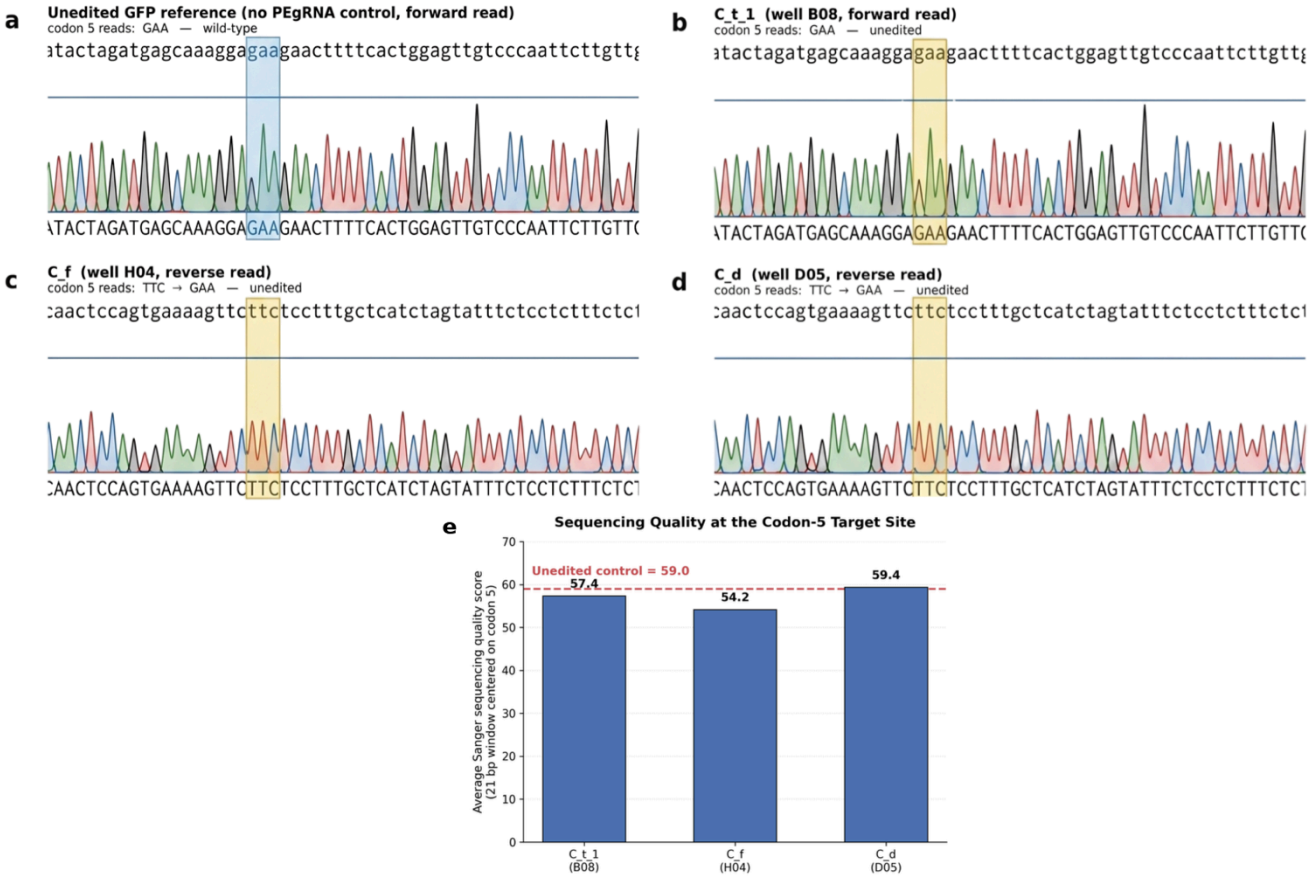


Figure 4: Sequencing confirmation at the GFP codon-5 target site. (a) Chromatogram of the unedited GFP reference (no PEgRNA control, forward read), showing wild type codon 5 (GAA). (b) Chromatogram of sample C_t_1 (well B08, forward read), showing unedited codon 5 (GAA). (c) Chromatogram of sample C_f (well H04, reverse read), showing unedited codon 5 (TTC on reverse strand, GAA on coding strand). (d) Chromatogram of sample C_d (well D05, reverse read), showing unedited codon 5 (TTC on reverse strand, GAA on coding strand). (e) Average Sanger sequencing quality score (21 bp window centered on codon 5) for each sample, compared to the unedited control (dashed line, 59.0).

4. Discussion

Three lines of evidence were presented above. These included growth and bulk fluorescence over time (Fig. 2), colony-level fluorescence quantification (Fig. 3), and Sanger sequencing at the codon 5 target site (Fig. 4). Across these three lines of evidence, no molecular evidence of the intended GAA-to-TAG stop codon edit was obtained. Bacterial growth (OD_{600})

converged to a similar range (0.14-0.16) across the Stop Codon construct and both negative controls by the final timepoint, while GFP fluorescence for the Stop Codon construct fell from 47,344 a.u. to 3,417 a.u. over the same period and converged with the CRISPR only and PEgRNA only controls, which remained between 7,879 and 18,018 a.u. throughout (Fig. 2). At the colony level, mean background corrected fluorescence differed by a factor of approximately 3.6 between Experiment Set A (mean 26,313 a.u., n = 6) and Sample C_f (mean 7,294 a.u., n = 6), with C_f additionally showing greater relative variability (coefficient of variation 49.9% versus 27.5% for Set A) (Fig. 3). Sanger sequencing of three Stop Codon colonies, C_t_1, C_f, and C_d, showed wild type GAA (or its reverse complement, TTC) at codon 5 in all three cases, matching the unedited no PEgRNA control, with average sequencing quality scores (54.2–59.4) comparable to the control (59.0) (Fig. 4).

Considered on its own, the difference in mean colony brightness between Experiment Set A and Sample C_f could be read as evidence of differing editing status between the two plates. However, this interpretation is complicated by colony density, which also differed markedly between the two plates: colonies in Set A were moderately spaced and individually distinguishable, while those in C_f were considerably denser and, in places, near confluent (Fig. 3a, b). Because fluorescence was quantified from manually drawn regions of interest around individual colonies, crowding on the C_f plate provides a plausible technical confound. Overlapping regions of interest, competition for nutrients or oxygen between adjacent colonies, and reduced accuracy of background correction could each lower or add noise to individual colony readings. These effects would occur independent of any underlying genotype (Chacón et al., 2018). The comparatively higher variability observed in C_f is more consistent with this kind of density related technical variation than with a straightforward genotype driven reduction in brightness. Because the two plates are independent platings rather than replicates of the same condition, the present data cannot separate a genuine biological difference from plate to plate technical variation.

A related limitation affects Fig. 2. At the first timepoint, the Stop Codon construct already showed both higher OD_{600} (0.11) and higher fluorescence (47,344 a.u.) than either control. Control OD_{600} values were roughly 2.4 times lower (0.045) at the same timepoint. Because fluorescence in this experiment was not normalized to OD, part of the early difference in raw fluorescence is attributable to differences in culture density rather than to per-cell GFP expression. By the final timepoint, fluorescence in all three conditions, including both negative controls that carry no target edit, converged to a similarly low range (3,417-3,631 a.u.) even as OD_{600} reached its highest value in every condition. This decline occurred uniformly across edited and unedited conditions alike. This pattern is more consistent with a general reduction in GFP signal as cultures approach stationary phase than with a signature specific to stop-codon-mediated loss of function. Possible causes of this general decline include reduced GFP synthesis, impaired chromophore maturation, or increased culture turbidity (El Itawi et al., 2026). Taken together, the OD confound at the first timepoint and the shared decline at the last limit how much Fig. 2 alone can indicate about editing status.

Sanger sequencing provided the only nucleotide level test of editing status in this study, and none of the three colonies sequenced carried the intended TAG stop codon at position 5, all three instead matched the wild type GAA sequence of the unedited control (Fig. 4a-d). Average sequencing quality across the 21 bp window centred on codon 5 remained within five points of the control for all three samples (Fig. 4e). This indicates that these were high-confidence base calls rather than low-quality reads. It also rules out poor sequencing as an explanation for the absence of a detected edit. This null result should, however, be interpreted alongside an important methodological difference from Tong et al. (2021b), whereas Tong et al. verified editing outcomes by sequencing 24 isolated single colonies per condition, C_t_1, C_f, and C_d were sequenced from bulk liquid culture. A Sanger read from a bulk culture reports only the dominant sequence present across

the sampled population. This approach is characteristically far less sensitive to a minority edited subpopulation than sequencing of isolated clones. An edited variant generally must approach a substantial fraction of the population, rather than being present in only a small number of cells, before it becomes visible above background in the chromatogram trace. Accordingly, the present result indicates that the wild type sequence was the dominant outcome among the cells sampled in each bulk culture, but it does not by itself establish that no cell within either culture carried the intended edit.

This methodological gap in detection sensitivity is especially relevant given the nature of the edit attempted here. Converting codon 5 from GAA to TAG requires two simultaneous nucleotide changes within the same codon, at the first and third positions, while the middle base remains unchanged. This differs from the single-nucleotide T-to-A substitution that Tong et al. (2021b) characterized across a full anhydrotetracycline dose-response series. It more closely resembles a two-base-pair substitution event that they separately validated at the GFP locus by whole-genome sequencing of a single clone. No comparable dose-response efficiency was reported for that two-base-pair substitution event. A directly matched efficiency figure for a two position substitution is therefore not available from their data. Nonetheless, among the edit types Tong et al. (2021b) did characterize systematically, single-nucleotide substitution was consistently the least efficient. It reached only about 2% at the highest inducer concentration tested and about 1% at a more moderate concentration (approximately 200–256 ng/mL). Edits requiring more than one simultaneous base change, their combinatorial editing category, also fell well below the efficiency of a comparable single-nucleotide deletion or insertion, at approximately 20% and 13% under the same two conditions, respectively. This compares to roughly 44% and 29% for a 1 bp deletion under the same two conditions.

This pattern indicates that substitution-type and multibase edits are markedly less efficient than insertions or deletions of similar size in this toolkit. Whether a two-nucleotide substitution within a single codon behaves more like the low single-substitution result or the intermediate combinatorial result cannot be determined without testing it directly. In either case, if the efficiency of the present edit fell anywhere in this lower range, a bulk-culture sample would plausibly contain only a small minority of edited cells. This proportion could fall below the level at which a Sanger read of that population would show anything other than the wild-type consensus. Read in this light, the absence of a detected edit in the present data is broadly consistent with the comparatively low efficiency reported for substitution and multibase editing events in this system, rather than necessarily indicating that no editing occurred. Notably, Tong et al.'s (2021b) own verification strategy, sequencing 24 isolated clones per condition, reflects a design built specifically to resolve editing outcomes at this kind of low per cell efficiency, one that a three sample bulk culture approach is not equipped to resolve.

Several limitations qualify these findings. Most centrally, and consistent with the detection-sensitivity argument above, editing status was assessed by Sanger sequencing of only three bulk-culture samples. This falls short of isolated single-colony sequencing at a scale comparable to Tong et al.'s 24 clones per condition. It also falls short of amplicon-based deep sequencing capable of resolving low-frequency variants within a population. Second, the dataset does not include a matched no tetracycline induction condition for the Stop Codon construct, so a paired induced versus uninduced comparison, of the kind used by Tong et al. (2021b) to establish dose dependent editing efficiency, could not be performed. Additional confounds affect the fluorescence-based measurements discussed above. Fluorescence in Fig. 2 was not normalized to OD_{600}. The two plates compared in Fig. 3 also differed substantially in colony density and represent independent platings rather than replicates of the same condition. Sample sizes were modest throughout. Six manually selected colonies were quantified per plate. Only two genuinely distinct Codon-plus-TET plate images were available in the dataset (an additional candidate pair was found to be a byte-identical duplicate file rather than an independent replicate).

Only three colonies were carried forward to sequencing. No positive control was included to confirm that the fluorescence and sequencing workflows used here were capable of detecting a known successful edit. Fluorescence and sequencing data also came from different cells, plate grown colonies for fluorescence and bulk liquid culture for sequencing, so colony brightness cannot be directly linked to the sequenced genotype. Finally, as with any GFP-based reporter assay, loss of fluorescence is only a proxy for gene disruption in E. coli. Results obtained on editing efficiency in this bacterial system do not necessarily transfer directly to mammalian neurons, the eventual context for NR2B (GRIN2B) targeting in ischemic stroke.

Together, these results indicate that no stop codon edit was detected at codon 5 of GFP among the samples sequenced. They also indicate that the present design, bulk-culture sequencing of a small sample without a matched uninduced control, would not have reliably detected editing occurring at the low efficiencies reported previously for substitution-type edits. Distinguishing between these possibilities will require one of two approaches: isolating and sequencing single colonies at a scale comparable to Tong et al.'s (2021b) screen, or applying amplicon-based deep sequencing to detect low-frequency edited variants. Either approach would also need to be paired with generating the missing no-TET control needed for a proper induced versus uninduced comparison. Testing a deletion-type edit, which showed the highest efficiency in the Tong et al. (2021b) toolkit, could additionally serve as a positive system validation control. This would confirm that the cloning, culturing, and sequencing workflow used here is capable of detecting a successful edit, before returning to the more technically demanding substitution required for stop codon insertion into NR2B. Establishing these methodological foundations in this bacterial reporter system is a necessary prerequisite for extending prime editing toward NR2B (GRIN2B) silencing in mammalian models of ischemic stroke.

5. Conclusion

This study established a Sanger sequencing and colony fluorescence based workflow for evaluating CRISPR prime editing of a stop codon at codon 5 of GFP in E. coli. Across three sequenced colonies, no evidence of the intended GAA to TAG edit was detected, and the accompanying fluorescence measurements were subject to confounds that limit their ability to indicate editing status on their own. Because this edit type is reported elsewhere as markedly less efficient than insertion or deletion type edits, and because only bulk-culture sequencing of three samples was performed, this null result is best read as inconclusive rather than as evidence against editing having occurred. Establishing a more sensitive verification workflow, through single-colony sequencing and a matched no-TET control, is a necessary next step before extending this approach toward NR2B (GRIN2B) silencing in ischemic stroke.

6. References

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I would like to thank my **parents**, who always support me in this research project. I would also like to thank **Y** and **Z** for their guidance and mentorship on this project, for training me in lab activities, data collection, and methodologies, and for reviewing and editing the paper.

Author Biography

X is a student at W. He plans to pursue a Bachelor's degree in neuroscience or informatics, with a specific interest in cognitive, neurological diseases, and genetics. He was awarded a bronze medal at the most prestigious national research competition in V for his neuroscience research on working memory, and has been running a free mentoring and manuscript review program for students preparing science fair projects, which has reached over 100 mentees across V.

Mentor Contribution Statement

Y, faculty mentor for this project, provided extensive feedback on the organization, language, and structure of the manuscript. He taught the author how to construct figures, including how to organize graphics for clarity and how to correctly indicate statistical significance and confidence levels on graphs, and facilitated the author's access to paywalled journal articles through his institutional access. **Z**, teaching assistant to Y, provided additional support throughout the

project. Together, Y and Z worked closely with the author on constructing the plasmid, troubleshooting experimental issues, and explaining the CRISPR prime editing system step by step as part of the laboratory activities. They also assisted extensively with collecting lab data, including OD and fluorescence intensity measurements, and tutored the author on using Fiji and Benchling to analyze the resulting datasets.

Title: Validating CRISPR Prime Editing for Targeted NR2B Inhibition in Ischemic Stroke Using an *E. coli* GFP Reporter System

Overall Comments

This study explores the use of a GFP reporter system in *E. coli* to evaluate CRISPR prime editing, with the longer-term goal of applying a similar strategy to NR2B inhibition in ischemic stroke. The topic is interesting, and the manuscript is generally organized in a logical way. I especially appreciated that the authors were careful in interpreting the negative results and discussed several technical limitations rather than overstating the findings. However, the main concern is that the current title and overall framing appear stronger than what the experimental data can support. The study does not directly test NR2B or an ischemic stroke model, and the intended GFP stop-codon edit was not detected by sequencing. Therefore, the manuscript would benefit from presenting the work more clearly as an evaluation of a bacterial reporter system and an initial feasibility study. Several experimental and presentation issues should also be addressed before the conclusions can be fully supported.

Major Comments

1. **Result**: The Results section currently reads more like a list of individual findings, which makes the overall logical flow difficult to follow. Although scientific writing should be objective, it should still tell a coherent story. For each figure or experiment, I suggest following a clear sequence: briefly explain why the experiment was performed, what approach was used, what the main finding was, and what the finding suggests or means in the context of the study. This structure will help the reader understand how each result connects to the overall research question.
   Also, I would avoid phrases such as “Figure 4 presents...” Instead, describe the scientific finding directly and cite the relevant figure at the end of the sentence or paragraph (Fig. 4). This will make the Results section sound more formal and consistent with scientific writing.

2. **Title and overall scope:** The current title suggests validation of prime editing for NR2B inhibition in ischemic stroke. However, NR2B was not directly targeted, and the experiments were performed using GFP in *E. coli*. I suggest focusing the title and main conclusions on evaluation of the GFP reporter system, while presenting NR2B/stroke as a potential future application.

3. **Interpretation of the editing results :** None of the three sequenced samples contained the expected TAG stop codon. Therefore, terms such as “validation” should be used cautiously. The authors appropriately discuss the limitations of bulk-culture Sanger sequencing and the possibility of low-frequency editing, but the conclusions should consistently reflect that successful editing was not demonstrated in the current experiment.

4. **Fluorescence analysis and OD_{600} normalization :** In Figure 2, the starting OD_{600} values differed considerably among conditions, while raw fluorescence was compared directly. Normalizing GFP fluorescence to OD_{600} would provide a more meaningful

comparison of fluorescence relative to bacterial density. This is particularly important because the fluorescence eventually decreased to similar levels across all conditions.

5. The current experiments do not appear to be directly related to ischemic stroke, although the manuscript emphasizes stroke as an important disease context. If the authors wish to maintain this connection, additional evidence would be needed to demonstrate the relevance of the gene to ischemic stroke. For example, an *in silico* analysis using publicly available datasets could be used to examine whether the gene is altered in ischemic stroke and whether it is associated with relevant biological pathways. Alternatively, if such additional analyses are beyond the scope of the study, I suggest reducing the emphasis on ischemic stroke and focusing the manuscript on what is directly supported by the current experiments.

Minor Comments

1. **Introduction:** The Introduction contains several grammatical and awkwardly worded sentences. For example, the paragraph describing the different roles of GluN2A and GluN2B is difficult to follow in places and should be carefully edited for clarity.
2. **Line 39** remains one the leading causes -> one of XXXX
3. The authors should explain how the six colonies used for fluorescence quantification were selected. Random or predefined selection criteria would help reduce possible selection bias.
4. Where is Figure 1?
5. **Method:** The Methods would benefit from a clearer description of the number of biological and technical replicates. A brief statistical analysis section should also be included if statistical comparisons are intended.
6. **Figur3:** Figure 3c and 3d use different y-axis scales. Using the same scale, if possible, would make the difference between the two groups easier to evaluate visually.
7. The connection between the bacterial GFP reporter experiment and eventual NR2B editing in mammalian neurons should be stated more cautiously. The current experiment can establish or evaluate a technical workflow, but it does not directly demonstrate the feasibility or therapeutic effectiveness of NR2B editing in ischemic stroke.

Overall, this is an interesting preliminary study, and the authors have done a good job discussing the limitations of the current dataset. The manuscript would be considerably stronger if the claims and framing were brought into closer alignment with what was directly demonstrated experimentally.

Final Recommendation: Revise and resubmit

Title: Validating CRISPR Prime Editing for Targeted NR2B Inhibition in Ischemic Stroke Using an E. coli GFP Reporter System

Recommendation: Accept with major revisions

This manuscript investigates CRISPR prime editing as a potential strategy for targeted NR2B inhibition in ischemic stroke. Using an E. coli GFP reporter system as an initial proof-of-concept, the study tests whether prime editing can introduce a premature stop codon into GFP, with loss of fluorescence as a proxy for gene disruption. The author evaluates bacterial growth and fluorescence, quantifying fluorescence at the colony level, and using Sanger sequencing to assess whether the chosen GAA-to-TAG edit occurred.

The paper addresses an interesting and clinically relevant question, and the use of a bacterial GFP reporter system as an initial model for developing a more complex NR2B-targeted approach is very creative! I particularly appreciated the author's openness to critically interpreting their own negative experimental result, rather than overstate their findings. The Discussion thoughtfully considers several explanations for why the intended edit was not detected, including the use of bulk-culture sequencing, the small number of samples sequenced, and the potentially low efficiency of the two-nucleotide substitution. Importantly, the author distinguishes between no editing occurring and no editing being detected, which is an important scientific distinction.

While the overall study has merit and a really interesting research premise, the manuscript requires Major Revisions before it is ready for publication. Most key concerns involve the framing of the study, interpretation of the fluorescence data, and limitations of the experimental design rather than the broader scientific question.

Key strengths

- Creative use of a simple GFP reporter system as an initial step toward investigating a more complex therapeutic application of prime editing.
- The manuscript is transparent about the negative sequencing result of all three tested samples retaining the wild-type sequence rather than showing the intended TAG stop codon.
- The Discussion does a particularly good job considering methodological explanations for the findings rather than interpreting the absence of a detected edit as definitive evidence that editing did not occur.
- Inclusion of growth measurements, colony-level fluorescence analysis, and Sanger sequencing provides multiple approaches to evaluating the experimental outcome.
- The broader biological motivation for selectively targeting NR2B rather than globally inhibiting NMDA receptors is interesting and clearly establishes the potential significance of the longer-term research direction.

Major revisions

- The title and overall framing currently make a stronger claim than the experiments support. The study does not directly investigate NR2B or an ischemic stroke model, and the intended GFP edit was not successfully demonstrated. Therefore, describing the study as "Validating CRISPR Prime

Editing for Targeted NR2B Inhibition" may be misleading. I would recommend reframing the study more explicitly as a preliminary or feasibility experiment evaluating a GFP reporter workflow that could eventually be adapted to NR2B.

- The interpretation of the fluorescence data should be a little more cautious. For example, in Figure 2, fluorescence was not normalized to OD600, despite substantial differences in bacterial density between the Stop Codon construct and controls at the first timepoint. The manuscript appropriately acknowledges this limitation in the Discussion, but it makes the early fluorescence differences difficult to interpret as changes in GFP expression rather than differences in the number of bacteria present.
- Similarly, the colony-level fluorescence differences in Figure 3 should not be interpreted too strongly. Experiment Set A and Sample C_f differ substantially in colony density and represent independent platings rather than replicates of the same condition. The approximately 3.6-fold difference in mean fluorescence is interesting, but differences in crowding, nutrient availability, and background correction could contribute substantially to this result.
- The sequencing strategy is the most important methodological limitation. Only three samples underwent Sanger sequencing, and these were obtained from bulk liquid culture. As the author discusses, bulk Sanger sequencing primarily reflects the dominant sequence and may fail to detect a small edited subpopulation. The comparison to Tong et al., who sequenced 24 isolated colonies per condition, is particularly important. If additional experiments are feasible, sequencing a larger number of isolated colonies would substantially strengthen the study. If not, this limitation should remain central to the interpretation of the results.
- The manuscript should more clearly distinguish between the immediate experimental conclusion and the longer-term therapeutic hypothesis. The present experiment can evaluate the feasibility of a particular prime-editing workflow in GFP, but it cannot yet establish whether NR2B can be successfully edited, whether doing so would reduce excitotoxic neuronal injury, or whether this approach could ultimately function therapeutically in ischemic stroke. I would recommend consistently framing these as future directions rather than implications demonstrated by the present study.

Minor comments

- The manuscript would benefit from careful grammatical and stylistic editing. There are a few awkward or incomplete sentences, particularly in the Introduction, that occasionally make the scientific argument difficult to follow.
- Consider simplifying some of the longer paragraphs in the Discussion. The discussion of editing efficiency and comparison with Tong et al. is valuable, but parts could be condensed to make the central argument easier to follow.
- Figure 3 uses different y-axis scales for the two fluorescence plots. Using the same scale, or making the difference particularly prominent in the figure legend, would make comparison between the groups more intuitive.
- The Methods state that "positive clones identified by loss of GFP fluorescence were verified" by sequencing, which could imply that these clones were confirmed as successfully edited. Because sequencing ultimately showed the wild-type sequence, I would revise this language to distinguish candidate clones selected based on fluorescence from molecularly confirmed edited clones.

- The authos should ensure that all experimental data are included as an appendix or made available through a repository, as required by the journal's submission guidelines.

Overall, this is an interesting and thoughtful preliminary study addressing a potentially important application of CRISPR prime editing. I particularly appreciated the author's careful discussion of the negative sequencing result and recognition that failure to detect the intended edit does not necessarily establish that editing did not occur. With revisions that more clearly frame the study as a preliminary feasibility experiment, align the conclusions with what was directly demonstrated, and emphasize the limitations of the fluorescence and sequencing approaches, I believe the manuscript would be substantially strengthened.

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A GFP Reporter Workflow for Evaluating CRISPR Prime Editing in *E. coli*: A Feasibility Study Toward NR2B-Targeted Editing

Abstract

Ischemic stroke damages neurons in part through overactivation of NMDA receptors that contain the GluN2B (NR2B) subunit. Drugs that block the whole receptor have failed in clinical trials, so selective NR2B inactivation remains an unmet goal. CRISPR prime editing could install a loss-of-function edit in GRIN2B, but only if that edit can be reliably detected. This study tested such a verification workflow in a simple bacterial reporter system. A pegRNA was designed to change codon 5 of GFP in *Escherichia coli* from GAA to the stop codon TAG. Loss of fluorescence was used as a candidate screening proxy. Growth, bulk and colony fluorescence, and Sanger sequencing at the target site were assessed. All three matched the wild-type sequence, so none carried the intended edit. The fluorescence data were confounded by culture density and by colony size. No unedited GFP-expressing control was included, and sequencing was performed on pooled colonies rather than isolated ones. The result is therefore inconclusive rather than negative, and identifies detection sensitivity as the problem to solve before this approach is extended to GRIN2B.

Keywords: CRISPR prime editing, NR2B (*GRIN2B*), ischemic stroke, GFP reporter system, *Escherichia coli*, feasibility study, editing verification sensitivity

1. Introduction

Ischemic stroke remains one of the leading causes of mortality and disability worldwide, with around 7.8 million new cases and 3.6 million deaths in 2021 alone (Feigin et al., 2025). While reperfusion therapies such as mechanical thrombectomy restore blood flow, they do not directly address neuronal damage that follows the initial ischemic insult, leaving many

recovered patients with permanent side effects (Dammavalam et al., 2024). This has motivated years of study into neuroprotective strategies that target the cellular mechanisms of injury rather than blood flow alone.

A central driver of this secondary damage is glutamate excitotoxicity. During ischemic stroke, energy failure causes excessive glutamate release and reuptake dysfunction, leading to sustained overactivation of N-methyl-D-aspartate receptors (NMDARs) on postsynaptic neurons (Yu et al., 2023). Critically, not all NMDAR subtypes contribute equally to this damage. Receptors containing the GluN2B (NR2B) subunit are strongly associated with pro-death signalling, whereas GluN2A-containing receptors tend to promote neuronal survival. This functional divergence makes NR2B a more appealing therapeutic target than the receptor as a whole. It may also explain why pan-NMDAR antagonists have repeatedly failed in clinical trials, since blocking the receptor indiscriminately inhibits the protective GluN2A pathway alongside the harmful GluN2B one (Wu et al., 2023).

Achieving NR2B inhibition pharmacologically has proven difficult, motivating interest in precision genome editing tools that could mutate a single target gene with minimal off-target effects. CRISPR prime editing is one such tool since it uses a Cas9 nickase (Cas9n) fused to an engineered reverse transcriptase, guided by a prime editing guide RNA (pegRNA) without generating double-strand breaks or requiring a donor template (Anzalone et al., 2019). Because prime editing can make precise insertions, such as premature stop codons that shorten a protein and abolish its function, it offers a candidate route to targeted, functional inactivation of NR2B (*GRIN2B*), an approach that has not been tested in a neural or disease context.

Before such an approach can be tested in a mammalian or clinical context, it is necessary to establish whether prime editing can install a loss-of-function edit. It is equally necessary to establish whether a given workflow is sensitive enough to detect that edit when it occurs. Prior work has demonstrated this principle in *Escherichia coli* by using prime editing to insert premature stop codons into the gene encoding green fluorescent protein (GFP), with loss of fluorescence functioning as an easily scored proxy for successful gene disruption (Tong et al., 2021). The present study adapts this bacterial GFP based design as a first step toward the longer-term goal of NR2B-targeted prime editing. Using *E. coli* expressing GFP as a substitute target, we designed a pegRNA to insert a premature stop codon into the GFP coding sequence, hypothesizing that successful editing would silence GFP expression and be visually detectable as loss of fluorescence. Evaluating this approach in a simple, well-characterised system allows the verification workflow itself to be assessed before it is applied to a more demanding target. Extension to NR2B (*GRIN2B*) silencing in models of ischemic stroke remains a future objective rather than an outcome of the present work. The experimental design and the verification workflow are summarised in Figure 1.

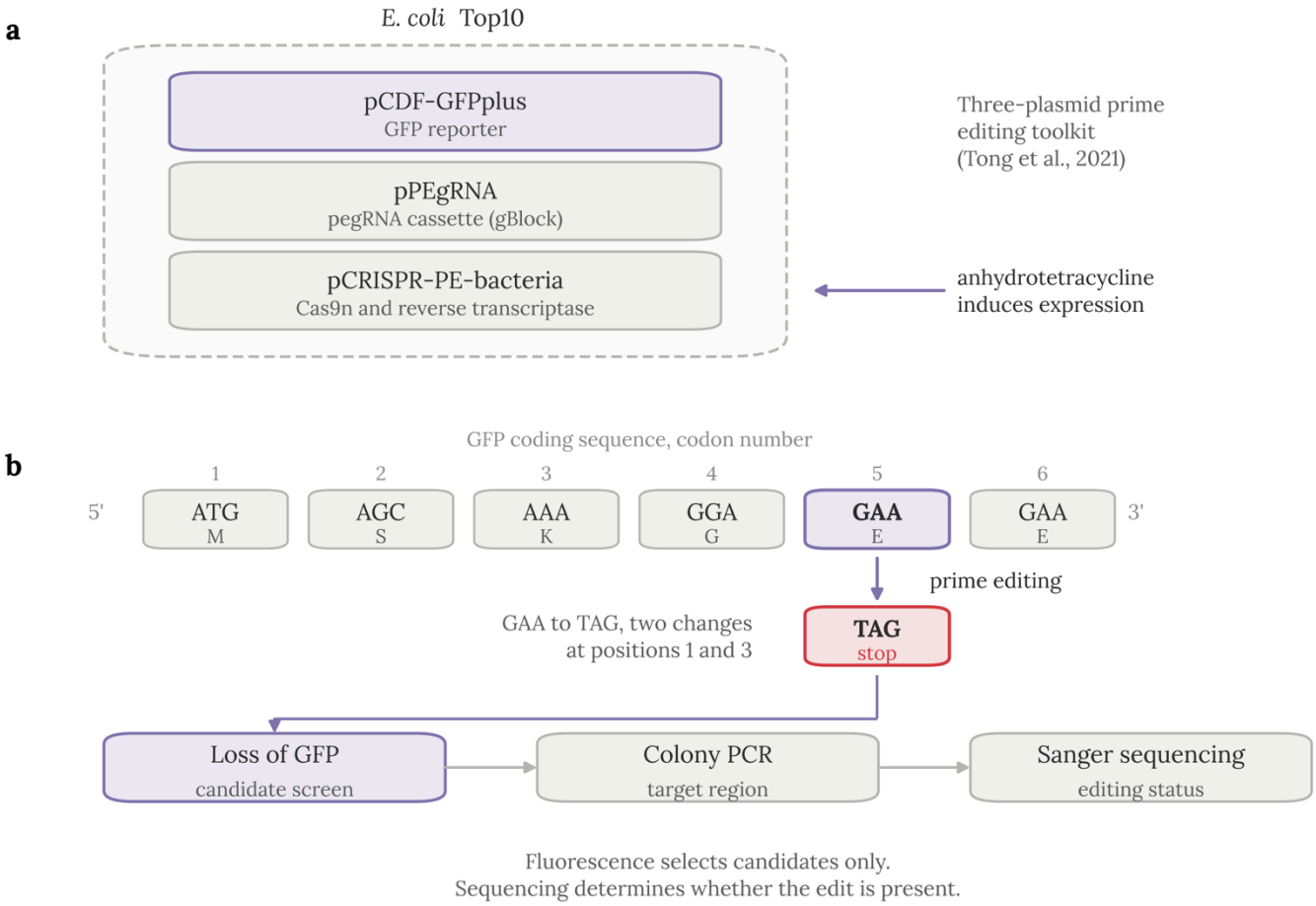


Figure 1: Experimental design and verification workflow. (a) The three-plasmid prime editing system in *Escherichia coli* Top10, comprising the pCDF-GFPplus reporter, the pPEgRNA plasmid carrying the pegRNA cassette, and pCRISPR-PE-bacteria encoding the Cas9 nickase and reverse transcriptase fusion, adapted from Tong et al. (2021). Expression of the editing machinery is induced with anhydrotetracycline. (b) The intended edit converts codon 5 of the GFP coding sequence from GAA to the stop codon TAG, which requires two nucleotide changes at the first and third positions. Candidate clones were screened by loss of GFP fluorescence, then assessed by colony PCR and Sanger sequencing across the target region.

2. Methods

2.1 Strains, Plasmids, Media, and Growth Conditions

Escherichia coli Top10 cells and the plasmids used in this study were adapted from the CRISPR-Prime Editing toolkit for E.

coli (Tong et al., 2021). Cultures were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (Fisher Scientific). Appropriate antibiotics were supplemented at the following working concentrations: spectinomycin (50 µg/mL), ampicillin (100 µg/mL), and chloramphenicol (25 µg/mL), with anhydrotetracycline (0–1 µg/mL) used to induce expression of the Cas9n–reverse transcriptase fusion. Two percent agar was used for agar plates. Antibiotics were purchased from Sigma-Aldrich (USA).Enzymes and cloning reagents were purchased from New England Biolabs (USA). Plasmid purification kits were purchased from Qiagen (Germany).

2.2 pegRNA Design and Plasmid Construction

A pegRNA targeting the GFP+ coding sequence was designed to insert a premature stop codon at the fifth codon position, following the standard prime editing guide RNA architecture (20-nucleotide protospacer, gRNA scaffold, reverse transcriptase template, and primer binding site, each ~13 nucleotides). The pegRNA cassette was synthesised as a gBlock and assembled into the empty pPEgRNA backbone by Gibson Assembly (NEB Gibson Assembly Master Mix, E2611S) using NEBuilder compatible overhangs. Plasmid backbones were amplified by PCR using Q5 High-Fidelity 2X Master Mix (NEB, M0492S).

2.3 Transformation and Clone Selection

Assembled plasmid DNA was introduced into chemically competent Top10 cells by heat-shock transformation, cells were equilibrated on ice with the Gibson Assembly product for 5 minutes, heat-shocked at 42°C for 30 seconds, returned to ice, recovered in SOC medium at 37°C for 1 hour, and plated on LB agar containing the appropriate antibiotic. Individual colonies were picked for downstream screening.

2.4 Plasmid Isolation and PCR Screening

Plasmid DNA was isolated from overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, 27104). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, 28104) and resolved on agarose gels (Sigma, A6013) stained with SYBR Safe (Thermo Fisher, S33102) in 1X TAE buffer, alongside a 1 Kb Plus DNA Ladder (Thermo Fisher, 10787018).

2.5 Fluorescence and Growth Measurements

Bacterial growth (OD_{600}) and GFP fluorescence intensity were measured using a SPARK multimode microplate reader in a 96-well plate format. Wells containing LB medium only (no bacteria) served as the blank control. Colonies were additionally imaged under UV illumination using a gel documentation system (SGC-W5000 Plus, Servicebio) to visually assess fluorescence.

2.6 Fluorescence Image Analysis

Fluorescence images were quantified using Fiji (ImageJ). A region of interest was drawn around each of six individual colonies per plate using the ROI Manager. A seventh region of interest was placed on an area of agar with no colonies, to serve as a background measurement. For every region the Area, Mean, Min, Max, IntDen, and RawIntDen measures were

recorded. Background-corrected RawIntDen was calculated by subtracting the background region's RawIntDen from each colony value. The background region was not matched in area to the colony regions. It covered 289 pixels against 306 to 484 pixels for Experiment Set A, and 81 pixels against 96 to 154 pixels for Sample C_f. A fixed total was therefore subtracted from regions of differing size, which under-corrects larger regions and over-corrects smaller ones. A size-independent measure was calculated for this reason, as the colony mean grey value minus the background mean grey value. The six colonies on each plate were picked without reference to their brightness, and no formal randomisation procedure was used. Measurements were exported to Microsoft Excel for analysis. Per-colony values are given in Table S1.

Replication was as follows. Bulk growth and fluorescence were measured once per condition at each timepoint, so Figure 2 represents a single biological replicate with no technical replicates. Colony fluorescence was quantified for six colonies on each of two independent platings. No inferential statistical tests were performed, because the design does not support them. Summary values are reported as means, ranges and coefficients of variation.

2.7 Assessment of Editing Status by Sanger Sequencing

Candidate clones were selected on the basis of reduced GFP fluorescence and assessed at the DNA level by colony PCR followed by Sanger sequencing across the target region, in order to determine whether the intended stop codon was present at codon 5 of the GFP coding sequence. Selection on fluorescence identifies candidates only. It does not establish that an edit occurred.

2.8 Public Expression Data

Public expression data were used to establish whether the intended target gene is altered in ischemic brain tissue. The dataset GSE38037 was retrieved from the NCBI Gene Expression Omnibus (platform GPL3631). It comprises cerebral cortex from Sprague Dawley rats after transient middle cerebral artery occlusion (tMCAO, n = 4, GSM932851 to GSM932854) and from sham-operated controls (n = 4, GSM932847 to GSM932850). The array is two colour. Every sample, tMCAO and sham alike, was co-hybridised against a common reference pool of sham-operated cortex, so each value is a ratio against that shared reference. Differential expression between the two groups was computed with GEO2R. GEO2R fits a limma empirical-Bayes linear model to the series matrix and corrects for multiple testing across all 23,376 probes by the Benjamini-Hochberg procedure. No new experimental data were generated for this analysis.

3. Results

3.1 Growth and Fluorescence Measurements

Bacterial growth (OD_{600}) was monitored at three timepoints (10:45, 13:30, and 16:48) for the Stop Codon construct and the two plasmid-only controls (Fig. 2a). All three conditions grew comparably and reached similar final OD_{600} values of 0.14 to 0.16. The Stop Codon condition was a single culture measured at these three timepoints, so n = 1 biological replicate.

Raw GFP fluorescence in the Stop Codon construct fell across the day, from 47,344 a.u. at 10:45 to 37,891 a.u. at 13:30 and 3,417 a.u. at 16:48 (Fig. 2b). Both plasmid-only controls stayed low throughout between 7,879 and 18,018 a.u. By the final

timepoint, all three samples had converged to a low range of 3,417 to 3,631 a.u. This convergence occurred in the Stop Codon construct and in both controls alike, so it cannot be a signature of editing.

Neither control carries the GFP reporter plasmid. Their signal therefore reflects background autofluorescence and not unedited GFP expression. They serve as plasmid-only controls rather than as unedited-GFP comparators. The study includes no GFP-expressing condition that received no pegRNA. Without that condition there is no baseline from which fluorescence could be described as lost, so the fluorescence data cannot speak to editing status.

Fluorescence scales with the number of cells present, so each reading was divided by the OD_{600} of the same sample (Table 1). Normalised values for the Stop Codon construct were 428,452, 471,281 and 21,946 a.u./OD_{600} across the three timepoints. Relative to the mean of the two controls, the Stop Codon construct was 2.39 times higher at 10:45, 1.43 times higher at 13:30 and 0.88 times as high at 16:48. The equivalent raw ratios were 5.85, 2.18 and 0.96. Normalisation therefore removes more than half of the early difference. It also places the Stop Codon construct slightly below both controls at the final timepoint, where the raw data had the three conditions roughly equal. Because the controls carry no GFP, the difference that remains confirms GFP expression in the Stop Codon construct and does not bear on editing status.

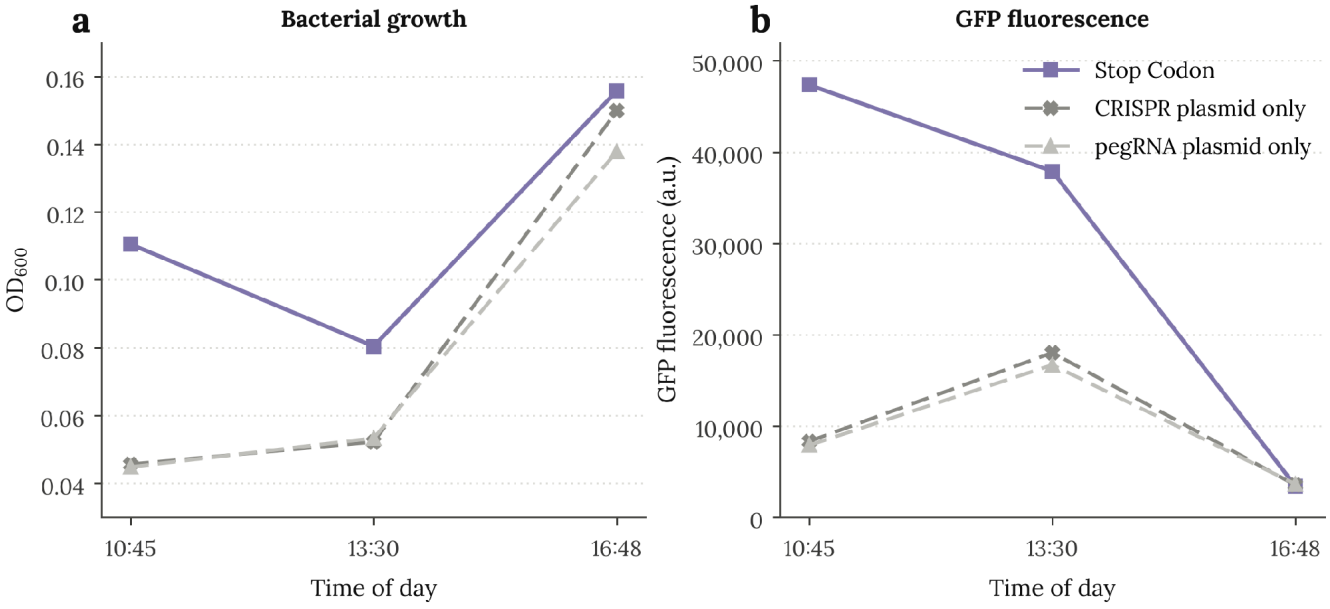


Figure 2: Bacterial growth and GFP fluorescence for the Stop Codon construct and the plasmid-only controls. (a) OD_{600} and (b) raw GFP fluorescence at three timepoints (10:45, 13:30 and 16:48) for the Stop Codon construct, the CRISPR plasmid only control and the pegRNA plasmid only control. Each condition is a single culture measured at the three time points, so n = 1 biological replicate with no technical replicates. Neither control carries the GFP reporter plasmid, so the control signal reflects background autofluorescence rather than unedited GFP expression. Fluorescence normalised to culture density is given in Table 1.

Table 1: GFP fluorescence normalised to culture density (a.u./OD_{600}). Blank-corrected fluorescence divided by the OD_{600} of the same sample at the same timepoint, for the Stop Codon construct and the two plasmid-only controls at each of the three timepoints.

Condition	**10:45**	**13:30**	**16:48**
Stop Codon	428,452	471,281	21,946
CRISPR plasmid only	182,259	345,172	23,485
pegRNA plasmid only	175,871	312,964	26,350

Plate images and colony fluorescence for two independent platings are shown in Fig. 3. The plate for Experiment Set A (Fig. 3a) carried colonies at moderate density that were individually distinguishable. The plate for Sample C_f (Fig. 3b) was markedly denser, with several regions approaching confluence.

Background-corrected RawIntDen was then measured for six colonies on each plate. In Experiment Set A (Fig. 3c) values ranged from 19,747 to 40,270, with a mean of 26,313. In Sample C_f (Fig. 3d) values ranged from 3,128 to 12,542, with a mean of 7,294. The two means differ by a factor of about 3.6. RawIntDen is the sum of grey values inside a region of interest, so it scales with the area of that region. Dividing RawIntDen by the mean grey value recovers the pixel count of each region. Colonies in Set A averaged 355 pixels and those in C_f averaged 124 pixels, a difference of 2.85 times in area. Most of the 3.6-fold difference in RawIntDen is therefore a difference in colony size rather than in brightness.

A size-independent measure was calculated as the colony mean grey value minus the background mean grey value. On this measure Set A averaged 65.1 and C_f averaged 41.9, a difference of 1.55 times. The two groups also overlap. C_f colonies 4 and 6 (66.9 and 62.1) were brighter than the two dimmest colonies in Set A (56.8 and 56.0). Variability was much higher in C_f, with a coefficient of variation of 48.2% against 12.9% for Set A. Per-colony values and pixel counts are given in Table S1.

These two plates are two separate platings of the same construct that differed substantially in colony density, so plate to plate technical variation cannot be separated from any real difference in brightness. The six colonies on each plate were picked without reference to their brightness, and no formal randomisation procedure was used. This comparison is therefore descriptive and cannot be read as a difference in editing status.

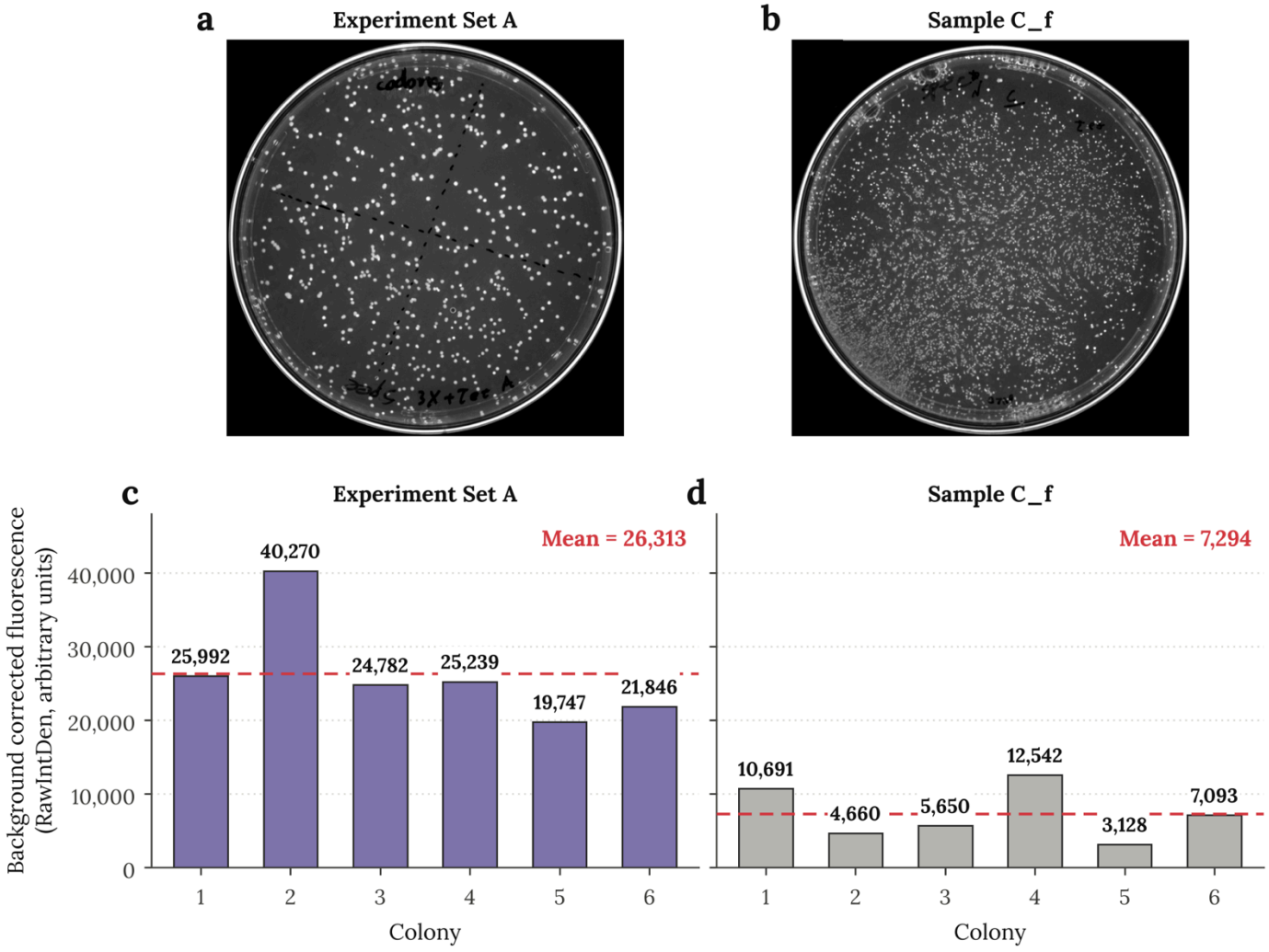


Figure 3: Plate density and colony fluorescence for two independent platings of the Stop Codon construct under anhydrotetracycline induction. (a) Plate image for Experiment Set A, showing colonies at moderate density. (b) Plate image for Sample C_f, showing markedly higher density with regions approaching confluence. (c, d) Background-corrected RawIntDen for six colonies from each plate, Experiment Set A in (c) and Sample C_f in (d), plotted on a common y-axis. RawIntDen is the sum of grey values within a region of interest and therefore scales with colony area, which differs 2.85-fold between the two plates. The size-independent per-pixel measure differs only 1.55-fold. Per-colony values, region areas and per-pixel intensities are given in Table S1. The two plates are separate platings of the same construct and differed substantially in colony density.

3.2 Editing Status at the Target Site

Three Stop Codon samples were sequenced across the codon 5 target site to test directly whether the intended edit was present. Each was compared with an unedited no pegRNA reference (Fig. 4a). The three test colonies, C_t_1 (well B08, forward read, Fig. 4b), C_f (well H04, reverse read, Fig. 4c), and C_d (well D05, reverse read, Fig. 4d), read GAA, TTC, and TTC at codon 5, respectively (TTC being the reverse complement of GAA on the reverse strand), matching the sequence in Fig. 4a. None of the three colonies shows the TAG sequence. In addition, average Sanger sequencing quality scores across the 21 bp window centred on codon 5 (Fig. 4e) were 57.4 for C_t_1, 54.2 for C_f, and 59.4 for C_d, compared with 59.0 for the unedited control (dashed red line). C_t_1, C_f, and C_d were sequenced from bulk liquid culture, whereas Tong et al. (2021) sequenced isolated single colonies.

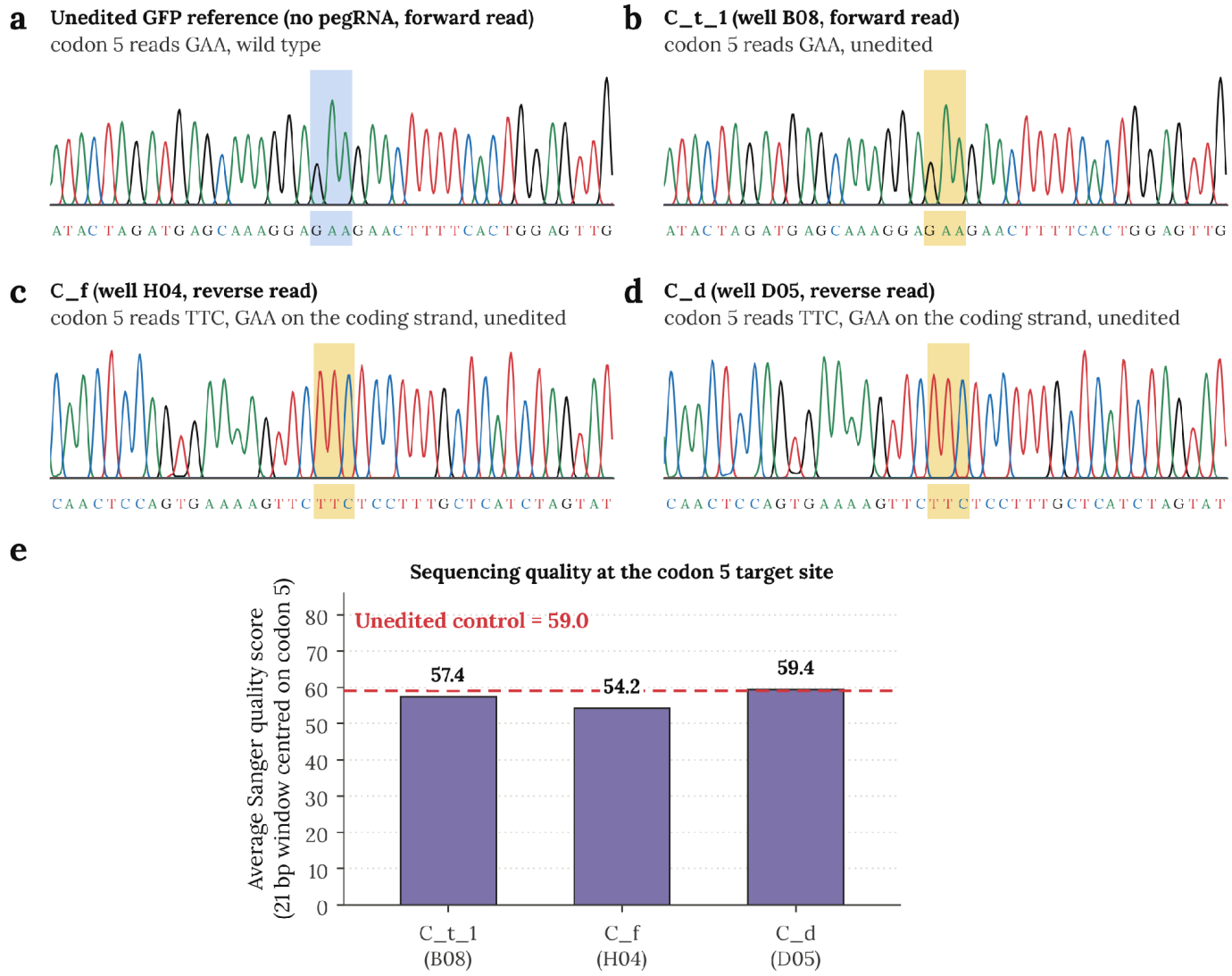


Figure 4: Sanger sequencing at the GFP codon-5 target site. (a) Chromatogram of the unedited GFP reference (no pegRNA control, forward read), showing wild-type codon 5 (GAA). (b) Chromatogram of sample C_t_1 (well B08, forward read), showing unedited codon 5 (GAA). (c) Chromatogram of sample C_f (well H04, reverse read), showing unedited codon 5 as TTC on the reverse strand, corresponding to GAA on the coding strand. (d) Chromatogram of sample C_d (well D05, reverse read), showing unedited codon 5 as TTC on the reverse strand. (e) Mean Sanger sequencing quality score across a 21 bp window centred on codon 5 for each sample, with the unedited control shown as a dashed line at 59.0. None of the three samples carries the intended TAG codon. All three were sequenced from bulk liquid culture rather than isolated

single colonies.

3.3 *Grin2b* Expression in Ischemic Cortex

In GSE38037, *Grin2b* was modestly higher in ischemic cortex than in sham cortex (probe 10020292529, $\log_2$ fold change +0.18, equivalent to a 1.13-fold increase, $p = 0.014$, adjusted $p = 0.049$). Other NMDA receptor subunit transcripts rose to a similar or greater degree. These were *Grin1* (+0.27, adjusted $p = 0.005$), *Grin2a* (+0.34, adjusted $p = 0.009$), *Grin2c* (+0.21, adjusted $p = 0.006$) and *Grin3a* (+0.29, adjusted $p = 0.020$). *Grin2d* and *Grin3b* were unchanged (Fig. 5). In this dataset, 6,919 of 23,376 probes (29.6%) reached an adjusted p below 0.05. That high proportion is consistent with the low within-group variance expected of a common-reference two-colour design. Statistical significance alone therefore provides limited evidence of specificity, and *Grin2b* ranked 6,850th by p-value. These data are consistent with coordinated engagement of NMDA receptor subunit expression after cerebral ischemia. They do not show selective *Grin2b* dysregulation.

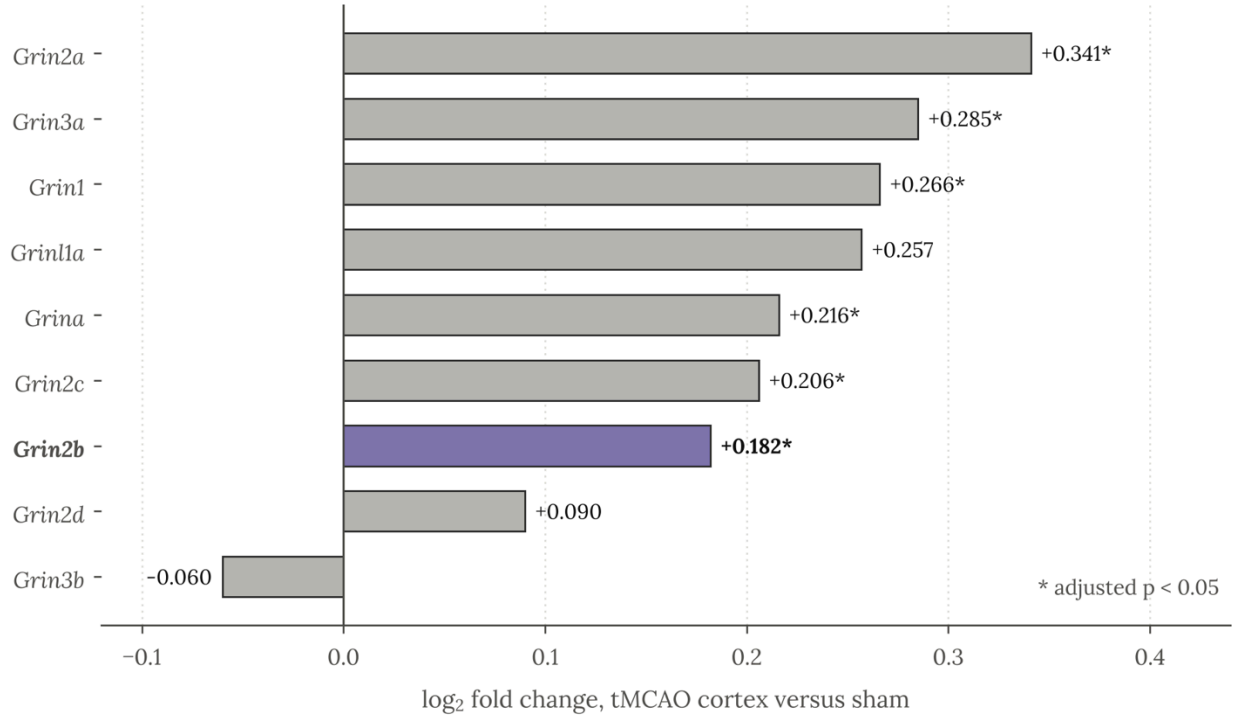


Figure 5: NMDA receptor subunit transcript changes in rat ischemic cortex. Differential expression of NMDA receptor subunit and subunit-associated transcripts in GSE38037 (transient middle cerebral artery occlusion versus sham, rat cerebral cortex, four samples per group), computed with GEO2R. Bars show $\log_2$ fold change. *Grin2b*, the target of the editing strategy evaluated in this study, is highlighted. Asterisks mark an adjusted p below 0.05 after Benjamini–Hochberg correction. *Grin2b* shows the smallest significant increase among the subunit transcripts measured.

4. Discussion

Public expression data indicate that *Grin2b* is transcriptionally higher in ischemic cortex. This confirms that the gene is altered in the disease context that motivates this work. The effect is small, and comparable or larger increases occur in the other NMDA receptor subunits. These data therefore do not identify *GRIN2B* as uniquely dysregulated. The case for subunit-selective intervention rests instead on the divergent downstream signalling of GluN2A-containing and GluN2B-containing receptors (Wu et al., 2023; Yu et al., 2023). Transcript abundance does not address that question. The relevance of this analysis to the present study is narrower still. It establishes that the eventual target is disease-relevant, while the editing and verification questions examined here remain unresolved. The public expression analysis used a rodent model of ischemia rather than human stroke tissue, and the gene is *Grin2b* in rat rather than *GRIN2B* in human. It is also a transcript-level observation, which does not establish that receptor-level signalling changes in proportion.

Three lines of evidence were presented above. These were growth and bulk fluorescence over time (Fig. 2), colony fluorescence on two plates (Fig. 3), and Sanger sequencing at the codon 5 target site (Fig. 4). None of them provides molecular evidence of the intended GAA to TAG edit. Growth was comparable across the Stop Codon construct and both plasmid-only controls. The fall in fluorescence at the final timepoint occurred in all three conditions alike, including the two that carry no GFP. Sequencing showed the wild-type codon in all three samples tested. Each line of evidence is taken in turn below.

Considered on its own, the difference in mean colony brightness between Experiment Set A and Sample C_f could be read as evidence of differing editing status between the two plates. However, this interpretation is complicated by colony density, which also differed markedly between the two plates: colonies in Set A were moderately spaced and individually distinguishable, while those in C_f were considerably denser and, in places, near confluent (Fig. 3a, b). Because fluorescence was quantified from manually drawn regions of interest around individual colonies, crowding on the C_f plate provides a plausible technical confound. Overlapping regions of interest, competition for nutrients or oxygen between adjacent colonies, and reduced accuracy of background correction could each lower or add noise to individual colony readings. These effects would occur independent of any underlying genotype (Chacón et al., 2018). The comparatively higher variability observed in C_f is more consistent with this kind of density related technical variation than with a straightforward genotype driven reduction in brightness. Because the two plates are separate platings of the same construct and were not matched for density, the present data cannot separate a genuine biological difference from plate to plate technical variation.

A related limitation affects Fig. 2. At the first timepoint, the Stop Codon construct already showed both higher OD_{600} (0.11) and higher fluorescence (47,344 a.u.) than either control. Control OD_{600} values were roughly 2.4 times lower (0.045) at the same timepoint. Because the fluorescence plotted in Fig. 2b is raw rather than normalised, part of the early difference in that panel is attributable to differences in culture density rather than to per-cell GFP expression. The normalised values in Table 1 remove most of it. By the final timepoint, fluorescence in all three conditions, including both plasmid-only controls, which carry no GFP reporter, converged to a similarly low range (3,417-3,631 a.u.) even as OD_{600} reached its highest value in every condition. This decline occurred uniformly across edited and unedited conditions alike. This pattern is more consistent with a general reduction in GFP signal as cultures approach stationary phase than with a signature specific to stop-codon-mediated loss of function. Possible causes of this general decline include reduced GFP synthesis, impaired chromophore maturation, or increased culture turbidity (El Itawi et al., 2026). Taken together, the OD confound at the first

timepoint and the shared decline at the last limit how much Fig. 2 alone can indicate about editing status.

Sanger sequencing provided the only nucleotide level test of editing status in this study, and none of the three colonies sequenced carried the intended TAG stop codon at position 5. All three instead matched the wild-type GAA sequence of the unedited control (Fig. 4a-d). Average sequencing quality across the 21 bp window centred on codon 5 remained within five points of the control for all three samples (Fig. 4e). This indicates that these were high-confidence base calls rather than low-quality reads. It also rules out poor sequencing as an explanation for the absence of a detected edit. This null result should, however, be interpreted alongside an important methodological difference from Tong et al. (2021), whereas they verified editing outcomes by sequencing 24 isolated single colonies per condition, C_t_1, C_f, and C_d were sequenced from bulk liquid culture. A Sanger read from a bulk culture reports only the dominant sequence present across the sampled population. This approach is characteristically far less sensitive to a minority edited subpopulation than sequencing of isolated clones. An edited variant generally must approach a substantial fraction of the population, rather than being present in only a small number of cells, before it becomes visible above background in the chromatogram trace. Accordingly, the present result indicates that the wild type sequence was the dominant outcome among the cells sampled in each bulk culture, but it does not by itself establish that no cell within either culture carried the intended edit.

The nature of the edit attempted here makes that sensitivity gap more important. Converting codon 5 from GAA to TAG requires two nucleotide changes within one codon, at the first and third positions. Tong et al. (2021) characterised a single-nucleotide substitution across a full anhydrotetracycline dose-response series, but reported no comparable series for a two-base substitution, so a directly matched efficiency figure is not available. Among the edit types they did characterise, substitution was consistently the least efficient. It reached only about 2% at the highest inducer concentration and about 1% at a moderate one. Their combinatorial category, which requires more than one simultaneous base change, reached about 20% and 13% under the same two conditions, against roughly 44% and 29% for a 1 bp deletion. If the efficiency of the present edit falls anywhere in that range, a bulk culture would contain only a small minority of edited cells, probably below the level at which a Sanger consensus would show anything but the wild-type sequence. The absence of a detected edit is therefore as consistent with low editing efficiency as with no editing at all.

Several limitations qualify these findings. Most centrally, and consistent with the detection-sensitivity argument above, editing status was assessed by Sanger sequencing of only three bulk-culture samples. This falls short of isolated single-colony sequencing at a scale comparable to Tong et al.'s 24 clones per condition. It also falls short of amplicon-based deep sequencing capable of resolving low-frequency variants within a population. Second, the dataset does not include a matched no tetracycline induction condition for the Stop Codon construct, so a paired induced versus uninduced comparison, of the kind used by Tong et al. (2021) to establish dose dependent editing efficiency, could not be performed. Additional confounds affect the fluorescence-based measurements discussed above. The two plates compared in Fig. 3 also differed substantially in colony density and were not matched replicates. Sample sizes were modest throughout. Six colonies were quantified per plate. Only two genuinely distinct Codon-plus-TET plate images were available in the dataset (an additional candidate pair was found to be a byte-identical duplicate file rather than an independent replicate). Only three colonies were carried forward to sequencing. No positive control was included to confirm that the fluorescence and sequencing workflows used here were capable of detecting a known successful edit. Fluorescence and sequencing data also came from different cells, plate grown colonies for fluorescence and bulk liquid culture for sequencing, so colony brightness cannot be directly linked to the sequenced genotype. Finally, as with any GFP-based reporter assay, loss of fluorescence is only a proxy for gene disruption in *E. coli*. Results obtained on editing efficiency in this bacterial system do

not necessarily transfer directly to mammalian neurons, the eventual context for NR2B (*GRIN2B*) targeting in ischemic stroke.

Together, these results indicate that no stop codon edit was detected at codon 5 of GFP among the samples sequenced. They also indicate that the present design, bulk-culture sequencing of a small sample without a matched uninduced control, would not have reliably detected editing occurring at the low efficiencies reported previously for substitution-type edits. Distinguishing between these possibilities will require one of two approaches: isolating and sequencing single colonies at a scale comparable to Tong et al.'s (2021) screen, or applying amplicon-based deep sequencing to detect low-frequency edited variants. Either approach would also need to be paired with generating the missing no-TET control needed for a proper induced versus uninduced comparison. Testing a deletion-type edit, which showed the highest efficiency in the Tong et al. (2021) toolkit, could additionally serve as a positive system validation control. This would confirm that the cloning, culturing, and sequencing workflow used here is capable of detecting a successful edit, before returning to the more technically demanding substitution required for stop codon insertion into NR2B. Establishing these methodological foundations in this bacterial reporter system is a necessary prerequisite for extending prime editing toward NR2B (*GRIN2B*) silencing in mammalian models of ischemic stroke.

5. Conclusion

This study implemented and evaluated a Sanger sequencing and colony fluorescence workflow for assessing CRISPR prime editing of a stop codon at codon 5 of GFP in *E. coli*. The sensitivity of that workflow proved to be the factor limiting what the data can show. Across three sequenced colonies, no evidence of the intended GAA to TAG edit was detected, and the accompanying fluorescence measurements were subject to confounds that limit their ability to indicate editing status on their own. Because this edit type is reported elsewhere as markedly less efficient than insertion or deletion type edits, and because only bulk-culture sequencing of three samples was performed, this null result is best read as inconclusive rather than as evidence against editing having occurred. Establishing a more sensitive verification workflow, through single-colony sequencing and a matched no-TET control, is a necessary next step before extending this approach toward NR2B (*GRIN2B*) silencing in ischemic stroke.

6. Data Availability

The public expression dataset analysed in this study is available from the NCBI Gene Expression Omnibus under accession GSE38037 (platform GPL3631). Differential expression was computed with GEO2R using its default settings, comparing four tMCAO samples (GSM932851 to GSM932854) with four sham-operated controls (GSM932847 to GSM932850). The full GEO2R output table, the OD_{600} and fluorescence readings behind Figure 2 and Table 1, and the Fiji region-of-interest measurements behind Figure 3 and Table S1 are available from the author on request. No new high-throughput sequencing data were generated in this study.

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X is a student at W. He plans to pursue a Bachelor's degree in neuroscience or informatics, with a specific interest in cognitive, neurological diseases, and genetics. He was awarded a bronze medal at the most prestigious national research competition in V for his neuroscience research on working memory, and has been running a free mentoring and manuscript review program for students preparing science fair projects, which has reached over 100 mentees across V.

Mentor Contribution Statement

Y, faculty mentor for this project, provided extensive feedback on the organisation, language, and structure of the

manuscript. He taught the author how to construct figures, including how to organise graphics for clarity and how to correctly indicate statistical significance and confidence levels on graphs, and facilitated the author's access to paywalled journal articles through his institutional access. **Z**, teaching assistant to Y, provided additional support throughout the project. Together, Y and Z worked closely with the author on constructing the plasmid, troubleshooting experimental issues, and explaining the CRISPR prime editing system step by step as part of the laboratory activities. They also assisted extensively with collecting lab data, including OD and fluorescence intensity measurements, and tutored the author on using Fiji and Benchling to analyse the resulting datasets.

8. Appendix

Table S1: Per-colony fluorescence measurements for Figure 3. Background corrected RawIntDen, region-interest area in pixels, and background-corrected mean intensity for each of the six colonies measured on each plate, with plate means and the background region on each plate. Region area was recovered as RawIntDen divided by mean grey value. Background-corrected mean intensity is the colony mean grey value minus the background mean grey value.

Plate	Colony	RawIntDen	ROI area (px)	Mean Intensity
Experiment Set A	1	25,992	340	68.8
	2	40,270	484	62.7
	3	24,782	306	78.2
	4	25,239	336	68.0
	5	19,747	320	56.8
	6	21,846	342	56.0
	Mean	**26,313**	**355**	**65.1**
	Background ROI	14,714	289	-
Sample C_f	1	10,691	154	47.5
	2	4,660	132	17.4
	3	5,650	120	32.0
	4	12,542	144	66.9
	5	3,128	96	25.4
	6	7,093	100	62.1

	Mean	**7,294**	**124**	**41.9**
	Background ROI	3,747	81	-

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A GFP Reporter Workflow for Evaluating CRISPR Prime Editing in *E. coli*: A Feasibility Study Toward NR2B-Targeted Editing

Abstract

~~Validating CRISPR Prime Editing for Targeted NR2B Inhibition in Ischemic Stroke Using an *E. coli* GFP Reporter System~~

¶

Keywords: CRISPR prime editing, NR2B (GRIN2B), ischemic stroke, GFP reporter system, *Escherichia coli*, feasibility study, editing verification sensitivity~~gene editing verification~~

1. Introduction

Ischemic stroke remains one of the leading causes of mortality and disability worldwide, with around 7.8 million new cases and 3.6 million deaths in 2021 alone (Feigin et al., 2025). While reperfusion therapies such as mechanical thrombectomy restore blood flow, they do not directly address neuronal damage that follows the initial ischemic insult, leaving many recovered patients with permanent side effects (Dammavalam et al., 2024). This has motivated years of study into neuroprotective strategies that target the cellular mechanisms of injury rather than blood flow alone.

A central driver of this secondary damage is glutamate excitotoxicity. During ischemic stroke, energy failure causes excessive glutamate release and reuptake dysfunction, leading to sustained overactivation of N-methyl-D-aspartate receptors (NMDARs) on postsynaptic neurons (Yu et al., 2023). Critically, not all NMDAR subtypes contribute equally to this damage. Receptors containing~~For instance~~, the GluN2B (NR2B) subunit ~~that~~ are strongly associated with pro-death signalling, whereas GluN2A-~~-~~containing receptors tend to promote neuronal survival. This ~~variance~~ functional divergence makes~~identifies~~ NR2B a more ~~as an~~ appealing ~~for specific~~ therapeutic target than the receptor as a whole. It may also~~,~~ ~~in~~

contrast to explain why pan-NMDAR antagonists, which have repeatedly failed in clinical trials, since blocking the receptor indiscriminately. Possibly, that because they inhibits both the harmful GluN2B pathway and the protective GluN2A pathway alongside the harmful GluN2B one simultaneously (Wu et al., 2023).

Achieving NR2B inhibition pharmacologically has proven difficult, motivating interest in precision genome editing tools that could mutate a single target gene with minimal off-target effectsdislocation. CRISPR prime editing is one such tool since it uses impaired a Cas9 nickase (Cas9n) fused to an engineered reverse transcriptase, guided by a prime editing guide RNA (pegRNA) without generating double-strand breaks or requiring a donor template (Anzalone et al., 2019). Because prime editing can make precise insertions, such as premature stop codons that shorten a protein and abolish its function, it offers provides a candidate route promising approach to targeted, functional inactivation of NR2B (GRIN2B), an approach that has not been tested in a neural or disease context. as a treatment for ischemic stroke.

Before such an approach can be tested in a mammalian or clinical context, it is necessary important to establish whether prove that prime editing can reliably install a lossa loss-of-function edit. It is and equally necessary to establish whether a given workflow is sensitive enough to detect that edit when it occursthat this edit can be verified. Prior work has demonstrated this principle in *Escherichia coli* by using prime editing to insert premature stop codons into the gene encoding green fluorescent protein (GFP), with loss of fluorescence functioning as an easily scored proxy for successful gene disruption (Tong et al., 2021). (Tong et al., 2021a). The present study adapts this bacterial GFP based design as a first step toward the longer-term goal of NR2B-targeted prime editing. Using *E. coli* expressing GFP as a substitute target, we designed a pegRNA to insert a premature stop codon into the GFP coding sequence, hypothesizing that successful editing would silence GFP expression and be visually detectable as loss of fluorescence. Evaluating Showing that this approach method works in a simple, well-studied characterised system allows the verification workflow itself to be assessed before it is applied to a more demanding target. Extension to NR2B (*GRIN2B*) silencing in models of ischemic stroke remains a future objective rather than an outcome of the present work. The experimental design and the verification workflow are summarised in Figure 1.like *E. coli* gives us a reliable way to test and screen for successful edits that can subsequently be extended to NR2B silencing in ischemic stroke models.

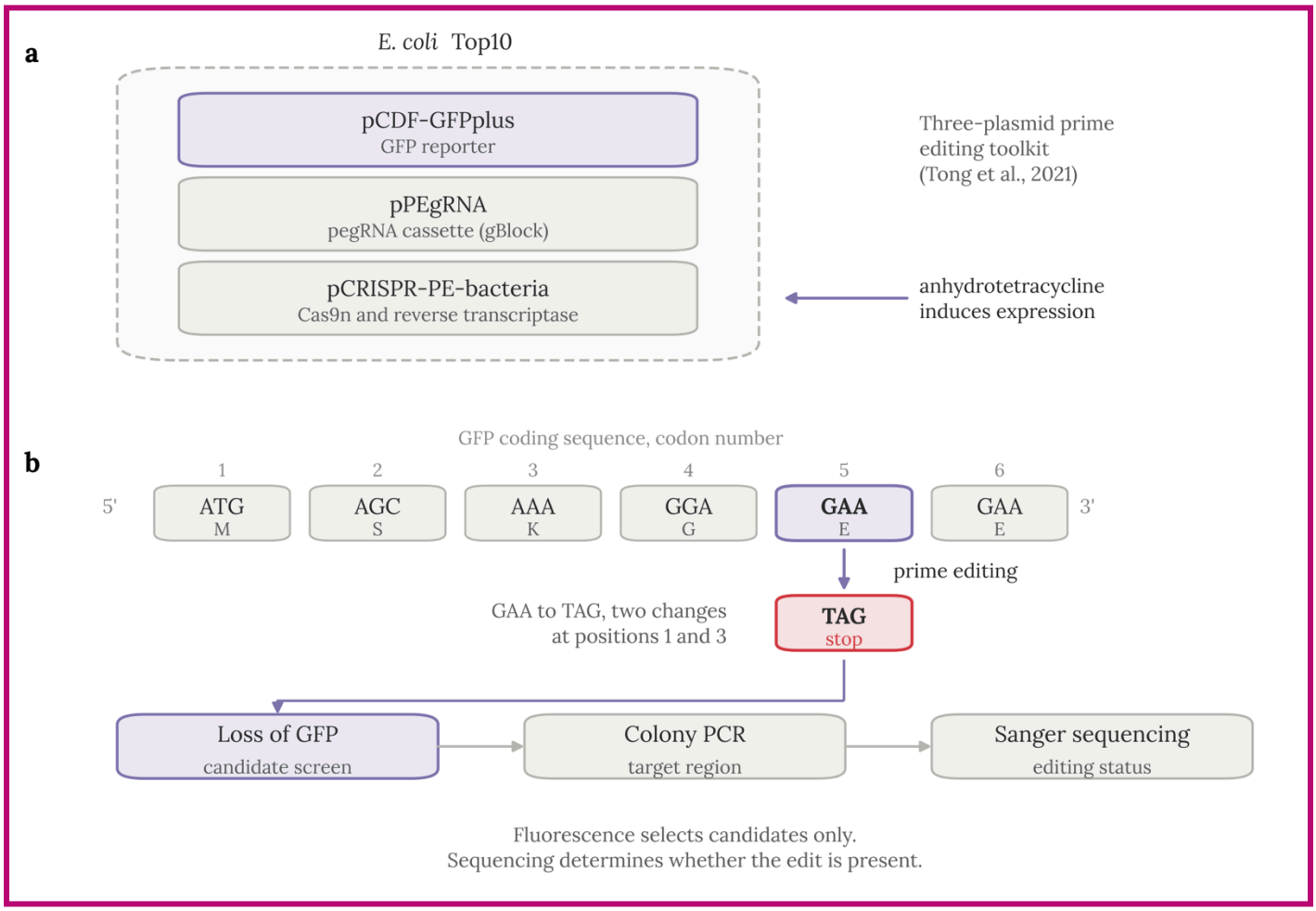


Figure 1: Experimental design and verification workflow. (a) The three-plasmid prime editing system in *Escherichia coli* Top10, comprising the pCDF-GFPplus reporter, the pPEgRNA plasmid carrying the pegRNA cassette, and pCRISPR-PE-bacteria encoding the Cas9 nickase and reverse transcriptase fusion, adapted from Tong et al. (2021). Expression of the editing machinery is induced with anhydrotetracycline. (b) The intended edit converts codon 5 of the GFP coding sequence from GAA to the stop codon TAG, which requires two nucleotide changes at the first and third positions. Candidate clones were screened by loss of GFP fluorescence, then assessed by colony PCR and Sanger sequencing across the target region.

2. Methods

2.1 Strains, Plasmids, Media, and Growth Conditions

Escherichia coli Top10 cells and the plasmids used in this study were adapted from the CRISPR-Prime Editing toolkit for *E. coli* (Tong et al., 2021). Cultures were grown at 37°C in Luria-Bertani (LB) broth or on LB agar (Fisher Scientific). Appropriate antibiotics were supplemented at the following working concentrations: spectinomycin (50 µg/mL), ampicillin (100 µg/mL), and chloramphenicol (25 µg/mL), with anhydrotetracycline (0–1 µg/mL) used to induce expression of the Cas9n–reverse transcriptase fusion. Two percent agar was used for agar plates. Antibiotics were purchased from Sigma-Aldrich (USA). Enzymes and cloning reagents were purchased from New England Biolabs (USA). Plasmid purification kits were purchased from Qiagen (Germany).

2.2 pegRNA Design and Plasmid Construction

A pegRNA targeting the GFP+ coding sequence was designed to insert a premature stop codon at the fifth codon position, following the standard prime editing guide RNA architecture (20-nucleotide protospacer, gRNA scaffold, reverse transcriptase template, and primer binding site, each ~13 nucleotides). The pegRNA cassette was synthesized as a gBlock and assembled into the empty pPEgRNA backbone by Gibson Assembly (NEB Gibson Assembly Master Mix, E2611S) using NEBuilder compatible overhangs. Plasmid backbones were amplified by PCR using Q5 High-Fidelity 2X Master Mix (NEB, M0492S).

2.3 Transformation and Clone Selection

Assembled plasmid DNA was introduced into chemically competent Top10 cells by heat-shock transformation, cells were equilibrated on ice with the Gibson Assembly product for 5 minutes, heat-shocked at 42°C for 30 seconds, returned to ice, recovered in SOC medium at 37°C for 1 hour, and plated on LB agar containing the appropriate antibiotic. Individual colonies were picked for downstream screening.

2.4 Plasmid Isolation and PCR Screening~~Verification~~

Plasmid DNA was isolated from overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, 27104). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, 28104) and resolved on agarose gels (Sigma, A6013) stained with SYBR Safe (Thermo Fisher, S33102) in 1X TAE buffer, alongside a 1 Kb Plus DNA Ladder (Thermo Fisher, 10787018).

2.5 Fluorescence and Growth Measurements

Bacterial growth (OD_{600}) and GFP fluorescence intensity were measured using a SPARK multimode microplate reader in a 96-well plate format. Wells containing LB medium only (no bacteria) served as the blank control. Colonies were additionally imaged under UV illumination using a gel documentation system (SGC-W5000 Plus, Servicebio) to visually assess fluorescence.

2.6 Fluorescence Image Analysis

~~Fluorescence images were quantifi~~Fluorescence images were quantified using Fiji (ImageJ). A region of interest was drawn

around each of six individual colonies per plate using the ROI Manager. A seventh region of interest was placed on an area of agar with no colonies, to serve as a background measurement. For every region the Area, Mean, Min, Max, IntDen, and RawIntDen measures were recorded. Background-corrected RawIntDen was calculated by subtracting the background region's RawIntDen from each colony value. The background region was not matched in area to the colony regions. It covered 289 pixels against 306 to 484 pixels for Experiment Set A, and 81 pixels against 96 to 154 pixels for Sample C_f. A fixed total was therefore subtracted from regions of differing size, which under-corrects larger regions and over-corrects smaller ones. A size-independent measure was calculated for this reason, as the colony mean grey value minus the background mean grey value. The six colonies on each plate were picked without reference to their brightness, and no formal randomisation procedure was used. Measurements were exported to Microsoft Excel for analysis. Per-colony values are given in Table S1.ed using Fiji (ImageJ). Regions of interest were manually drawn around individual colonies, and fluorescence intensity was extracted along a line profile and exported to Microsoft Excel for analysis.

Replication was as follows. Bulk growth and fluorescence were measured once per condition at each timepoint, so Figure 2 represents a single biological replicate with no technical replicates. Colony fluorescence was quantified for six colonies on each of two independent platings. No inferential statistical tests were performed, because the design does not support them. Summary values are reported as means, ranges and coefficients of variation.

2.7 Assessment of Editing Confirmation Status by Sanger Sequencing

Positive Candidate clones were selected on the basis of reduced identified by loss of GFP fluorescence and were verified assessed at the DNA level by colony PCR followed by Sanger sequencing across the target region, in order to determine whether confirming the intended stop codon insertion was present at the fifth codon 5 of the GFP coding sequence. Selection on fluorescence identifies candidates only. It does not establish that an edit occurred.

2.8 Public Expression Data

Public expression data were used to establish whether the intended target gene is altered in ischemic brain tissue. The dataset GSE38037 was retrieved from the NCBI Gene Expression Omnibus (platform GPL3631). It comprises cerebral cortex from Sprague Dawley rats after transient middle cerebral artery occlusion (tMCAO, n = 4, GSM932851 to GSM932854) and from sham-operated controls (n = 4, GSM932847 to GSM932850). The array is two-colour. Every sample, tMCAO and sham alike, was co-hybridised against a common reference pool of sham-operated cortex, so each value is a ratio against that shared reference. Differential expression between the two groups was computed with GEO2R. GEO2R fits a limma empirical-Bayes linear model to the series matrix and corrects for multiple testing across all 23,376 probes by the Benjamini-Hochberg procedure. No new experimental data were generated for this analysis.

3. Results

3.1 Bacterial Growth and Fluorescence Measurements Intensity¶

Bacterial growth (OD_{600}) was monitored ~~acro~~at~~ss~~ three timepoints (10:45 ~~AM~~, 13:30 ~~PM~~, and 164:48 ~~PM~~) ~~to assess bacterial growth~~ for the Stop Codon construct and ~~relative to~~ the two ~~CRISPR plasmid only and pegRNA~~ plasmid-only controls (Fig. 2a). All three conditions ~~showed~~ grew comparably~~e~~ and ~~growth trajectories,~~ reached ~~ing~~ similar final OD_{600} values of 0.14 to 0.16~~(0.14–0.16) by the last timepoint~~. T~~ Subsequently, t~~he Stop Codon condition ~~onstruct showed~~ was a single culture measured at these~~a decline in GFP fluorescence across the~~ three ~~measured~~ timepoints, so n = 1 biological replicate.

Raw GFP fluorescence in the Stop Codon construct fell across the day, ~~f~~rom 47,344 a.u. at 10:45 ~~AM~~ to 37,891 a.u. at 13:30 ~~PM~~ and 3,417 a.u. at 164:48 ~~PM~~ (Fig. 2b). Both ~~negative controls, CRISPR~~ plasmid-only ~~controls and pegRNA plasmid only~~ stayed~~, remained at consistently~~ low ~~fluorescence~~ throughout between ~~the day~~ (7,879 and ~~–~~18,018 a.u.~~)~~. By the final timepoint, all three samples had converged to a ~~similar,~~ low ~~fluorescence~~ range of ~~(~~3,417 to ~~–~~3,631 a.u.~~)~~ This convergence occurred in the Stop Codon construct and in both controls alike, so it cannot be a signature of editing.~~.~~

Neither control carries the GFP reporter plasmid. Their signal therefore reflects background autofluorescence and not unedited GFP expression. They serve as plasmid-only controls rather than as unedited-GFP comparators. The study includes no GFP-expressing condition that received no pegRNA. Without that condition there is no baseline from which fluorescence could be described as lost, so the fluorescence data cannot speak to editing status.

Fluorescence scales with the number of cells present, so each reading was divided by the OD_{600} of the same sample (Table 1). Normalised values for the Stop Codon construct were 428,452, 471,281 and 21,946 a.u./OD_{600} across the three timepoints. Relative to the mean of the two controls, the Stop Codon construct was 2.39 times higher at 10:45, 1.43 times higher at 13:30 and 0.88 times as high at 16:48. The equivalent raw ratios were 5.85, 2.18 and 0.96. Normalisation therefore removes more than half of the early difference. It also places the Stop Codon construct slightly below both controls at the final timepoint, where the raw data had the three conditions roughly equal. Because the controls carry no GFP, the difference that remains confirms GFP expression in the Stop Codon construct and does not bear on editing status.

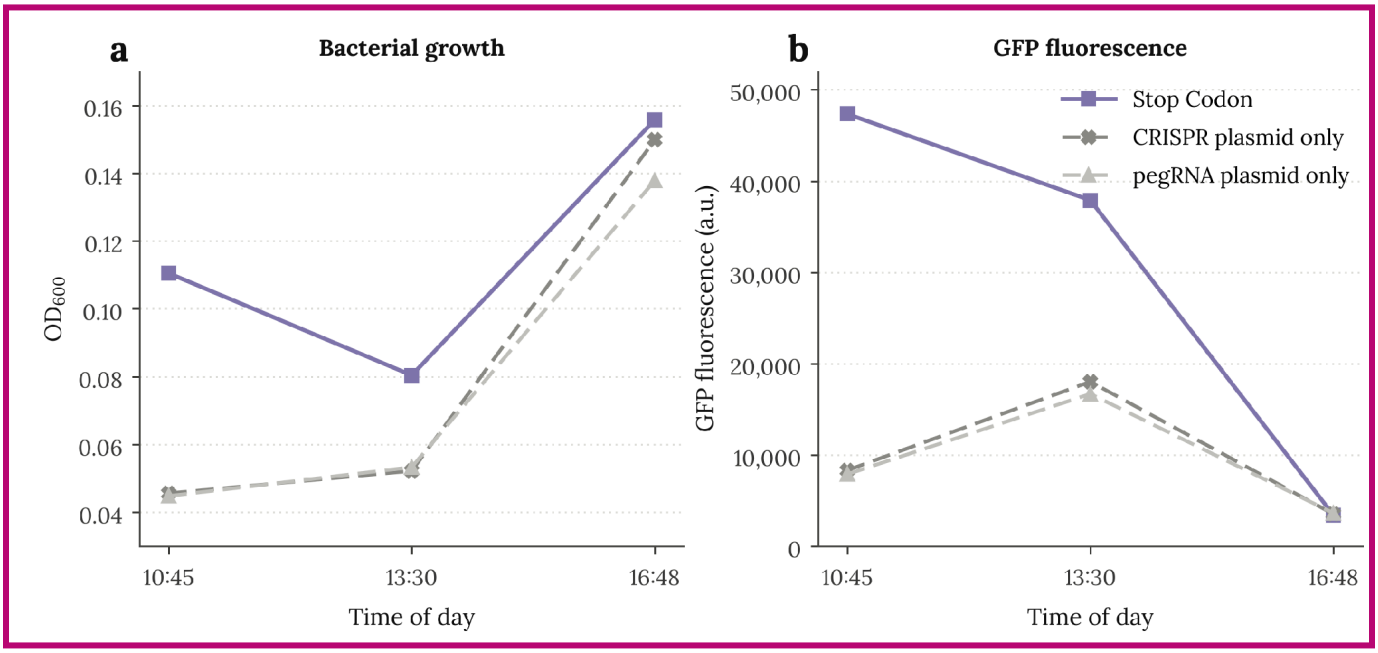
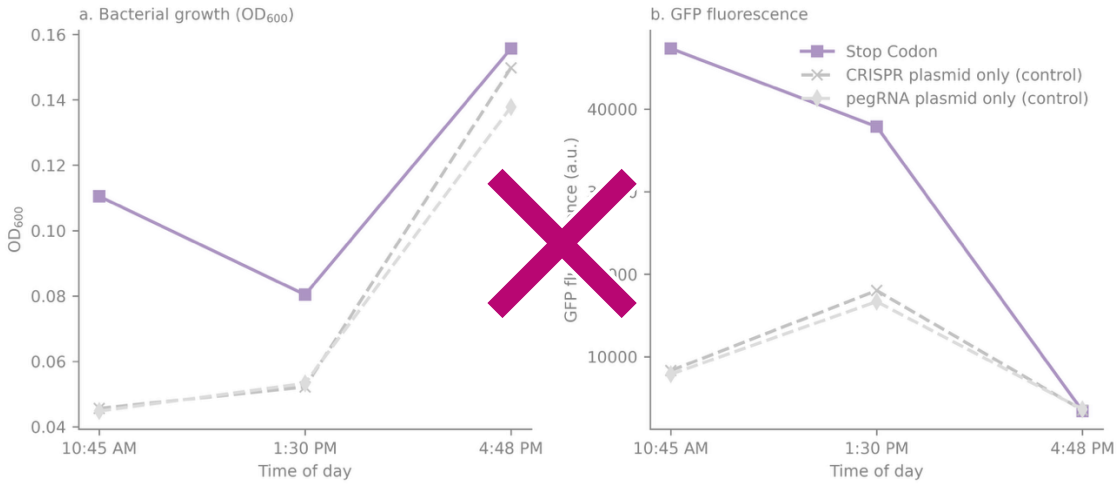


Figure 2: Bacterial growth and GFP fluorescence for the Stop Codon construct and the plasmid-only controls. (a) OD_{600} and (b) raw GFP fluorescence at three timepoints (10:45, 13:30 and 16:48) for the Stop Codon construct, the CRISPR plasmid only control and the pegRNA plasmid only control. Each condition is a single culture measured at the three time points, so n = 1 biological replicate with no technical replicates. Neither control carries the GFP reporter plasmid, so the control signal reflects background autofluorescence rather than unedited GFP expression. Fluorescence normalised to culture density is given in Table 1.

Table 1: GFP fluorescence normalised to culture density (a.u./OD_{600}). Blank-corrected fluorescence divided by the OD_{600} of the same sample at the same timepoint, for the Stop Codon construct and the two plasmid-only controls at each of the three timepoints.

Condition	**10:45**	**13:30**	**16:48**
Stop Codon	428,452	471,281	21,946
CRISPR plasmid only	182,259	345,172	23,485
pegRNA plasmid only	175,871	312,964	26,350

¶



¶

Figure 2: Bacterial growth and GFP fluorescence for the Stop Codon construct and plasmid only controls. (a) OD_{600} growth curves and (b) GFP fluorescence over three time points for the Stop Codon construct compared to CRISPR plasmid only and pegRNA plasmid only controls.¶

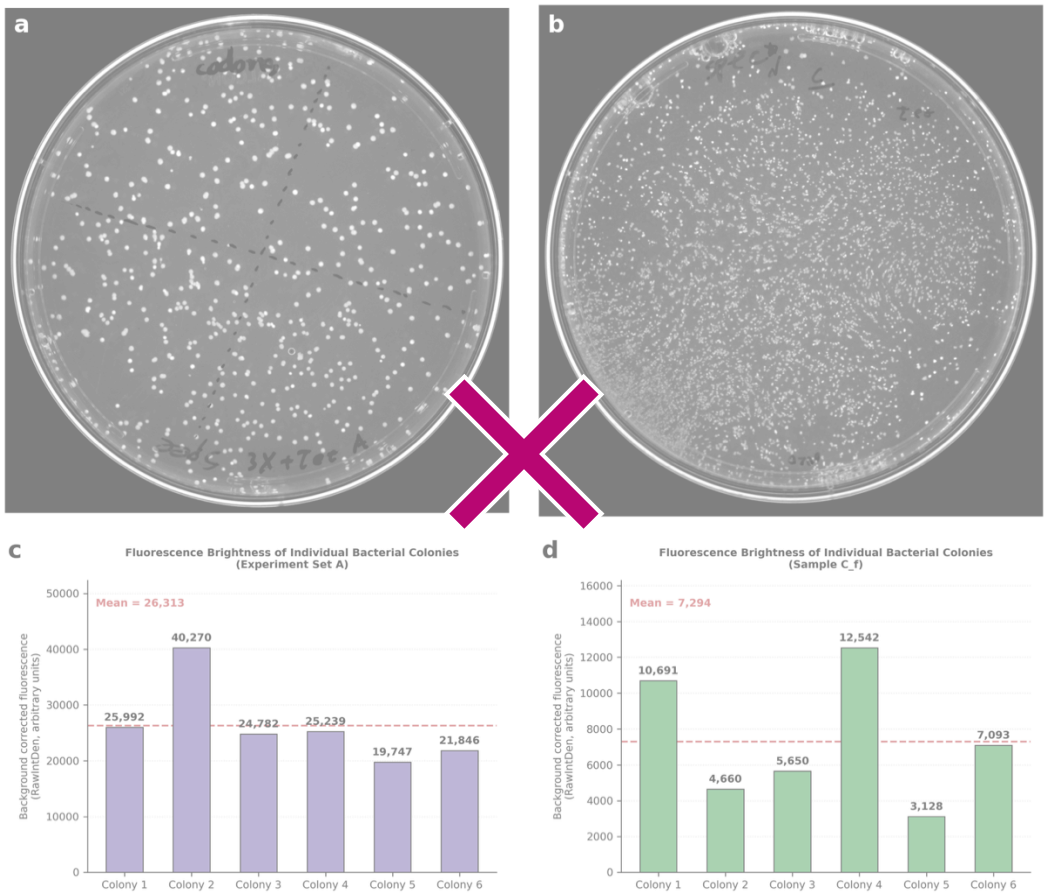
PFigure 3 represents plate images and corresponding colony fluorescence quantification for two independent platings are shown in Fig. 3. bacterial colony samples grown under different selection conditions. The agar plate for Experiment Set A (Fig. 3a) carried displayed colonies at moderate density that were individually distinguishable. across the surface, whereas Tthe plate for Sample C_f (Fig. 3b) showed was markedly higher denser colony density, with several regions approaching confluence.

Background-corrected fluorescence intensity (RawIntDen) was then measured for six individually selected colonies from on each plate. In Experiment Set A (Fig. 3c), values ranged from 19,747 to 40,270, across Colonies 1–6, yielding with a mean of 26,313. I, with Colony 2 showing the highest fluorescence and Colony 5 the lowest, in Sample C_f (Fig. 3d) , values ranged from 3,128 to 12,542, yielding with a mean of 7,294. The two means differ by a factor of about 3.6. RawIntDen is the sum of grey values inside a region of interest, so it scales with the area of that region. Dividing RawIntDen by the mean grey value recovers the pixel count of each region. Colonies in Set A averaged 355 pixels and those in C_f averaged 124 pixels, a difference of 2.85 times in area. Most of the 3.6-fold difference in RawIntDen is therefore a difference in colony size rather than in brightness.

A size-independent measure was calculated as the colony mean grey value minus the background mean grey value. On this measure Set A averaged 65.1 and C_f averaged 41.9, a difference of 1.55 times. The two groups also overlap. C_f colonies 4 and 6 (66.9 and 62.1) were brighter than the two dimmest colonies in Set A (56.8 and 56.0). Variability was much higher in C_f, with a coefficient of variation of 48.2% against 12.9% for Set A. Per-colony values and pixel counts are given in Table

~~S1., with Colony 4 showing the highest fluorescence and Colony 5 again the lowest. Fig. 3c, d are plotted on different y-axis scales (0–50,000 and 0–16,000, respectively), and the mean fluorescence value in Fig. 3c is approximately 3.6 times that in Fig. 3d.~~

These two plates are two separate platings of the same construct that differed substantially in colony density, so plate to plate technical variation cannot be separated from any real difference in brightness. The six colonies on each plate were picked without reference to their brightness, and no formal randomisation procedure was used. This comparison is therefore descriptive and cannot be read as a difference in editing status.



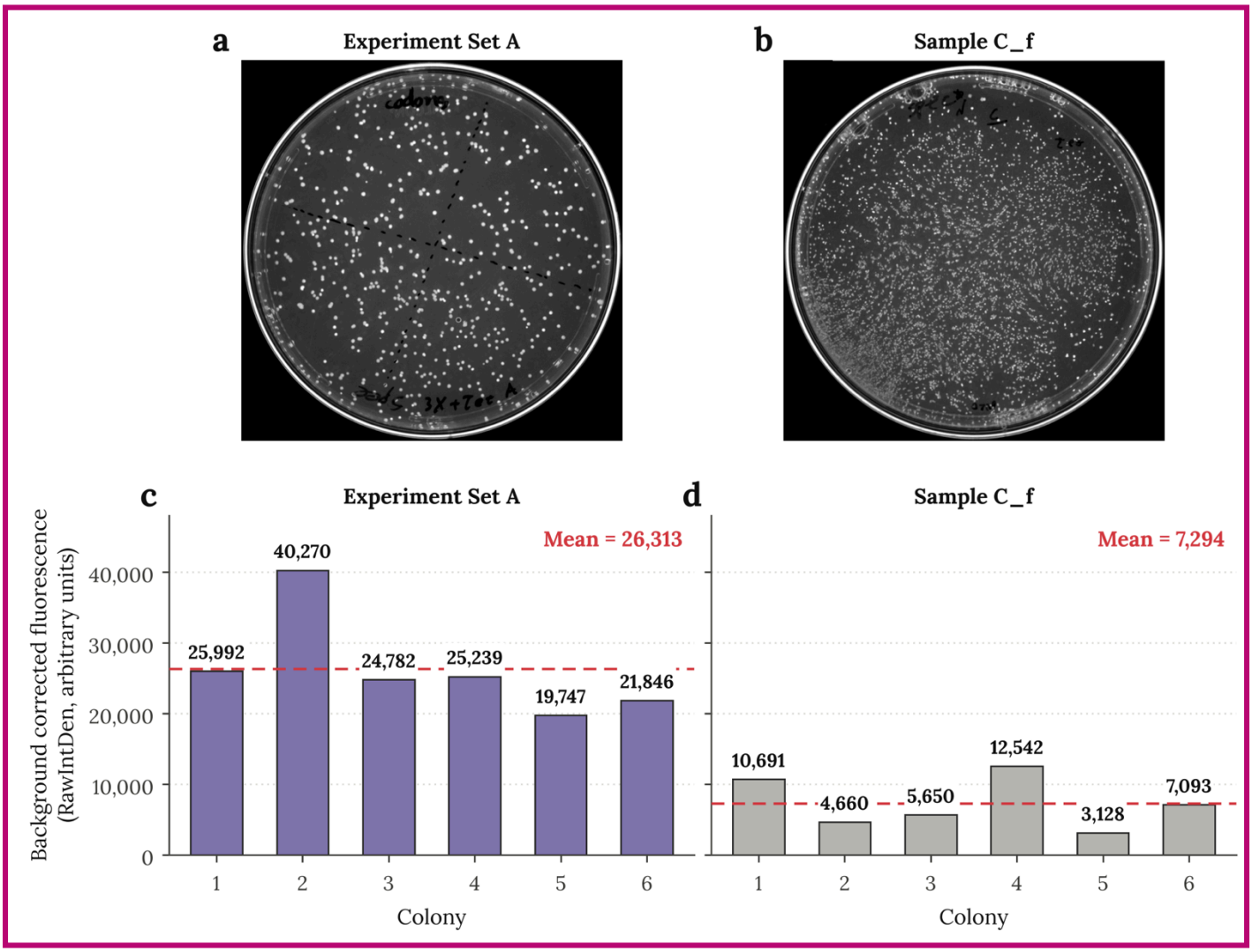
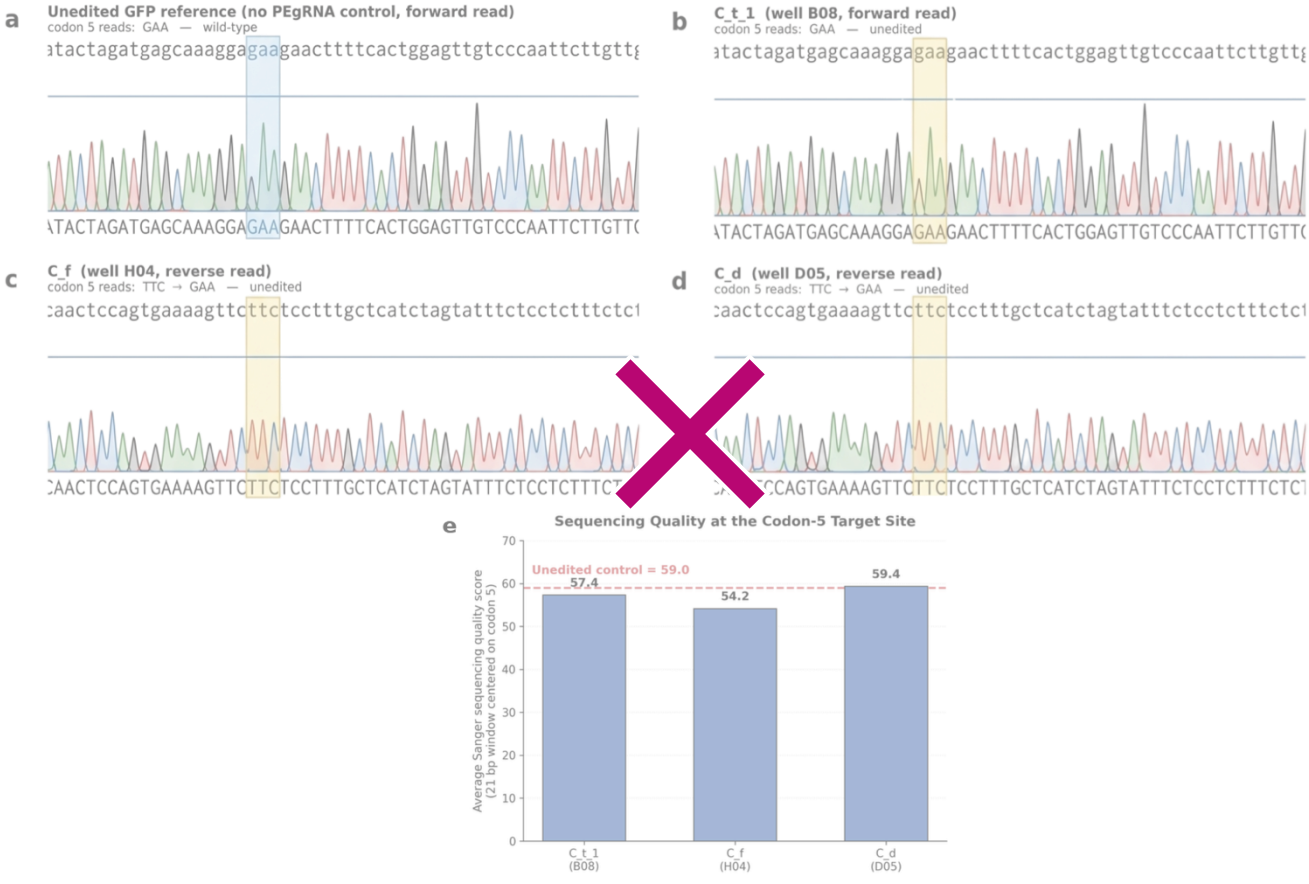


Figure 3: Plate density and cColony fluorescence ~~in Codon construct~~ for two independent platings of the Stop Codon construct ~~es~~ under anhydrotetracycline ~~TET~~ induction. (a) P~~Representative p~~late image for ~~of Codon construct colonies, TET induced (~~Experiment Set A, showing colonies at moderate density~~)~~. (b) ~~Representative P~~plate image for ~~of Codon construct colonies, TET induced (S~~sample C_f, showing markedly higher density with regions approaching confluence~~)~~. (c, d) Background-corrected RawIntDen for six colonies from each plate, Experiment Set A in (c) and Sample C_f in (d), plotted on a common y-axis. RawIntDen is the sum of grey values within a region of interest and therefore scales with colony area, which differs 2.85-fold between the two plates. The size-independent per-pixel measure differs only 1.55-fold. Per-colony values, region areas and per-pixel intensities are given in Table S1. The two plates are separate platings of the same construct and differed substantially in colony density.~~Quantified fluorescence brightness of individual colonies from~~

Experiment Set A (n = 6). (d) Quantified fluorescence brightness of individual colonies from sample C_f (n = 6).

3.2 Editing Status at the Target SiteSequencing Confirmation

Figure 4 presents SangerThree Stop Codon samples were sequenced across ing chromatograms and quality scores at the codon 5 target site for three Stop Codon coloniesto test directly whether the intended edit was present. Each was compared with an The unedited GFP reference (no pePEgRNA referencecontrol, (Fig. 4a). reads GAA at codon 5. The three test colonies, C_t_1 (well B08, forward read, Fig. 4b), C_f (well H04, reverse read, Fig. 4c), and C_d (well D05, reverse read, Fig. 4d), read GAA, TTC, and TTC at codon 5, respectively (TTC being the reverse complement of GAA on the reverse strand), matching the sequence in Figure 4a. None of the three colonies shows the TAG sequence. In addition, average Sanger sequencing quality scores across the 21 bp window centered on codon 5 (Fig. 4e) were 57.4 for C_t_1, 54.2 for C_f, and 59.4 for C_d, compared with 59.0 for the unedited control (dashed red line). C_t_1, C_f, and C_d were sequenced from bulk liquid culture, whereas Tong et al. (2021b) sequenced isolated single colonies.



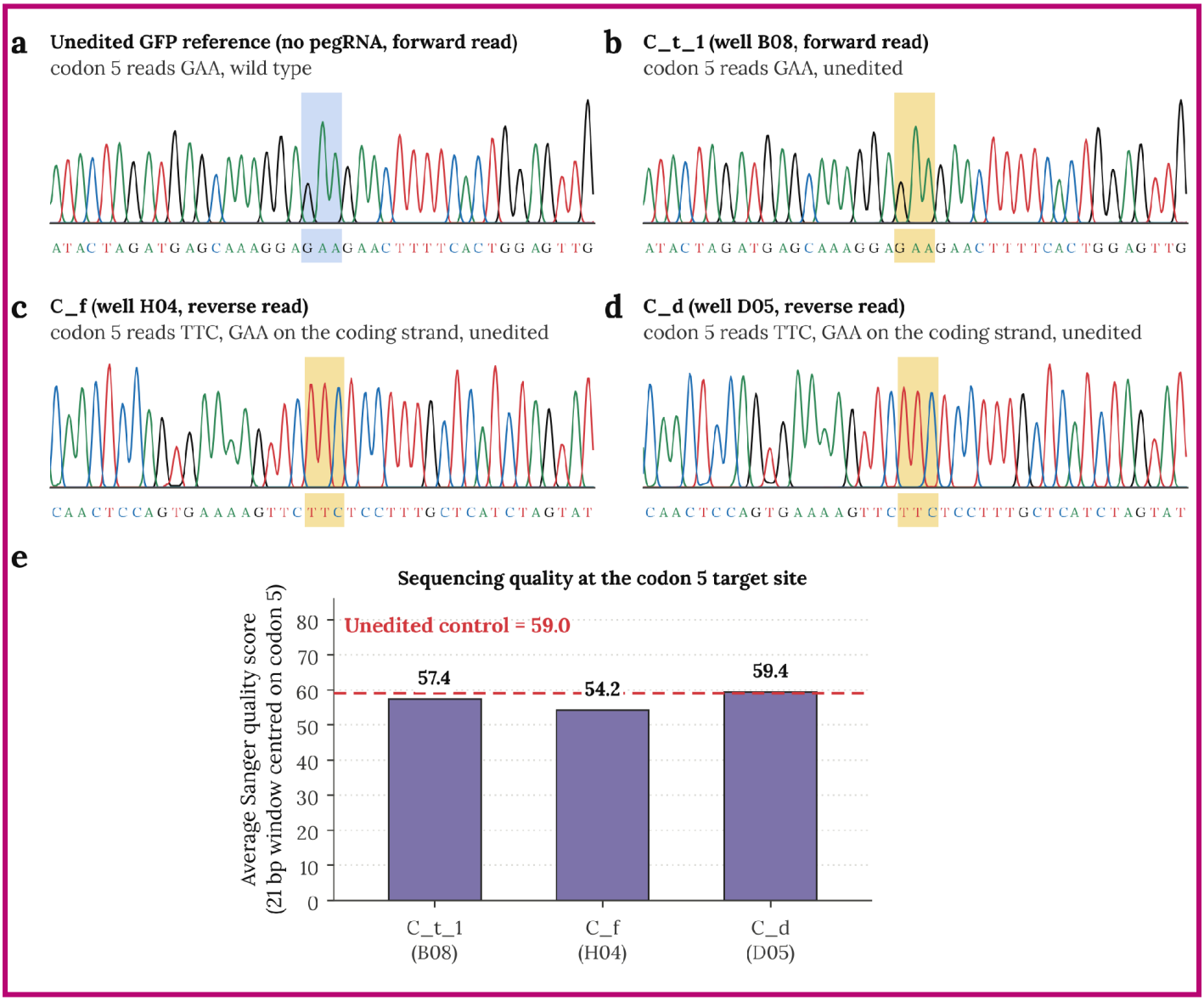


Figure 4: Sanger s~~S~~equencing ~~confirmation~~ at the GFP codon-5 target site. (a) Chromatogram of the unedited GFP reference (no pe~~PE~~gRNA control, forward read), showing wild-~~-~~type codon 5 (GAA). (b) Chromatogram of sample C_t_1 (well B08, forward read), showing unedited codon 5 (GAA). (c) Chromatogram of sample C_f (well H04, reverse read), showing unedited codon 5 as ~~(~~TTC on the reverse strand, corresponding to GAA on the coding strand~~)~~. (d) Chromatogram of sample C_d (well D05, reverse read), showing unedited codon 5 as ~~(~~TTC on the reverse strand~~, GAA on coding strand~~). (e) Mean ~~Average~~ Sanger sequencing quality score across a ~~(~~21 bp window centered on codon 5~~)~~ for each sample, ~~compared to~~

with the unedited control shown as a (dashed line at, 59.0). None of the three samples carries the intended TAG codon. All three were sequenced from bulk liquid culture rather than isolated single colonies.

3.3 *Grin2b* Expression in Ischemic Cortex

In GSE38037, *Grin2b* was modestly higher in ischemic cortex than in sham cortex (probe 10020292529, $\log_2$ fold change +0.18, equivalent to a 1.13-fold increase, $p = 0.014$, adjusted $p = 0.049$). Other NMDA receptor subunit transcripts rose to a similar or greater degree. These were *Grin1* (+0.27, adjusted $p = 0.005$), *Grin2a* (+0.34, adjusted $p = 0.009$), *Grin2c* (+0.21, adjusted $p = 0.006$) and *Grin3a* (+0.29, adjusted $p = 0.020$). *Grin2d* and *Grin3b* were unchanged (Fig. 5). In this dataset, 6,919 of 23,376 probes (29.6%) reached an adjusted p below 0.05. That high proportion is consistent with the low within-group variance expected of a common-reference two-colour design. Statistical significance alone therefore provides limited evidence of specificity, and *Grin2b* ranked 6,850th by p-value. These data are consistent with coordinated engagement of NMDA receptor subunit expression after cerebral ischemia. They do not show selective *Grin2b* dysregulation.

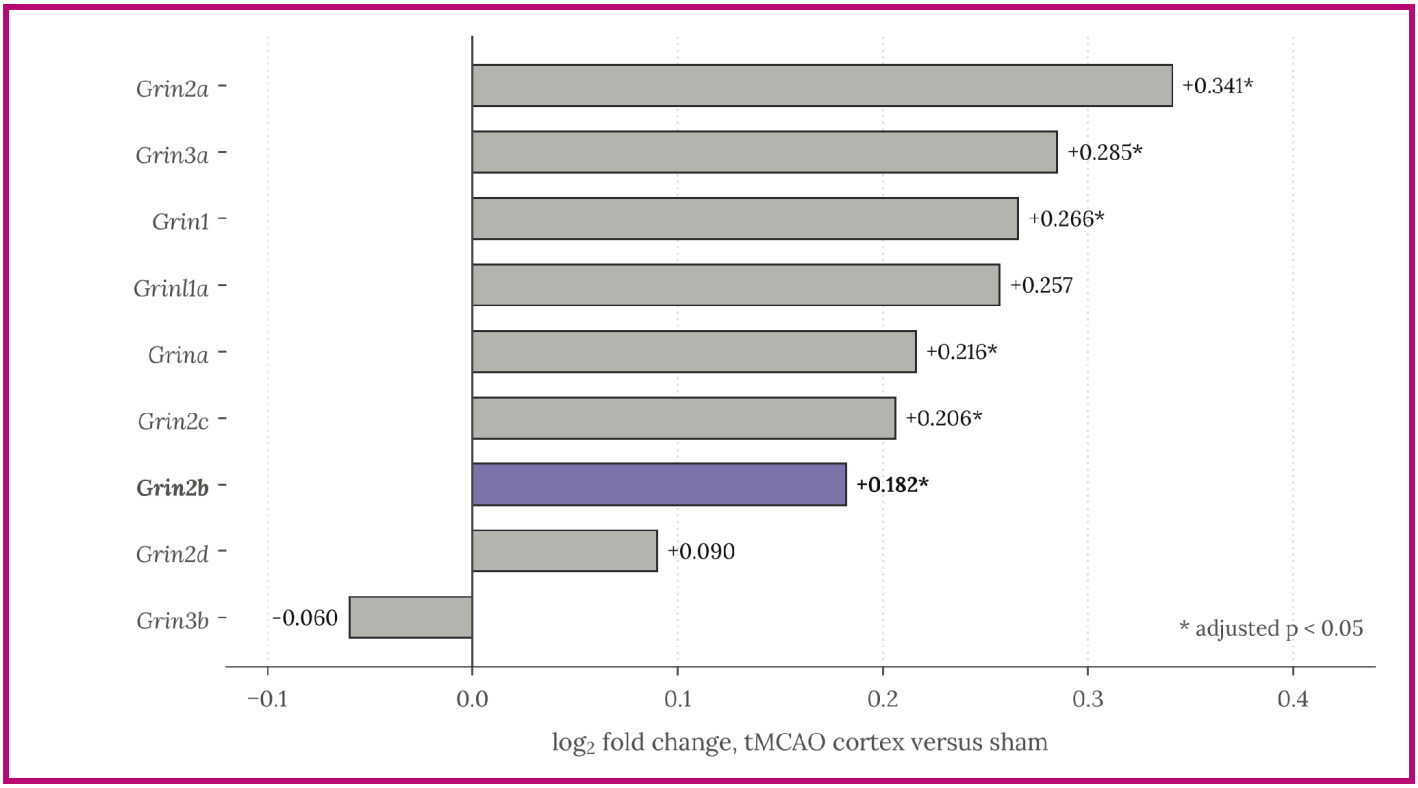


Figure 5: NMDA receptor subunit transcript changes in rat ischemic cortex. Differential expression of NMDA receptor subunit and subunit-associated transcripts in GSE38037 (transient middle cerebral artery occlusion versus sham, rat cerebral cortex, four samples per group), computed with GEO2R. Bars show $\log_2$ fold change. *Grin2b*, the target of the editing strategy evaluated in this study, is highlighted. Asterisks mark an adjusted p below 0.05 after Benjamini-Hochberg

correction. *Grin2b* shows the smallest significant increase among the subunit transcripts measured.

4. Discussion

Public expression data indicate that *Grin2b* is transcriptionally higher in ischemic cortex. This confirms that the gene is altered in the disease context that motivates this work. The effect is small, and comparable or larger increases occur in the other NMDA receptor subunits. These data therefore do not identify *GRIN2B* as uniquely dysregulated. The case for subunit-selective intervention rests instead on the divergent downstream signalling of GluN2A-containing and GluN2B-containing receptors (Wu et al., 2023; Yu et al., 2023). Transcript abundance does not address that question. The relevance of this analysis to the present study is narrower still. It establishes that the eventual target is disease-relevant, while the editing and verification questions examined here remain unresolved. The public expression analysis used a rodent model of ischemia rather than human stroke tissue, and the gene is *Grin2b* in rat rather than *GRIN2B* in human. It is also a transcript-level observation, which does not establish that receptor-level signalling changes in proportion.

Three lines of evidence were presented above. These ~~included~~ were growth and bulk fluorescence over time (Fig. 2), colony~~-level~~ fluorescence ~~quantification~~ on two plates (Fig. 3), and Sanger sequencing at the codon 5 target site (Fig. 4). ~~Across these three lines of evidence~~None of them provides~~, no~~ molecular evidence of the intended GAA ~~-~~to ~~-~~TAG ~~stop codon edit was obtained~~. ~~Bacterial G~~growth was comparable ~~(OD_{600}) converged to a similar range (0.14–0.16)~~ across the Stop Codon construct and both plasmid~~negative~~-only ~~-~~controls~~. by the final timepoint, while GFP fluorescence for the Stop Codon construct~~ The fall in fluorescence at the final timepoint occurred in all three conditions alike, including the two that carry no GFP. Sequencing showed the wild-type codon in all three samples tested. Each line of evidence is taken in turn below~~.fell from 47,344 a.u. to 3,417 a.u. over the same period and converged with the CRISPR-only and PEgRNA-only controls, which remained between 7,879 and 18,018 a.u. throughout (Fig. 2). At the colony level, mean background-corrected fluorescence differed by a factor of approximately 3.6 between Experiment Set A (mean 26,313 a.u., n = 6) and Sample C_f (mean 7,294 a.u., n = 6), with C_f additionally showing greater relative variability (coefficient of variation 49.9% versus 27.5% for Set A) (Fig. 3). Sanger sequencing of three Stop Codon colonies, C_t_1, C_f, and C_d, showed wild-type GAA (or its reverse complement, TTC) at codon 5 in all three cases, matching the unedited no-PEgRNA control, with average sequencing quality scores (54.2–59.4) comparable to the control (59.0) (Fig. 4).~~

Considered on its own, the difference in mean colony brightness between Experiment Set A and Sample C_f could be read as evidence of differing editing status between the two plates. However, this interpretation is complicated by colony density, which also differed markedly between the two plates: colonies in Set A were moderately spaced and individually distinguishable, while those in C_f were considerably denser and, in places, near confluent (Fig. 3a, b). Because fluorescence was quantified from manually drawn regions of interest around individual colonies, crowding on the C_f plate provides a plausible technical confound. Overlapping regions of interest, competition for nutrients or oxygen between adjacent colonies, and reduced accuracy of background correction could each lower or add noise to individual colony readings. These effects would occur independent of any underlying genotype (Chacón et al., 2018). The comparatively higher variability observed in C_f is more consistent with this kind of density related technical variation than with a straightforward genotype driven reduction in brightness. Because the two plates are ~~independent~~ separate platings ~~rather than replicates~~ of the same ~~condition~~construct and were not matched for density, the present data cannot separate a genuine biological difference from plate to plate technical variation.

A related limitation affects Fig. 2. At the first timepoint, the Stop Codon construct already showed both higher OD_{600} (0.11) and higher fluorescence (47,344 a.u.) than either control. Control OD_{600} values were roughly 2.4 times lower (0.045) at the same timepoint. Because the fluorescence plotted in Fig. 2b is ~~this experiment was not normalized to OD, part of the early difference in~~ raw rather than normalised, part of the early ~~fluorescence is attributable to~~ difference ~~s~~ in that panel is attributable to differences in culture density rather than to per-cell GFP expression. The normalised values in Table 1 remove most of it. By the final timepoint, fluorescence in all three conditions, including both plasmid-only ~~negative~~ controls, which ~~that~~ carry no ~~target edit~~GFP reporter, converged to a similarly low range (3,417-3,631 a.u.) even as OD_{600} reached its highest value in every condition. This decline occurred uniformly across edited and unedited conditions alike. This pattern is more consistent with a general reduction in GFP signal as cultures approach stationary phase than with a signature specific to stop-codon-mediated loss of function. Possible causes of this general decline include reduced GFP synthesis, impaired chromophore maturation, or increased culture turbidity (El Itawi et al., 2026) ~~(El Itawi et al., 2026)~~. Taken together, the OD confound at the first timepoint and the shared decline at the last limit how much Fig. 2 alone can indicate about editing status.

Sanger sequencing provided the only nucleotide level test of editing status in this study, and none of the three colonies sequenced carried the intended TAG stop codon at position 5. ~~,~~ A~~a~~ll three instead matched the wild-~~-~~type GAA sequence of the unedited control (Fig. 4a-d). Average sequencing quality across the 21 bp window centred on codon 5 remained within five points of the control for all three samples (Fig. 4e). This indicates that these were high-confidence base calls rather than low-quality reads. It also rules out poor sequencing as an explanation for the absence of a detected edit. This null result should, however, be interpreted alongside an important methodological difference from Tong et al. (2021~~b~~), whereas ~~Tong et al.~~they verified editing outcomes by sequencing 24 isolated single colonies per condition, C_t_1, C_f, and C_d were sequenced from bulk liquid culture. A Sanger read from a bulk culture reports only the dominant sequence present across the sampled population. This approach is characteristically far less sensitive to a minority edited subpopulation than sequencing of isolated clones. An edited variant generally must approach a substantial fraction of the population, rather than being present in only a small number of cells, before it becomes visible above background in the chromatogram trace. Accordingly, the present result indicates that the wild type sequence was the dominant outcome among the cells sampled in each bulk culture, but it does not by itself establish that no cell within either culture carried the intended edit.

~~This methodological gap in detection sensitivity is especially relevant given~~ T~~t~~he nature of the edit attempted here makes that sensitivity gap more important. Converting codon 5 from GAA to TAG requires two ~~simultaneous~~ nucleotide changes within ~~the same~~ one codon, at the first and third positions~~, while the middle base remains unchanged. This differs from the single-nucleotide T-to-A substitution that~~ Tong et al. (2021~~b~~) characteri~~s~~zed a single-nucleotide substitution across ~~across~~ a full anhydrotetracycline dose-response series, but reported~~. It more closely resembles a two-base-pair substitution event that they separately validated at the GFP locus by whole-genome sequencing of a single clone.~~ n~~N~~o comparable series for ~~dose-response~~a ~~efficiency was reported for that~~ two-base~~-pair~~ substitution ~~event.~~, so ~~a~~A directly matched efficiency figure ~~for a two position substitution~~ is ~~therefore~~ not available ~~from their data. Nonetheless~~ A~~,~~ among the edit types they ~~Tong et al. (2021b)~~ did characteri~~s~~ze ~~systematically, single-nucleotide~~ substitution was consistently the least efficient. It reached only about 2% at the highest inducer concentration ~~tested~~ and about 1% at a ~~more~~ moderate ~~concentration (approximately 200–256 ng/mL~~ one~~)~~. ~~Edits requiring more than one simultaneous base change,~~ T~~t~~heir combinatorial ~~editing~~ category, which requires more than one simultaneous base change, reached about ~~also fell well below the efficiency~~

of a comparable single-nucleotide deletion or insertion, at approximately 20% and 13% under the same two conditions, against, respectively. This compares to roughly 44% and 29% for a 1 bp deletion. If the efficiency of the present edit falls anywhere in that range, a bulk culture would contain only a small minority of edited cells, probably below the level at which a Sanger consensus would show anything but the wild-type sequence. The absence of a detected edit is therefore as consistent with low editing efficiency as with no editing at all. under the same two conditions.

This pattern indicates that substitution-type and multibase edits are markedly less efficient than insertions or deletions of similar size in this toolkit. Whether a two-nucleotide substitution within a single codon behaves more like the low single-substitution result or the intermediate combinatorial result cannot be determined without testing it directly. In either case, if the efficiency of the present edit fell anywhere in this lower range, a bulk-culture sample would plausibly contain only a small minority of edited cells. This proportion could fall below the level at which a Sanger read of that population would show anything other than the wild-type consensus. Read in this light, the absence of a detected edit in the present data is broadly consistent with the comparatively low efficiency reported for substitution and multibase editing events in this system, rather than necessarily indicating that no editing occurred. Notably, Tong et al.'s (2021b) own verification strategy, sequencing 24 isolated clones per condition, reflects a design built specifically to resolve editing outcomes at this kind of low per cell efficiency, one that a three sample bulk culture approach is not equipped to resolve.¶

Several limitations qualify these findings. Most centrally, and consistent with the detection-sensitivity argument above, editing status was assessed by Sanger sequencing of only three bulk-culture samples. This falls short of isolated single-colony sequencing at a scale comparable to Tong et al.'s 24 clones per condition. It also falls short of amplicon-based deep sequencing capable of resolving low-frequency variants within a population. Second, the dataset does not include a matched no tetracycline induction condition for the Stop Codon construct, so a paired induced versus uninduced comparison, of the kind used by Tong et al. (2021b) to establish dose dependent editing efficiency, could not be performed. Additional confounds affect the fluorescence-based measurements discussed above. Fluorescence in Fig. 2 was not normalized to OD_{600}. The two plates compared in Fig. 3 also differed substantially in colony density and represent independent platings rather than replicates of the same conditionwere not matched replicates. Sample sizes were modest throughout. Six manually selected colonies were quantified per plate. Only two genuinely distinct Codon-plus-TET plate images were available in the dataset (an additional candidate pair was found to be a byte-identical duplicate file rather than an independent replicate). Only three colonies were carried forward to sequencing. No positive control was included to confirm that the fluorescence and sequencing workflows used here were capable of detecting a known successful edit. Fluorescence and sequencing data also came from different cells, plate grown colonies for fluorescence and bulk liquid culture for sequencing, so colony brightness cannot be directly linked to the sequenced genotype. Finally, as with any GFP-based reporter assay, loss of fluorescence is only a proxy for gene disruption in *E. coli*. Results obtained on editing efficiency in this bacterial system do not necessarily transfer directly to mammalian neurons, the eventual context for NR2B (GRIN2B) targeting in ischemic stroke.

Together, these results indicate that no stop codon edit was detected at codon 5 of GFP among the samples sequenced. They also indicate that the present design, bulk-culture sequencing of a small sample without a matched uninduced control, would not have reliably detected editing occurring at the low efficiencies reported previously for substitution-type edits. Distinguishing between these possibilities will require one of two approaches: isolating and sequencing single colonies at a scale comparable to Tong et al.'s (2021b) screen, or applying amplicon-based deep sequencing to detect

low-frequency edited variants. Either approach would also need to be paired with generating the missing no-TET control needed for a proper induced versus uninduced comparison. Testing a deletion-type edit, which showed the highest efficiency in the Tong et al. (2021b) toolkit, could additionally serve as a positive system validation control. This would confirm that the cloning, culturing, and sequencing workflow used here is capable of detecting a successful edit, before returning to the more technically demanding substitution required for stop codon insertion into NR2B. Establishing these methodological foundations in this bacterial reporter system is a necessary prerequisite for extending prime editing toward NR2B (GRIN2B) silencing in mammalian models of ischemic stroke.

5. Conclusion

This study implemented and evaluated a Sanger sequencing and colony fluorescence workflow for assessing CRISPR prime editing of a stop codon at codon 5 of GFP in *E. coli*. The sensitivity of that workflow proved to be the factor limiting what the data can show. Across three sequenced colonies, no evidence of the intended GAA to TAG edit was detected, and the accompanying fluorescence measurements were subject to confounds that limit their ability to indicate editing status on their own. Because this edit type is reported elsewhere as markedly less efficient than insertion or deletion type edits, and because only bulk-culture sequencing of three samples was performed, this null result is best read as inconclusive rather than as evidence against editing having occurred. Establishing a more sensitive verification workflow, through single-colony sequencing and a matched no-TET control, is a necessary next step before extending this approach toward NR2B (GRIN2B) silencing in ischemic stroke.

6. Data Availability

The public expression dataset analysed in this study is available from the NCBI Gene Expression Omnibus under accession GSE38037 (platform GPL3631). Differential expression was computed with GEO2R using its default settings, comparing four tMCAO samples (GSM932851 to GSM932854) with four sham-operated controls (GSM932847 to GSM932850). The full GEO2R output table, the OD_{600} and fluorescence readings behind Figure 2 and Table 1, and the Fiji region-of-interest measurements behind Figure 3 and Table S1 are available from the author on request. No new high-throughput sequencing data were generated in this study.

~~This study established a Sanger sequencing and colony fluorescence based workflow for evaluating CRISPR prime editing of a stop codon at codon 5 of GFP in *E. coli*. Across three sequenced colonies, no evidence of the intended GAA to TAG edit was detected, and the accompanying fluorescence measurements were subject to confounds that limit their ability to indicate editing status on their own. Because this edit type is reported elsewhere as markedly less efficient than insertion or deletion type edits, and because only bulk-culture sequencing of three samples was performed, this null result is best read as inconclusive rather than as evidence against editing having occurred. Establishing a more sensitive verification workflow, through single-colony sequencing and a matched no-TET control, is a necessary next step before extending this approach toward NR2B (GRIN2B) silencing in ischemic stroke.¶~~

~~7.~~ References

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Acknowledgements

I would like to thank my **parents**, who always support me in this research project. I would also like to thank **Y** and **Z** for their guidance and mentorship on this project, for training me in lab activities, data collection, and methodologies, and for reviewing and editing the paper.

Author Biography

X is a student at W. He plans to pursue a Bachelor's degree in neuroscience or informatics, with a specific interest in cognitive, neurological diseases, and genetics. He was awarded a bronze medal at the most prestigious national research competition in V for his neuroscience research on working memory, and has been running a free mentoring and manuscript review program for students preparing science fair projects, which has reached over 100 mentees across V.

Mentor Contribution Statement

Y, faculty mentor for this project, provided extensive feedback on the organisation, language, and structure of the manuscript. He taught the author how to construct figures, including how to organise graphics for clarity and how to correctly indicate statistical significance and confidence levels on graphs, and facilitated the author's access to paywalled journal articles through his institutional access. **Z**, teaching assistant to Y, provided additional support throughout the project. Together, Y and Z worked closely with the author on constructing the plasmid, troubleshooting experimental issues, and explaining the CRISPR prime editing system step by step as part of the laboratory activities. They also assisted extensively with collecting lab data, including OD and fluorescence intensity measurements, and tutored the author on using Fiji and Benchling to analyse the resulting datasets.

8. Appendix

Table S1: Per-colony fluorescence measurements for Figure 3. Background corrected RawIntDen, region-interest area in pixels, and background-corrected mean intensity for each of the six colonies measured on each plate, with plate means and the background region on each plate. Region area was recovered as RawIntDen divided by mean grey value. Background-corrected mean intensity is the colony mean grey value minus the background mean grey value.

Plate	Colony	RawIntDen	ROI area (px)	Mean Intensity
Experiment Set A	**1**	25,992	340	68.8
	2	40,270	484	62.7
	3	24,782	306	78.2
	4	25,239	336	68.0
	5	19,747	320	56.8
	6	21,846	342	56.0
	Mean	26,313	355	65.1
	Background ROI	14,714	289	-
Sample C_f	1	10,691	154	47.5
	2	4,660	132	17.4
	3	5,650	120	32.0

	4	12,542	144	66.9
	5	3,128	96	25.4
	6	7,093	100	62.1
	Mean	**7,294**	**124**	**41.9**
	Background ROI	**3,747**	81	-

9. ¶

Anzalone, A. V., Randolph, P. B., Davis, J. R., Sousa, A. A., Koblan, L. W., Levy, J. M., Chen, P. J., Wilson, C., Newby, G. A., Raguram, A., & Liu, D. R. (2019). Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, *576*(7785), 149–157. https://doi.org/10.1038/s41586-019-1711-4¶

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Mentor Contribution Statement¶

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Response to Reviewer 1

Manuscript: "A GFP Reporter Workflow for Evaluating CRISPR Prime Editing in *E. coli*: A Feasibility Study Toward NR2B-Targeted Editing "
Submission ID: 100308
Date: October 2026

Dear Reviewer 1,

Thank you for reading my paper so carefully, and for being kind about it. Your main point was that my title and my framing promised more than my experiments actually showed. You were right, and fixing that helped the paper more than anything else I did. The paper is now about testing a GFP reporter workflow in bacteria. NR2B and stroke are the reason I care about the problem and the direction I want to go next, not something I tested here. I also did the *in silico* analysis you suggested, normalised the fluorescence to OD_{600}, drew the figure 1 that was missing, and rewrote the Results so each part says why I did the experiment before saying what it showed. I answer each of your comments below. Section numbers refer to the revised paper, and every change is marked in the tracked changes file.

Major Comment 1: Structure of the Results section

> *"The Results section currently reads more like a list of individual findings, which makes the overall logical flow difficult to follow. Although scientific writing should be objective, it should still tell a coherent story. For each figure or experiment, I suggest following a clear sequence: briefly explain why the experiment was performed, what approach was used, what the main finding was, and what the finding suggests or means in the context of the study. … Also, I would avoid phrases such as 'Figure 4 presents…' Instead, describe the scientific finding directly and cite the relevant figure at the end of the sentence or paragraph (Fig. 4)."*

Response: I rewrote the Results in exactly that order. I took out every "Figure X presents" and "Figure X represents", and figures are now cited at the end of the sentence that states the finding. Each part now starts with why I did the experiment. Section 3.2 opens "Three Stop Codon samples were sequenced across the codon 5 target site to test directly whether the intended edit was present." Section 3.3 opens by saying why I looked at a public datasets at all. I also gave the three parts real headings, so you can follow the argument from the headings alone.

Major Comment 2: Title and overall scope

> *"The current title suggests validation of prime editing for NR2B inhibition in ischemic stroke. However, NR2B was not directly targeted, and the experiments were performed using GFP in E. coli. I suggest focusing the title and main conclusions on evaluation of the GFP reporter system, while presenting NR2B/stroke as a potential future application."*

Response: I agree. The new title is "A GFP Reporter Workflow for Evaluating CRISPR Prime Editing in E. coli: A Feasibility Study Toward NR2B-Targeted Editing". I rewrote the abstract and the Conclusion to match it, so NR2B and stroke now appear as my reason for doing the work and as future work, not as something I tested. The introduction now ends with "Extension to NR2B (*GRIN2B*) silencing in models of

ischemic stroke remains a future objective rather than an outcome of the present work." Reviewer 2 asked for the same thing, so this one change answers you both.

Major Comment 3: Interpretation of the editing results

> *"None of the three sequenced samples contained the expected TAG stop codon. Therefore, terms such as 'validation' should be used cautiously. … the conclusions should consistently reflect that successful editing was not demonstrated in the current experiment."*

Response: You are right, and I went looking for the problem word by word. I searched the whole paper for validate, validated, validation, and confirm, and I removed or replaced every one of them where I had not actually shown the thing I was claiming.

The abstract now says that all three sequenced samples matched the wild-type sequence, so none of them carried the edit I wanted. The Conclusion says the same thing plainly and names the real problem, which is that my workflow was not sensitive enough. It reads "The sensitivity of that workflow proved to be the factor limiting what the data can show."

I still call the result inconclusive rather than negative, and I explain why. My design could not have caught editing at the low rates other people report for this kind of edit, so a null result does not tell me that editing failed.

Major Comment 4: Fluorescence analysis and OD_{600} normalisation

> *"In Figure 2, the starting OD600 values differed considerably among conditions, while raw fluorescence was compared directly. Normalizing GFP fluorescence to OD600 would provide a more meaningful comparison of fluorescence relative to bacterial density. This is particularly important because the fluorescence eventually decreased to similar levels across all conditions."*

Response: This was the most useful thing anyone told me, because it changed what my own data say. I divided every reading by the OD_{600} of the same sample at the same timepoint. The normalised numbers are now in Section 3.1 and in a new Table 1.

Normalising removed more than half of the early difference. Compared with the average of my two controls, the Stop Codon construct was 2.39 times higher at the first timepoint instead of 5.85 times higher. At the last timepoint it actually slightly below controls, where the raw number had all three about equal. I report that as it is. Section 3.1 now says that the difference which is left only shows that GFP was being made, and says nothing about whether an edit happened. Reviewer 2 raised the same point.

Major Comment 5: Relevance of the target gene to ischemic stroke

> *"The current experiments do not appear to be directly related to ischemic stroke, although the manuscript emphasizes stroke as an important disease context. If the authors wish to maintain this connection, additional evidence would be needed to demonstrate the relevance of the gene to ischemic stroke. For example, an in silico analysis using publicly available datasets could be used to examine whether the gene is altered in ischemic stroke…"*

Response: I took the first of your two options and did the *in silico* analysis. It is now a new Methods Section 2.8, a new Results Section 3.3, a new Figure 5 and a new paragraph in the Discussion. I used GEO

dataset GSE38037, which is rat cortex after transient middle cerebral artery occlusion, four stroke samples against four sham samples. *Grin2b* is a little higher in the ischemic cortex. The probe is 10020292529, the $\log_2$ fold change is +0.18, which is a 1.13-fold increase, and the adjusted *p*-value is 0.049.

I was careful not to make this sound stronger than it is, because that is the same mistake your other comments caught. So I put the two weak points in the Results myself instead of leaving them for a reader to find. First, 6,919 of the 23,376 probes pass the same cut-off in this dataset, which is 29.6%, and *Grin2b* does. Section 2.8 also explains that this array is a common-reference two-colour design, which is probably why the variation inside each group is so small.

Because of that, I describe the result as the whole NMDA receptor family being switched on together, not as *GRIN2B* being picked out on its own. My reason for still choosing NR2B is now in the Discussion, and it is about what the subunit does downstream rather than about how much of it is made.

Minor Comment 1: Clarity of the Introduction

> *"The Introduction contains several grammatical and awkwardly worded sentences. For example, the paragraph describing the different roles of GluN2A and GluN2B is difficult to follow in places and should be carefully edited for clarity."*

Response: I went through the whole Introduction and rewrote that paragraph. It now reads "Receptors containing the GluN2B (NR2B) subunit are strongly associated with pro-death signalling, whereas GluN2A-containing receptors tend to promote neuronal survival. This functional divergence makes NR2B a more appealing therapeutic target than the receptor as a whole." I also broke up the long sentences elsewhere in the Introduction.

Minor Comment 2: Line 39

> *"Line 39 remains one the leading causes -> one of XXXX"*

Response: Fixed. It now reads "Ischemic stroke remains one of the leading causes of mortality and disability worldwide."

Minor Comment 3: How the six colonies were selected

> *"The authors should explain how the six colonies used for fluorescence quantification were selected. Random or predefined selection criteria would help reduce possible selection bias."*

Response: This is now in Section 2.6 and again in Section 3.1, where I make the comparison. I wrote down what I actually did rather than claiming a procedure I did not follow. The text says "The six colonies on each plate were picked without reference to their brightness, and no formal randomisation procedure was used." Section 3.1 then says that the comparison is only descriptive and cannot be read as a difference in editing status. I would rather admit that than pretend I randomised when I did not.

Minor Comment 4: Where is Figure 1?

> *"Where is Figure 1?"*

Response: It was missing. I am sorry about that. I drew a new Figure 1 and added it, instead of renumbering the figures I already had. Panel a shows the three plasmid prime editing system in *E. coli*

Top10, and panel b shows the codon 5 edit I was aiming for together with the three steps I used to check for it. The introduction now ends with “The experimental design and the verification workflow are summarised in Figure 1.”

Minor Comment 5: Replicates and a statistical analysis section

> *“The Methods would benefit from a clearer description of the number of biological and technical replicates. A brief statistical analysis section should also be included if statistical comparisons are intended.”*

Response: I added a short paragraph at the end of Section 2.6 that says exactly how many replicates there were, and it says plainly that I ran no statistical tests. It reads “Replication was as follows. Bulk growth and fluorescence were measured once per condition at each timepoint, so Figure 2 represents a single biological replicate with no technical replicates. Colony fluorescence was quantified for six colonies on each of two independent platings. No inferential statistical tests were performed, because the design does not support them. Summary values are reported as means, ranges and coefficients of variation.” I thought about adding tests, but with one culture per condition there is nothing a test could honestly tell you. Section 3.1 therefore gives means, ranges and coefficients of variation only.

Minor Comment 6: Figure 3c and 3d y-axes

> *“Figur3: Figure 3c and 3d use different y-axis scales. Using the same scale, if possible, would make the difference between the two groups easier to evaluate visually.”*

Response: Both panels now use the same y-axis. I redrew the whole of Figure 3, and the caption also gives the numbers, which are a 2.85-fold difference in colony area and a 1.55-fold difference once size is taken out. Reviewer 2 asked for the same thing.

Minor Comment 7: Caution about extending from bacteria to neurons

> *“The connection between the bacterial GFP reporter experiment and eventual NR2B editing in mammalian neurons should be stated more cautiously. The current experiment can establish or evaluate a technical workflow, but it does not directly demonstrate the feasibility or therapeutic effectiveness of NR2B editing in ischemic stroke.”*

Response: Agreed. Every forward-looking sentence in the Discussion and the Conclusion is now written as something I want to do next, not something I have shown. The limitations paragraph says “Results obtained on editing efficiency in this bacterial system do not necessarily transfer directly to mammalian neurons, the eventual context for NR2B (GRIN2B) targeting in ischemic stroke.” The conclusion ends by saying that a more sensitive way of checking for the edit has to come first, before anyone tries this on *GRIN2B*.

Thank you again for the time you spent on this. The reframing you asked for made the paper both more honest and easier to read, and the *in silico* analysis you suggested gave the stroke part of my argument a foundation it did not have before.

Sincerely,

[Researcher name]

Response to Reviewer 2

Manuscript: "A GFP Reporter Workflow for Evaluating CRISPR Prime Editing in *E. coli*: A Feasibility Study Toward NR2B-Targeted Editing "
Journal: Convergence Journal
Date: October 2026

Dear Reviewer 2,

Thank you for such a detailed review, and for the encouraging comments. Your main point was that my framing claimed more than my experiments actually showed. You were right, and I have changed that everywhere. The tittle is new, the fluorescence normalised to OD_{600}, the figure 3 comparison now comes with its confound measured instead of just mentioned, and every therapeutic claim is now written as future work. I was glad you thought I handled the null result carefully, and I tried to keep that while revising rather than tidy it away. I answer each of your comments below. Section numbers refer to the revised paper, and every change is marked in the tracked-changes file.

Major Comment 1: Title and overall framing

> *"The title and overall framing currently make a stronger claim than the experiments support. The study does not directly investigate NR2B or an ischemic stroke model, and the intended GFP edit was not successfully demonstrated. Therefore, describing the study as 'Validating CRISPR Prime Editing for Targeted NR2B Inhibition' may be misleading. I would recommend reframing the study more explicitly as a preliminary or feasibility experiment evaluating a GFP reporter workflow that could eventually be adapted to NR2B."*

Response: I agree, and I reframed the paper exactly the way you describe. The word validating is gone from the title and from the body. The new title is "A GFP Reporter Workflow for Evaluating CRISPR Prime Editing in E. coli: A Feasibility Study Toward NR2B-Targeted Editing". The abstract now says "This study tested such a verification workflow in a simple bacterial reporter system." The Introduction and the Conclusion treat NR2B and stroke as my motivation and my next step. Reviewer 1 made the same point, so this change answers you both.

Major Comment 2: Fluorescence not normalised to OD_{600}

> *"The interpretation of the fluorescence data should be a little more cautious. For example, in Figure 2, fluorescence was not normalized to OD600, despite substantial differences in bacterial density between the Stop Codon construct and controls at the first timepoint. … it makes the early fluorescence differences difficult to interpret as changes in GFP expression rather than differences in the number of bacteria present.*

Response: Agreed, and doing the normalisation changed what the data look like. I divided every reading by the OD_{600} of the same sample at the same timepoint, and the normalised numbers are now in Section 3.1 and in a new Table 1. The early difference drops from 5.85-fold to 2.39-fold, and at the last timepoint the Stop Codon construct ends up slightly below both controls.

While I was checking this I found something I had missed in the submitted version, and it makes my fluorescence data weaker still. Neither of my controls carries the GFP reporter plasmid at all. So what they show is background glow from the cells, not unedited GFP. I never had a condition with GFP but no pegRNA, which means I had no baseline to compare against, so I cannot say fluorescence was lost. Section 3.1 says this straight out and concludes that the fluorescence data cannot tell you anything about editing status. Reviewer 1 raised the same point about normalisation.

Major Comment 3: Over interpretation of the Figure 3 comparison

> *"Similarly, the colony-level fluorescence differences in Figure 3 should not be interpreted too strongly. Experiment Set A and Sample C_f differ substantially in colony density and represent independent platings rather than replicates of the same condition. The approximately 3.6-fold difference in mean fluorescence is interesting, but differences in crowding, nutrient availability, and background correction could contribute substantially to this result."*

Response: You were right, and instead of just softening the wording I went back to my Fiji measurements and worked out how big the problem actually is. RawIntDen adds up the grey values inside a region, so a bigger colony gives a bigger number even at the same brightness. Dividing RawIntDen by the mean grey value gives me back the area. My Set A colonies averaged 355 pixels and my C_f colonies averaged 124, which is 2.85 times bigger.

So I made a measure that does not depend on size, the colony mean grey value minus the background mean grey value. On that measure the two plates differ only 1.55 times, and the two groups overlap. Two of the C_f colonies were brighter than two dimmest colonies in Set A. Section 3.1 now reports the 3.6-fold difference as mostly a difference in colony size rather than in brightness, and says that two plates were separate platings of the same construct with very different densities, so I cannot separate plate to plate variation form a real difference.

I put that warning right where the comparison first appears, instead of saving it for the Discussion. I also added every colony measurement as Table S1 in the Appendix, so you can check my re-analysis yourself. Section 2.6 now admits one more thing, which is that my background region was not the same as the colony regions, so subtracting it under-corrects the bigger ones.

Major Comment 4: Sequencing strategy

> *"The sequencing strategy is the most important methodological limitation. Only three samples underwent Sanger sequencing, and these were obtained from bulk liquid culture. … If additional experiments are feasible, sequencing a larger number of isolated colonies would substantially strengthen the study. If not, this limitation should remain central to the interpretation of the results."*

Response: You are right that this is my biggest weakness. I could not do more sequencing, because the laboratory programme I did this work has finished and I no longer have access to a bench. So I took your second option and kept this limitation at the centre of the paper instead of pushing it to the side.

It now appears in four places. The abstract says the result is inconclusive rather than negative, and names detection sensitivity as the thing to fix. The Discussion uses it as the main explanation for why I found nothing, and compares my three bulk-culture samples with the 24 clones per condition that Tong

et al. (2021) screened. It is the first item in my list of limitations. The conclusion says that single-colony sequencing and a matched no-TET control are the first two things a follow-up study would have to do.

Major Comment 5: Separating the result from the therapeutic hypothesis

> *"The manuscript should more clearly distinguish between the immediate experimental conclusion and the longer-term therapeutic hypothesis. The present experiment can evaluate the feasibility of a particular prime-editing workflow in GFP, but it cannot yet establish whether NR2B can be successfully edited, whether doing so would reduce excitotoxic neuronal injury, or whether this approach could ultimately function therapeutically in ischemic stroke. I would recommend consistently framing these as future directions rather than implications demonstrated by the present study."*

Response: Agreed, and I applied this everywhere. No sentence in the revised paper claims a treatment implication form what I did. The introduction says that the extension to NR2B (*GRIN2B*) silencing in models of ischemic stroke remains a future objective rather than an outcome of the present work.

I held the new *in silico* analysis to the same rule. The Discussion says its relevance here is narrower still, because all it shows is that my eventual target is disease-relevant, while the editing and verification questions I actually set out to answer are still open.

Minor Comment 1: Grammar and style

> *"The manuscript would benefit from careful grammatical and stylistic editing. There are a few awkward or incomplete sentences, particularly in the Introduction, that occasionally make the scientific argument difficult to follow."*

Response: I edited the whole paper, and the Introduction most of all. I rewrote the incomplete sentences in the GluN2A and GluN2B paragraph and broke up the long sentences elsewhere. Reviewer 1 pointed the same paragraph.

Minor Comment 2: Condense the Discussion

> *"Consider simplifying some of the longer paragraphs in the Discussion. The discussion of editing efficiency and comparison with Tong et al. is valuable, but parts could be condensed to make the central argument easier to follow."*

Response: My comparison with Tong et al. took three paragraphs and now takes one. I also cut down the summary paragraph at the start of the Discussion, because it was repeating numbers I had already given in the Results. The Discussion is about 300 words shorter, and my main argument about detection sensitivity now sits in one place instead of being spread out.

Minor Comment 3: Figure 3 y-axis scales

> *"Figure 3 uses different y-axis scales for the two fluorescence plots. Using the same scale, or making the difference particularly prominent in the figure legend, would make comparison between the groups more intuitive."*

Response: I did both things you suggested. Both panels now share one scale, and the caption states the 2.85-fold difference in colony area and the 1.55-fold difference once size is taken out. Reviewer 1 asked for the same change.

Minor Comment 4: Methods wording on verification

> *"The Methods state that 'positive clones identified by loss of GFP fluorescence were verified' by sequencing, which could imply that these clones were confirmed as successfully edited. Because sequencing ultimately showed the wild-type sequence, I would revise this language to distinguish candidate clones selected based on fluorescence from molecularly confirmed edited clones."*

Response: Agreed, and thank you for catching it. That sentence contradicted my own Results. I rewrote Section 2.7 so that picking one and checking a clone are clearly two different steps, and the words positive and confirming are gone. It now reads "Candidate clones were selected on the basis of reduced GFP fluorescence and assessed at the DNA level by colony PCR followed by Sanger sequencing across the target region, in order to determine whether the intended stop codon was present at codon 5 of the GFP coding sequence. Selection on fluorescence identifies candidates only. It does not establish that an edit occurred."

Minor Comment 5: Data availability

> *"The authos should ensure that all experimental data are included as an appendix or made available through a repository, as required by the journal's submission guidelines."*

Response: I added a Data Availability section after the Conclusion, and I put the per-colony measurements behind Figure 3 into the Appendix as Table S1 so that my re-analysis can be checked. The statement gives the GEO accession and platform for the public dataset and names the eight samples I compared. It also says that the GEO2R output table, the OD_{600} and fluorescence readings behind Figure 2 and Table 1, and the Fiji region of interest measurements behind Figure 3 and Table S1 are available from me on request, and that I generated no new high throughput sequencing data.

Thank you again for the time you spent on this. Your comments on Figure 2 and Figure 3 sent me back to my raw measurements, and what came out of that is now one of the most useful parts of the paper.

Sincerely,

[Researcher name]

Final Feedback Referee 2 100308

I really appreciate the author's thoughtful and comprehensive responses to my initial review. The revised manuscript has improved substantially, particularly in its scientific framing, interpretation of results, and acknowledgment of methodological limitations. I was especially pleased to see that the author took the feedback seriously and revisited the original data rather than simply revising the language. Most importantly, the revised title and overall framing now appropriately describe the study as a preliminary feasibility evaluation of a GFP reporter workflow in E. coli, rather than suggesting that NR2B-targeted editing or therapeutic applications have been validated. This is an important improvement that makes the manuscript's conclusions much more consistent with the experimental findings.

I also appreciate the additional analyses addressing my concerns about fluorescence measurements. Normalizing fluorescence to OD600 and accounting for colony size in Figure 3 substantially strengthen the interpretation of these results. The author's acknowledgment that the controls lacked the GFP reporter plasmid is particularly important, as it clarifies why the observed fluorescence differences cannot establish editing status. The revised discussion of sequencing limitations is also much stronger. Although additional experiments would ideally include single-colony sequencing and appropriate controls, I understand that these are not currently feasible. The author has appropriately incorporated these limitations into the interpretation of the results and proposed reasonable next steps for future research.

The newly added Grin2b expression analysis provides useful biological context, although I would encourage the author to continue distinguishing these findings from direct evidence supporting NR2B-targeted intervention. Similarly, the manuscript should consistently emphasize that the feasibility of the verification workflow has been explored, but its ability to reliably detect successful editing remains unestablished.

Overall, I am pleased with the revisions and believe the author has addressed my major concerns effectively. The remaining limitations are now transparently discussed and do not necessarily require additional experiments for a preliminary feasibility study. I would support "acceptance with minor edits," primarily focused on maintaining precise language and ensuring consistency throughout the revised manuscript.