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Peer Review File

Prime assembly with linear DNA donors enables large genomic insertions

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
This manuscript presents a Prime Assembly (PA) strategy for inserting large DNA donor fragments by leveraging ends designed to overlap with flaps generated by twin prime editing (twinPE), thereby circumventing the need for double-strand breaks and recombinases. The approach represents a significant advance in large-fragment genomic insertion and holds promise for clinical applications. I suggest to accept this paper after revision and below are specific comments and suggestions for the authors' consideration.
Major:
1. Quantification of Insertion Efficiency:
The reported efficiency of large-fragment insertion (Fig. 4E, 4F) a is assessed primarily by gel-based analysis. The authors are encouraged to include quantitative evaluations—such as droplet digital PCR (ddPCR) or next-generation sequencing (NGS)—to provide more rigorous and sensitive measures of insertion efficiency.
2. Comparative Benchmarking:
A head-to-head comparison with existing tools for large-fragment integration (e.g., engineered R2 retrotransposons, evoCAST) is important. The comparison can address insertion efficiency, specificity, and maximum fragment size to contextualize the performance of PA.
3. Validation in Diverse Cell Types:
To demonstrate broader applicability, the authors are suggested to validate the PA system in a wider range of cellular contexts.
4. Parameter Optimization:
Additional optimization of key parameters—including the choice of prime editing tools (e.g., the recently reported PE7), flap homology length or GC content—would strengthen the utility and reproducibility of the method. A more systematic assessment would help establish robust methods.
Minor
1. Rationale for Multi-Donor Strategy:
While the manuscript proposes a multi-donor approach using linear donors with homologous sequences, the observed efficiency is lower than with single donors. The authors are suggested to discuss:
o The practical benefits of using multiple donors over a single donor.
o Whether there is a trade-off between donor number and efficiency, and if so, what the optimal or maximal number of donors might be.
2. Discussion about the combination with other methods:
Previous studies also showed that enhancing the pegRNA stability can increase PE efficiency (PMID: 35351879, 34608327). Thus, whether these strategies can be combined with PA to further increase PA's editing efficiency deserves more discussions.

Referee #2

(Remarks to the Author)

This manuscript by Liu et al. introduces Prime Assembly (PA), a very exciting and novel genome editing technique for the targeted insertion of large DNA fragments. The method cleverly utilizes the 3' flaps generated by twin prime editing to direct the integration of a linear DNA donor with relatively short homologous ends. The authors demonstrate the versatility of PA by successfully inserting DNA fragments ranging from 0.1 kb to an impressive 11.3 kb. The efficiency and precision of PA were enhanced by an inhibitor of the non-homologous end joining (NHEJ) pathway, AZD-7648. Interestingly, PA can mediate a Gibson-like assembly of multiple overlapping DNA fragments directly within the cell. This work provides a strategy for large gene insertion that avoids double-strand breaks (DSBs) and exogenous recombinases.
The conclusions are exciting in the field of genome editing, especially for therapeutic purposes, but they could be better supported by more compelling quantitative data.
Major comments
1. Genome-wide off-target effects should be thoroughly evaluated for at least three independent examples of PA, especially given that PA is therapeutically relevant. These PA examples should include a dsDNA donor without 3' overhang and one with a 3' overhang.
2. Quantitative profiling of PA would assist readers of the paper in designing PA for their experiments. For example, what are the optimal conditions (e.g. GC content, length, etc.) for the 3' flap?
3. Dependency on cell cycle and/or HDR is important. The authors conclude that PA is independent of cell cycle progression or HDR; "PA was not affected by nocodazole, which indicates that PA is independent of cell cycle progression (Extended Data Fig. 3). The latter observation, along with the efficiency of PA during RAD51 inhibition, suggests that PA does not rely on the canonical HDR machinery." However, the data shown in Extended Data Fig. 3 are not quantitative. The authors need to provide compelling quantitative data with statistical analysis to support this important conclusion.
4. Please provide quantitative data with statistical analysis supporting "Compared to the unmodified dsDNA donor, the odsDNA donor slightly reduced the frequency of insertion (Figs. 1d and 2e) but resulted in slightly increased precision (Extended Data Fig. 4e).". Furthermore, please provide exact quantitative values in place of the term "slightly" to allow clearer interpretation and comparison.

5. The use of split donors is excellent. Can the authors define the optimal conditions (e.g. lengths, GC contents, melting temperatures, etc.) for the overlapping sequences?

6. Regarding the conclusion "Consistent with results from two-segment PA, insertion efficiency improved upon AZD-7648 treatment (Fig. 3f).", please provide quantitative data. In addition, how many independent experiments were conducted?

Minor comments
1. Line 68, "indels/SNPs comprised 12%–17% of reads (Extended Data Fig. 2a and 2b).", extended Data Fig. 2a, b show only indels. The authors should include the percentage of reads with SNPs separately to support this statement.
2. Extended Data Fig. 4a and 4e are cited before Extended Data Fig. 3. The numbering of figures should follow the order of citation in the text.
3. Scale bars should be included in microscopic pictures (e.g. Extended Data Fig. 5a)
4. "PA also replaced a 1kb HEK3 region with a 1.3kb Halo-tag using PECas9 or PEmax (Extended Data Fig. 5b).". What is the difference between PEcas9 and PEmax? Does Cas9 refer to the Cas9 nuclease without reverse transcriptase? Please provide quantitative data for DMD and CAR insertion.
5. The authors mainly used nocodazole, which arrests cell cycles at the G2/M phase, to determine the necessity of cell cycle progression for PA. However, given that most cells in the human body are in the G0/G1 phase rather than the G2/M phase when their cell cycles are not progressing, using molecules that arrest the cell cycle at the G0/G1 phase would be more relevant to therapeutic applications.

Referee #3

(Remarks to the Author)
In their manuscript, Bin Liu et al. developed a strategy for targeted genomic insertion of large DNA fragments by using paired prime editors to generate fresh flaps homologous to the donor DNA. Since the discovery of sequence-specific endonucleases, numerous efforts have been made to achieve site-specific integration of large DNA fragments by introducing single- or double-stranded breaks into the genome and supplying various types of donor DNA. These approaches leverage both homology-dependent and homology-independent DNA repair pathways. Such efforts have led to the development of knock-in strategies based on NHEJ, MMEJ, SSA, and HDR, enabling either precise or imprecise insertions. To further enhance efficiency and accuracy, additional factors, such as exonucleases, single-stranded DNA-binding proteins, and prime editors, have been employed to generate single-stranded overhangs at the target site or within the donor DNA, or to promote donor invasion. Notably, a similar strategy was previously reported in the PAINT system, which used a prime editor to install 3′ flaps within the donor DNA that are homologous to the target genomic site (DOI: 10.1038/s41421-023-00552-0). Given this precedent, it is reasonable to expect that a similar design, installing a homologous flap at the genomic site, would also be effective.

1. To demonstrate the advantages and novelty of prime assembly, it should be benchmarked in parallel against existing similar strategies, such as HITI, PITCH, and PAINT etc. The parallel comparison should also include other recently reported knock-in approaches, particularly those based on prime editing, such as the evolved PASSIGE (with eeBxb1) and evoCAST.

2. In Fig. 3, it is unclear why multiple donors were used, given that a single long donor should be sufficient for targeted insertion. The use of multiple PCR-derived donor fragments with short homologous regions is expected to activate not only homology-dependent repair pathways, such as MMEJ, but also the non-homologous end joining (NHEJ) pathway. Therefore, the authors should perform more sensitive assays, such as high-throughput sequencing (HTS) of the junction regions, to determine whether NHEJ-mediated indels are present among the outcomes of the multiple-donor prime assembly. Additionally, there is a possibility that donor 1 was directly joined to donor 3 via NHEJ. To gain deeper insight into the characteristics of multiple-donor prime assembly, the AZD (–) control group should also be included.
3. For the detection of targeted integration, only PCR-based methods were employed, which are indirect and insufficient to provide conclusive evidence. PCR can only detect the presence of mosaic sequences containing genome and donor derived sequences. In addition to target integration of donor into the genome, there is also a possibility that genomic sequences flanking target site were integrated into the donor via endogenous DNA repaired mechanisms. Therefore, additional methodologies, such as Southern blotting or fluorescence in situ hybridization, would offer more direct and reliable detection of donor sequence integration at the intended genomic locus.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have further improved their study and my previous comments have been fully addressed. I recommend to accept this revised manuscript.

Referee #2

(Remarks to the Author)
The authors successfully addressed many of my comments. In particular, the genome-wide off-target evaluation result is great. It substantially strengthens the manuscript. However, the manuscript remains preliminary in some aspects described below.

Major comments
1. In their new data, the authors provide only relative prime assembly efficiency (e.g., Extended Fig. 1d, 1e, and 7b). Providing absolute prime assembly efficiency would be more informative for readers. Related to this issue, I would recommend providing absolute prime assembly efficiency for Fig. 2b as well.

2. Based on my comment, the authors now provide quantitative data for only some of the factors shown in Extended Data Fig. 4a. Quantitative data are still lacking for factors such as Mirin, NU7026, olaparib, and ART558.

3. Regarding my comment “The use of split donors is excellent. Can the authors define the optimal conditions (e.g. lengths, GC content, melting temperatures, etc.) for the overlapping sequences?”, the authors have not provided a solid guideline to design overlapping sequences due to their limited number of tested parameters. In addition to testing many more data points for each parameter, I would recommend changing one parameter at a time while fixing other parameters so that the authors can determine the effect of that parameter (except for melting temperature, which is determined by the GC content and lengths). The authors should provide reliable guidelines (based on systematic single-parameter optimization) so that readers can effectively use the prime assembly.

4. Regarding my comment, “Regarding the conclusion ‘Consistent with results from two-segment PA, insertion efficiency improved upon AZD-7648 treatment (Fig. 3f),’ please provide quantitative data. In addition, how many independent experiments were conducted?”, the authors now provide quantitative data. However, the number of replicates is only two, and it is not clear whether the difference is statistically significant. In its current form, the difference appears unlikely to reach statistical significance, particularly for the PA-3 donor.

5. The authors provide statistical analysis for only some of the newly added data. The authors should provide statistical analysis for all of the quantitative data.

6. The authors also now provide Southern blotting data as reviewer-only information. The authors should include it in the manuscript.

Minor comments
1. The authors show the number of replicates “n” for the new data as well as the initially included data. However, what does “n” represent? Does it mean the number of independent transfections or the number of independent sequencing runs? This should be clarified in all figure legends.

Referee #3

(Remarks to the Author)

I appreciate the authors' efforts to address my previous concerns. I am pleased to see that the rebuttal now includes Southern blot evidence for the targeted knock-in, as this method provides more direct and definitive validation than conventional PCR or ddPCR.
However, several issues remain unresolved. First, the positive band in the PA+AZD lane—although visually clearer than that in the PA-only lane—appears substantially weaker than would be expected based on the ddPCR quantification shown in Fig. 2. Second, the observed band size (approximately 2027 bp) is noticeably larger than the predicted size of 1871 bp. These discrepancies raise concerns about both the authenticity and the frequency of the reported knock-in events. At present, the Southern blot results do not convincingly support the ddPCR findings. I recommend that the authors conduct additional analyses to clarify these inconsistencies and to more rigorously confirm the presence and abundance of the targeted knock-in.

Version 2:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors have satisfactorily addressed most of my previous comments, including the provision of absolute efficiencies, additional statistical analyses, inclusion of Southern blot data in the manuscript, and clarification of replicate definitions. However, I have remaining concerns regarding the optimization of PA and the discussion of inhibitor results.

Major

1. I appreciate the authors' efforts to conduct optimization experiments for the split-donor overlap sequences, as I had requested. However, I remain concerned that the current dataset is insufficient to provide the "reliable guidelines based on systematic optimization" that I had specifically asked for in the previous round. I had explicitly requested "many more data points for each parameter," yet the authors still tested an insufficient number of data points for each parameter. Only four data points (8, 16, 30, and 100 bp) were tested, with a 70-bp gap between 30 and 100 bp. No intermediate lengths (e.g., 45, 60, 75 bp) were evaluated, making it impossible to determine whether 30 bp is genuinely optimal or whether a longer overlap would perform better. The recommendation of "30–100 bp" is based on the absence of data rather than evidence of a performance plateau. In addition, testing at 20% intervals (10–90%) omits the 40% and 60% conditions flanking the reported optimum of 50%. Without these data points, the conclusion that 50% GC is optimal is not well substantiated. Moreover, recommending a single pin-point value of 50% GC is of limited practical utility, as the overlap sequence is constrained by the donor and genomic context, making it often difficult to achieve exactly 50% GC. What users need to know is the acceptable range of GC content, which the current data cannot define. In conclusion, a guideline of "30–100 bp overlap and 50% GC" is too broad and too pin-pointed, respectively, to be actionable. I would ask the authors to: (i) test intermediate overlap lengths (e.g., 45, 60, and 75 bp), and (ii) test 40% and 60% GC, with sufficient replicates for statistical power to provide more evidence-based practical guidelines.

Minor

1. Ext. Data Fig. 4b now shows that Mirin (MRN complex inhibitor) has a statistically significant effect on PA efficiency, yet this is not discussed in the main text.

Referee #3

(Remarks to the Author)
The revised manuscript has addressed my concerns regarding the existence and efficiency of the prime assembly. I have no further comments and believe the revised manuscript meets the requirements for publication.

Version 3:

Reviewer comments:

Referee #2

(Remarks to the Author)
The authors have successfully addressed all of my remaining concerns, which improved the manuscript. I think that the manuscript is now suitable for publication.

We thank the reviewers for their positive evaluations and thoughtful, constructive comments, which have substantially improved the quality of our manuscript. In this revision, we have performed additional experiments and expanded our discussion to address the major concerns. Specifically:

- We performed a comprehensive genome-wide off-target analysis across three donor formats of PA.
- We quantified PA insertion efficiencies by ddPCR for *TRAC*, *DMD*, and *IRES-AAT*.
- We benchmarked PA against evoCAST and PAINT 3.0 at the same target site.
- We systematically assessed multiple parameters of PA, including donor fragment number, GC content, prime editor variants (PE7 vs PE6c), and flap length.
- We investigated cell-cycle effects on PA using chemical inhibitors.
- We performed Southern blotting to validate targeted genomic integrations.

Detailed replies to each reviewer are provided below in blue font.

Referees' comments:

Referee #1 (Remarks to the Author):

This manuscript presents a Prime Assembly (PA) strategy for inserting large DNA donor fragments by leveraging ends designed to overlap with flaps generated by twin prime editing (twinPE), thereby circumventing the need for double-strand breaks and recombinases. The approach represents a significant advance in large-fragment genomic insertion and holds promise for clinical applications.

We are grateful to the reviewer for the positive assessment of our results.

I suggest to accept this paper after revision and below are specific comments and suggestions for the authors' consideration.

Major:
1. Quantification of Insertion Efficiency:
The reported efficiency of large-fragment insertion (Fig. 4E, 4F) a is assessed primarily by gel-based analysis. The authors are encouraged to include quantitative evaluations—such as droplet digital PCR (ddPCR) or next-generation sequencing (NGS)—to provide more rigorous and sensitive measures of insertion efficiency.

We thank the reviewer for this constructive suggestion. We have added ddPCR-based quantification of PA insertions, including *dystrophin* (**Fig. 4b**), *TRAC* (**Fig. 4f**), and *IRES-AAT* (**Fig. 3g, Extended Data Fig. 7**). These results provide sensitive and quantitative confirmation of our previous gel-based measurements.

2. Comparative Benchmarking:
A head-to-head comparison with existing tools for large-fragment integration (e.g., engineered R2 retrotransposons, evoCAST) is important. The comparison can address insertion efficiency, specificity, and maximum fragment size to contextualize the performance of PA.

Following the reviewer's advice, we conducted direct benchmarking of PA against evoCAST and PAINT 3.0 at the same target site (**Extended Data Fig. 6f**). PA achieves higher insertion efficiency while requiring fewer components, highlighting PA's simplicity and versatility.

3. Validation in Diverse Cell Types:
To demonstrate broader applicability, the authors are suggested to validate the PA system in a wider range of cellular contexts.

We agree and have now validated PA in Huh7, K562 and IMR90 cells (**Extended Data Figs. 4b, 6d-e**), demonstrating its applicability across distinct human cell types.

4. Parameter Optimization:
Additional optimization of key parameters—including the choice of prime editing tools (e.g., the recently reported PE7), flap homology length or GC content—would strengthen the utility and reproducibility of the method. A more systematic assessment would help establish robust methods.

We systematically evaluated these parameters. We now provide data on varying GC content (**Extended Data Fig. 1d**) and a comparison between PE7 and PE6c in the context of PA (**Extended Data Fig. 1e**). Together with our existing flap-length analysis (**Extended Data Fig. 1c**), these results identify key factors influencing PA performance.

Minor

1. Rationale for Multi-Donor Strategy:
While the manuscript proposes a multi-donor approach using linear donors with homologous sequences, the observed efficiency is lower than with single donors. The authors are suggested to discuss:
- The practical benefits of using multiple donors over a single donor.

We appreciate this insightful comment and have expanded the discussion:

Page 7: "Using multiple donors can overcome the synthesis and amplification limitations of long-range PCR. Although full-length donors are ideal for therapeutic delivery, in-cell assembly of multiple fragments is advantageous for functional genomic studies requiring combinatorial alleles—for example, five variants of donor 1 combined with five variants of donor 2 can generate 25 (5 x 5) insertion alleles in one experiment. For future in vivo applications, split donors may also facilitate delivery of large cargos using multiple viral or non-viral vectors, thereby bypassing vector size constraints such as the AAV capacity limit (4.7kb only)."

- Whether there is a trade-off between donor number and efficiency, and if so, what the optimal or maximal number of donors might be.

We thank the reviewer for the very constructive suggestions. We compared 1, 3, 4, and 6 donors of IRES-AAT (**Fig. 3g**). We observed that 4 and 6 donors reduce the insertion efficiency, although 6 donors still mediate PA insertion at low efficiency. These data suggest that 3 donors yield optimal performance.

2. Discussion about the combination with other methods:
Previous studies also showed that enhancing the pegRNA stability can increase PE efficiency (PMID: **35351879, 34608327**). Thus, whether these strategies can be combined with PA to further increase PA's editing efficiency deserves more discussions.

We have discussed this important point and cited the suggested references. Thank you.
Page 7: "*Strategies that enhance the pegRNA stability can be combined with PA to further increase PA's efficiency.*"

Referee #2 (Remarks to the Author):

This manuscript by Liu et al. introduces Prime Assembly (PA), a very exciting and novel genome editing technique for the targeted insertion of large DNA fragments. The method cleverly utilizes the 3' flaps generated by twin prime editing to direct the integration of a linear DNA donor with relatively short homologous ends. The authors demonstrate the versatility of PA by successfully inserting DNA fragments ranging from 0.1 kb to an impressive 11.3 kb. The efficiency and precision of PA were enhanced by an inhibitor of the non-homologous end joining (NHEJ) pathway, AZD-7648. Interestingly, PA can mediate a Gibson-like assembly of multiple overlapping DNA fragments directly within the cell. This work provides a strategy for large gene insertion that avoids double-strand breaks (DSBs) and exogenous recombinases.

The conclusions are exciting in the field of genome editing, especially for therapeutic purposes, but they could be better supported by more compelling quantitative data.

Major comments

1. Genome-wide off-target effects should be thoroughly evaluated for at least three independent examples of PA, especially given that PA is therapeutically relevant. These PA examples should include a dsDNA donor without 3' overhang and one with a 3' overhang.

We thank the reviewer for the very constructive suggestions. We previously reported a PE-tag pipeline[1] to profile the genome-wide specificity of PE. To determine the specificity of PA, we performed modified PE-tag using insertion-specific reverse primers in cells transfected with *AAVS1* PA (**Fig. 1f**). PA+dsDNA shows a specific insertion signal at *AAVS1* whereas a dsDNA only control shows low levels of random background signal (**Fig. 1f**).

We also performed PE-tag for PA + odsDNA (**Fig. 2f**) and PA + ssDNA (**Extended Data Fig. 5e**) and further validated these results by deep sequencing (**Extended Data Fig. 5f**).

2. Quantitative profiling of PA would assist readers of the paper in designing PA for their experiments. For example, what are the optimal conditions (e.g. GC content, length, etc.) for the 3' flap?

We thank the reviewer for this excellent suggestion. We have now added new data evaluating different GC contents of the flap sequences, showing that higher GC content (54%) correlates with increased insertion efficiency (**Extended Data Fig. 1d**). This finding provides practical design guidance for optimizing PA performance.

3. Dependency on cell cycle and/or HDR is important. The authors conclude that PA is independent of cell cycle progression or HDR; "PA was not affected by nocodazole, which indicates that PA is independent of cell cycle progression (Extended Data Fig. 3). The latter observation, along with the efficiency of PA during RAD51 inhibition, suggests that PA does not rely on the canonical HDR machinery." However, the data shown in Extended Data Fig. 3 are not quantitative. The authors need to provide compelling quantitative data with statistical analysis to support this important conclusion.

We appreciate the reviewer's insightful comment emphasizing the importance of this point. In the revised manuscript, we have performed quantitative ddPCR and flow cytometry (FACS) analysis of GFP knock-in efficiencies under treatments with CDK4/6 inhibitor (Palbociclib) and RAD51 inhibitor, which perturb cell-cycle progression and homologous recombination, respectively. The results (**new Extended Data Fig. 4**) demonstrate that PA efficiency remains unaffected by these inhibitors, confirming that PA is independent of both cell-cycle transit and canonical HDR pathways.

4. Please provide quantitative data with statistical analysis supporting "Compared to the unmodified dsDNA donor, the odsDNA donor slightly reduced the frequency of insertion (Figs. 1d and 2e) but resulted in slightly increased precision (Extended Data Fig. 4e).". Furthermore, please provide exact quantitative values in place of the term "slightly" to allow clearer interpretation and comparison.

We thank the reviewer for this helpful suggestion. We have now included exact quantitative values and corresponding statistical analyses in the revised manuscript. Specifically, insertion frequencies were 48.4% for dsDNA and 33.2% for odsDNA (**Figs. 1d and 2e**). In contrast, indel rates were markedly reduced when using odsDNA: 15.5% for dsDNA vs. 4.8% for odsDNA, and further decreased to 4.8% (AZD + dsDNA) and 0.6% (AZD + odsDNA) under NHEJ inhibition (**Extended Data Fig. 3e**). These quantitative data now replace the qualitative terms "slightly reduced" and "slightly increased precision" in the text.

5. The use of split donors is excellent. Can the authors define the optimal conditions (e.g. lengths, GC contents, melting temperatures, etc.) for the overlapping sequences?

We appreciate the reviewer's positive comments on our split-donor approach. Following the reviewer's suggestion, we have added new data to define the optimal parameters for the overlapping sequences (**Extended Data Fig. 7a**).

6. Regarding the conclusion "Consistent with results from two-segment PA, insertion efficiency improved upon AZD-7648 treatment (Fig. 3f)", please provide quantitative data. In addition, how many independent experiments were conducted?

We have now included quantitative ddPCR measurements (n = 2 independent experiments) confirming that AZD-7648 treatment reproducibly enhances insertion efficiency in the three overlapping segments (**Fig. 3f**). The corresponding quantitative data are presented in **Extended Data Fig. 7b**.

Minor comments

1. Line 68, "indels/SNPs comprised 12%–17% of reads (Extended Data Fig. 2a and 2b).", extended Data Fig. 2a, b show only indels. The authors should include the percentage of reads with SNPs separately to support this statement.

We used CRISPResso2 with the "ignore_substitutions" mode, meaning that the reported values represent indels only. We clarified this in the Methods section and removed "SNPs" from the text.

2. Extended Data Fig. 4a and 4e are cited before Extended Data Fig. 3. The numbering of figures should follow the order of citation in the text.
Thank you – we have reordered the Extended Data Figure numbers to follow the order of citation in the text.

3. Scale bars should be included in microscopic pictures (e.g. Extended Data Fig. 5a)
This figure is reordered as Extended Data Fig. 6a.

4. “PA also replaced a 1kb HEK3 region with a 1.3kb Halo-tag using PECas9 or PEmax (Extended Data Fig. 5b).”. What is the difference between PEcas9 and PEmax? Does Cas9 refer to the Cas9 nuclease without reverse transcriptase?
We defined PECas9 and Cas9 in the legends:
“using PECas9 (Cas9-RT fusion), Cas9 (Cas9 nuclease without RT) or PEmax”.

Please provide quantitative data for DMD and CAR insertion.
We now provide quantitative ddPCR data for DMD and CAR insertions (**Fig. 4b and Fig. 4f**).

5. The authors mainly used nocodazole, which arrests cell cycles at the G2/M phase, to determine the necessity of cell cycle progression for PA. However, given that most cells in the human body are in the G0/G1 phase rather than the G2/M phase when their cell cycles are not progressing, using molecules that arrest the cell cycle at the G0/G1 phase would be more relevant to therapeutic applications.

We thank the reviewer for this excellent suggestion. In addition to nocodazole, we have now included experiments using palbociclib, a CDK4/6 inhibitor that arrests cells at the G0/G1 phase. Consistent with the nocodazole results, PA remained functional in cells treated with palbociclib (**Extended Data Fig. 4b,c**), supporting the conclusion that PA functions independently of cell-cycle progression.

Referee #3 (Remarks to the Author):

In their manuscript, Bin Liu et al. developed a strategy for targeted genomic insertion of large DNA fragments by using paired prime editors to generate fresh flaps homologous to the donor DNA. Since the discovery of sequence-specific endonucleases, numerous efforts have been made to achieve site-specific integration of large DNA fragments by introducing single- or double-stranded breaks into the genome and supplying various types of donor DNA. These approaches leverage both homology-dependent and homology-independent DNA repair pathways. Such efforts have led to the development of knock-in strategies based on NHEJ, MMEJ, SSA, and HDR, enabling either precise or imprecise insertions. To further enhance efficiency and accuracy, additional factors, such as exonucleases, single-stranded DNA-binding proteins, and prime editors, have been employed to generate single-stranded overhangs at the target site or within the donor DNA, or to promote donor invasion. Notably, a similar strategy was previously reported in the PAINT system, which used a prime editor to install 3′ flaps within the donor DNA that are homologous to the target genomic site (DOI: 10.1038/s41421-023-00552-0). Given this precedent, it is reasonable to expect that a similar design, installing a homologous flap at the genomic site, would also be effective.

We thank the reviewer for the very constructive comments. We benchmarked PA against PAINT. PA shows much higher efficiency than PAINT (**Extended Data Fig. 6f**). Notably, PA (PE) requires fewer components than PAINT3.0 as PAINT utilizes both PE and SaCas9, posing additional challenges for efficient delivery. Below is the comparison of components in each editing system:

PA:	PE, two pegRNAs, donor
PAINT 3.0:	PE, pegRNA, donor, SaCas9, sgRNA
evoCAST:	Seven plasmids

In addition, we further show that PA is adaptable to a single single-stranded DNA (ssDNA) donor (**Extended Data Fig. 5**), which further simplifies the system and expands its therapeutic potential. Together with results from the two-ssDNA configuration, these data demonstrate that our PA system is mechanistically distinct from and exhibits clear advantages over PAINT and other related approaches.

1. To demonstrate the advantages and novelty of prime assembly, it should be benchmarked in parallel against existing similar strategies, such as HITI, PITCH, and PAINT etc. The parallel comparison should also include

other recently reported knock-in approaches, particularly those based on prime editing, such as the evolved PASSIGE (with eeBxb1) and evoCAST.

We appreciate this constructive suggestion. We have benchmarked PA against evoCAST and PAINT 3.0, and PA demonstrates much higher insertion efficiency than both systems (**Extended Data Fig. 6f**). Please also see Reviewer #1 comments for related benchmarking results.

Notably, PA requires fewer components than evoCAST and PAINT, highlighting its simplicity. Compared with the nuclease-based HITI approach, PA does not induce DSBs, reducing the risk of genomic deletions and translocations.

2. In Fig. 3, it is unclear why multiple donors were used, given that a single long donor should be sufficient for targeted insertion. The use of multiple PCR-derived donor fragments with short homologous regions is expected to activate not only homology-dependent repair pathways, such as MMEJ, but also the non-homologous end joining (NHEJ) pathway. Therefore, the authors should perform more sensitive assays, such as high-throughput sequencing (HTS) of the junction regions, to determine whether NHEJ-mediated indels are present among the outcomes of the multiple-donor prime assembly. Additionally, there is a possibility that donor 1 was directly joined to donor 3 via NHEJ. To gain deeper insight into the characteristics of multiple-donor prime assembly, the AZD (–) control group should also be included.

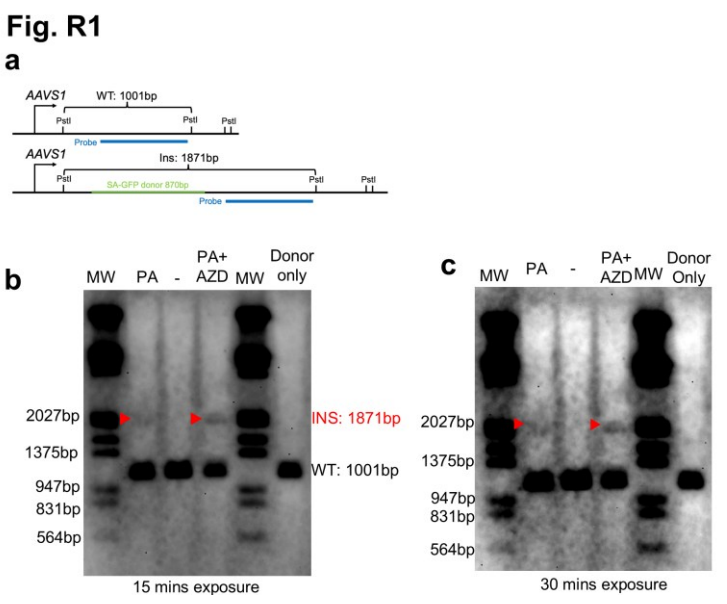
We thank the reviewer for this thoughtful comment and agree that a single long donor is often sufficient for targeted insertion. However, we also recognize that multiple donors offer distinct advantages in specific applications, such as delivery or combinatorial genomic studies. We have expanded the discussion on this topic, as also mentioned in our response to Reviewer #1.

Page 7: "Using multiple donors can overcome the synthesis and amplification limitations of long-range PCR. Although full-length donors are ideal for therapeutic delivery, in-cell assembly of multiple fragments is advantageous for functional genomic studies requiring combinatorial alleles—for example, five variants of donor 1 combined with five variants of donor 2 can generate 25 (5 x 5) insertion alleles in one experiment.

For future in vivo applications, split donors may also facilitate delivery of large cargos using multiple viral or non-viral vectors, thereby bypassing vector size constraints such as the AAV capacity limit (4.7kb only)."

In addition, we have performed high-throughput sequencing (HTS) of the donor junctions, which revealed a small proportion of NHEJ-mediated events (**Fig. 3d**). In the AZD (–) control group, 6.5% of reads contained indels, whereas AZD-7648 treatment reduced indels to 2%, indicating that NHEJ contributes minimally to multi-donor assembly and that NHEJ inhibition further enhances junctional precision.

3. For the detection of targeted integration, only PCR-based methods were employed, which are indirect and insufficient to provide conclusive evidence. PCR can only detect the presence of mosaic sequences containing genome and donor derived sequences. In addition to target integration of donor into the genome, there is also a possibility that genomic sequences flanking target site were integrated into the donor via endogenous DNA repaired mechanisms. Therefore, additional methodologies, such as Southern blotting or fluorescence in situ hybridization, would offer more direct and reliable detection of donor sequence integration at the intended genomic locus.



Reviewer Fig. R1. Southern blot detects PA insertion. (a) Restriction map before (top) and after (bottom) PA insertion. **(b-c)** Southern blot with 15 min. **(b)** and 30 min. **(c)** exposure. Red arrowheads denote expected on-target insertion bands. **"-"** denotes no-transfection control.

We previously reported a PE-tag pipeline to profile genome-wide specificity of PE[1]. To address this important concern, we performed a modified PE-tag analysis to evaluate the genome-wide specificity of PA (**Figs. 1g and 2f, Extended Data Fig. 5e**). PA shows overall high specificity at genome-wide scale using any donor DNA format, which is consistent with previous reports of prime editing–based technologies.

Furthermore, Southern blot analysis revealed a specific insertion band in the PA-treated samples (**Reviewer Fig. R1**), not present in either the no-transfection control or donor-only groups. Taken together, these results demonstrate that PA mediates highly specific integration of donor DNA at the intended genomic locus.

1. Liang SQ, Liu P, Ponnienselvan K, Suresh S, Chen Z, Kramme C, Chatterjee P, Zhu LJ, Sontheimer EJ, Xue W, Wolfe SA (2023) Genome-wide profiling of prime editor off-target sites in vitro and in vivo using PE-tag. *Nat Methods* **20**, 898-907. PMID: 37156841.

We thank the reviewers for their positive evaluations and thoughtful, constructive comments, which have substantially improved the quality of our manuscript. In this revision, we have performed additional experiments to address the concerns. Specifically:

- We provided the absolute efficiencies of Prime Assembly and increased the numbers of biological replicates.
- We systematically assessed overlap parameters and provided guidelines for split-donor PA.
- We quantified the effects of additional cell-cycle inhibitors on PA editing efficiency.
- We performed additional Southern blotting to address Reviewer 3's comments.

Detailed replies to each reviewer are provided below in blue font.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have further improved their study and my previous comments have been fully addressed. I recommend to accept this revised manuscript.

We are grateful to the reviewer for the positive assessment.

Referee #2 (Remarks to the Author):

The authors successfully addressed many of my comments. In particular, the genome-wide off-target evaluation result is great. It substantially strengthens the manuscript. However, the manuscript remains preliminary in some aspects described below.
We are grateful to the reviewer for the positive assessment of our results.

Major comments
1. In their new data, the authors provide only relative prime assembly efficiency (e.g., Extended Fig. 1d, 1e, and 7b). Providing absolute prime assembly efficiency would be more informative for readers. Related to this issue, I would recommend providing absolute prime assembly efficiency for Fig. 2b as well.
We thank the reviewer for this constructive suggestion. We have provided the absolute efficiencies of Prime Assembly in revised **Fig. 2b**, **Extended Data Fig. 1d, 1e and 7b**.

2. Based on my comment, the authors now provide quantitative data for only some of the factors shown in Extended Data Fig. 4a. Quantitative data are still lacking for factors such as Mirin, NU7026, olaparib, and ART558.
We quantified the effects of additional inhibitors. The new data are shown in **Extended Data Fig. 4b**.

3. Regarding my comment "The use of split donors is excellent. Can the authors define the optimal conditions (e.g. lengths, GC content, melting temperatures, etc.) for the overlapping sequences?", the authors have not provided a solid guideline to design overlapping sequences due to their limited number of tested parameters. In addition to testing many more data points for each parameter, I would recommend changing one parameter at a time while fixing other parameters so that the authors can determine the effect of that parameter (except for melting temperature, which is determined by the GC content and lengths). The authors should provide reliable guidelines (based on systematic single-parameter optimization) so that readers can effectively use the prime assembly.

We appreciate this insightful comment. Following the reviewer's advice, we conducted additional single-parameter optimization and added the results in **Extended Data Fig. 7a**.

*Page 5: "We fixed the overlap length at 30 bp and varied the GC content. The 50% GC content mediated the highest PA insertion (**Extended Data Fig. 7a**). Next, we fixed the GC content at 50% and varied the overlap length from 8bp to 100bp. We observed that the 30bp and 100bp overlaps showed higher PA efficiency than 8 or 16bp (**Extended Data Fig. 7a**). We recommend starting with an overlap length of 30–100 bp and a GC content of 50%."*

4. Regarding my comment, "Regarding the conclusion 'Consistent with results from two-segment PA, insertion efficiency improved upon AZD-7648 treatment (Fig. 3f),' please provide quantitative data. In addition, how many independent experiments were conducted?", the authors now provide quantitative data. However, the number of replicates is only two, and it is not clear whether the difference is statistically significant. In its current form, the difference appears unlikely to reach statistical significance, particularly for the PA-3 donor.
We provided *n* = 3-5 biological repeats for **Extended Data Fig. 7b**.

5. The authors provide statistical analysis for only some of the newly added data. The authors should provide statistical analysis for all of the quantitative data.
Following the reviewer's advice, we provided statistical analysis throughout the figures.

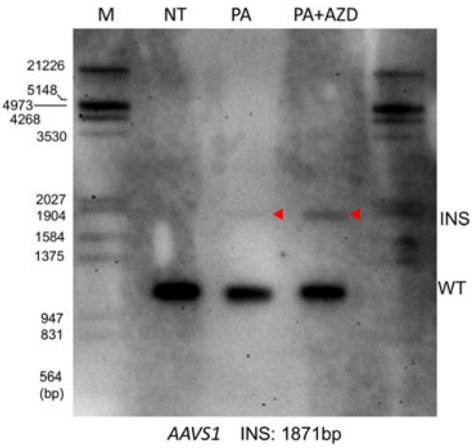
6. The authors also now provide Southern blotting data as reviewer-only information. The authors should include it in the manuscript.
We added Southern blotting data in **Extended Data Fig. 9**. Thank you.

Minor comments
1. The authors show the number of replicates "n" for the new data as well as the initially included data. However, what does "n" represent? Does it mean the number of independent transfections or the number of independent sequencing runs? This should be clarified in all figure legends.
We clarified that *n* represents independent transfections.
Figure legends: "Data and error bars indicate the mean and s.d. of independent biological replicates."

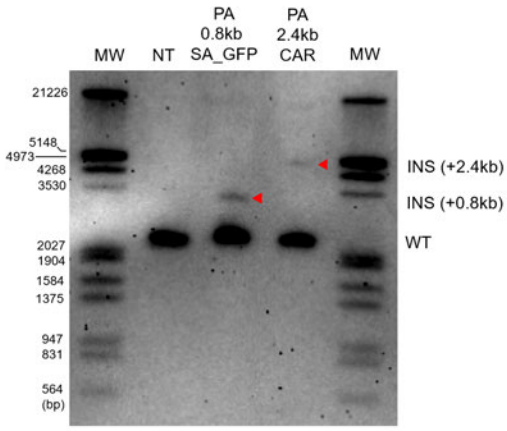
Referee #3 (Remarks to the Author):
I appreciate the authors' efforts to address my previous concerns. I am pleased to see that the rebuttal now includes Southern blot evidence for the targeted knock-in, as this method provides more direct and definitive validation than conventional PCR or ddPCR.

However, several issues remain unresolved. First, the positive band in the PA+AZD lane—although visually clearer than that in the PA-only lane—appears substantially weaker than would be expected based on the ddPCR quantification shown in Fig. 2. Second, the observed band size (approximately 2027 bp) is noticeably larger than the predicted size of 1871 bp. These discrepancies raise concerns about both the authenticity and the frequency of the reported knock-in events. At present, the Southern blot results do not convincingly support the ddPCR findings. I recommend that the authors conduct additional analyses to clarify these inconsistencies and to more rigorously confirm the presence and abundance of the targeted knock-in.

[Redaction]
Figure Includes
Third Party Material



Extended Data Fig. 9a. Southern blot at the *AAVS1* locus.



Extended Data Fig. 9c. Southern blot at the *TRAC* locus.

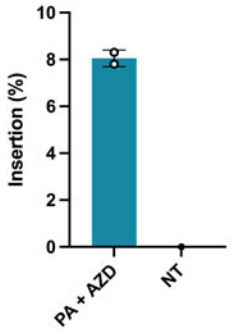
We thank the reviewer for the very constructive suggestions.

We used DNA Molecular Weight Marker III (Roche cat#:11218603910) (**Reviewer Fig. R1**). The two ladders at 2027bp and 1904bp did not separate well on the gel so they appeared as a thick band. We repeated the Southern blot with extended gel run time. The insertion band is slightly smaller than the 1904bp ladder (**Extended Data Fig. 9a**).

We performed additional Southern blot at the *TRAC* locus and detected the expected insertion bands for the 0.8kb and 2.4kb donors. We added the new data as **Extended Data Fig. 9c**.

These data resolve the size discrepancies noted by the reviewer and confirm the presence of the targeted knock-in. As for the abundance, however, although Southern blotting is only a semi-quantitative readout, band intensities are indeed somewhat weaker than expected given the ddPCR quantitations in **Fig. 1d** and **Fig. 4f**. However, comparing the two readouts at the quantitative level could be problematic, for at least two reasons:

1. To obtain sufficient genomic DNA for Southern blotting, the transfection-based editing had to be done at substantially larger scale: for most of our ddPCR experiments, 0.8 x 10^5 cells per well were transfected in a 12-well plate with individually-prepared reagents, whereas in the Southern experiment, we made a large transfection master mix for 36 wells per group. These distinct experimental conditions render direct quantitative comparisons with the earlier results difficult, as transfection efficiencies, editing efficiencies, or both may be lower at the larger scale. Indeed, ddPCR quantitation of gDNA from the Southern experiment indicates that the average insertion rate for +AZD was 8.1% (**Extended Data Fig. 9b**).



Extended Data Fig. 9b. ddPCR of the Southern blot DNA samples. PA+AZD: *AAVS1* +AZD**.** NT: no treatment.

2. Larger DNA fragments (insertion bands) can transfer less efficiently to membranes, leading to weaker signals compared to the wildtype band (Current Protocols,1987; Sapkota, 2021).

To reduce our reliance on ddPCR, we also quantified insertion of ssDNA donors by deep sequencing to provide an orthogonal readout (**Extended Data Fig. 5d)**. Furthermore, we note that PA efficiencies were measured by ddPCR in multiple experiments (each with 3-5 replicates) (**Figs. 1d, EDF.1d, 1e, and 6f**). We moved **Extended Data Fig. 6f** to **Fig. 1d** to better represent the average insertion rate by ddPCR and to provide direct comparison between PA with PAINT and evoCAST.

References:

Analysis of DNA sequences by blotting and hybridization. Current Protocols (1987).

Sapkota, Anupama "Southern Blot- Definition, Principle, Steps, Results, Applications". Microbe Notes. (2021).

We thank the reviewers for their positive evaluations and thoughtful, constructive comments, which have substantially improved the quality of our manuscript. In this revision, we performed additional experiments to address the remaining concerns. Specifically:

- We assessed additional overlap parameters and provided refined guidelines for split-donor PA (***Extended Data Fig. 7a-d***).

Detailed replies to each reviewer are provided below in blue font.

Referee #2:

The authors have satisfactorily addressed most of my previous comments, including the provision of absolute efficiencies, additional statistical analyses, inclusion of Southern blot data in the manuscript, and clarification of replicate definitions. However, I have remaining concerns regarding the optimization of PA and the discussion of inhibitor results.

Major

1. I appreciate the authors' efforts to conduct optimization experiments for the split-donor overlap sequences, as I had requested. However, I remain concerned that the current dataset is insufficient to provide the "reliable guidelines based on systematic optimization" that I had specifically asked for in the previous round. I had explicitly requested "many more data points for each parameter," yet the authors still tested an insufficient number of data points for each parameter. Only four data points (8, 16, 30, and 100 bp) were tested, with a 70-bp gap between 30 and 100 bp. No intermediate lengths (e.g., 45, 60, 75 bp) were evaluated, making it impossible to determine whether 30 bp is genuinely optimal or whether a longer overlap would perform better. The recommendation of "30–100 bp" is based on the absence of data rather than evidence of a performance plateau. In addition, testing at 20% intervals (10–90%) omits the 40% and 60% conditions flanking the reported optimum of 50%. Without these data points, the conclusion that 50% GC is optimal is not well substantiated. Moreover, recommending a single pin-point value of 50% GC is of limited practical utility, as the overlap sequence is constrained by the donor and genomic context, making it often difficult to achieve exactly 50% GC. What users need to know is the acceptable range of GC content, which the current data cannot define. In conclusion, a guideline of "30–100 bp overlap and 50% GC" is too broad and too pin-pointed, respectively, to be actionable. I would ask the authors to: (i) test intermediate overlap lengths (e.g., 45, 60, and 75 bp), and (ii) test 40% and 60% GC, with sufficient replicates for statistical power to provide more evidence-based practical guidelines.

We thank the reviewer for the very constructive suggestions. We repeated experiments with additional parameters and biological replicates to more precisely define optimal split-donor PA guidelines.

(i) We added new data with intermediate fragment lengths between 30 and 100 bp. Because the split donors were produced via PCR amplification of a plasmid template, it was not feasible to generate precisely 60 or 75 bp overlaps while maintaining a consistent GC content. Therefore, we used 45, 61, and 76 bp overlaps, as these parameters are as close as possible to those suggested by the reviewer. Please note that the exact length of the 100bp group in the previous revision was 96bp, and this has now been corrected.

PA efficiency was slightly higher with 30, 45, and 61 bp overlaps than with 16 or 76 bp overlaps (**Extended Data Fig. 7b**). A 96 bp overlap provides efficiency comparable to 30–61 bp overlaps but requires a larger DNA cargo. An overlap length of ~30 bp represents a practical starting point, as it supports robust PA editing while minimizing the required DNA cargo length.

(ii) We tested 40% and 60% GC content. The data indicate that 40-60% GC content mediates higher PA insertion efficiency compared to the other conditions tested (**Extended Data Fig. 7a**), thereby supporting a broader and more flexible GC range than the previously noted 50%.

We also include nanopore sequencing analyses of editing products for these datasets, demonstrating that insertion accuracy does not change significantly across all tested G/C and length variants. These new experiments, as suggested by the reviewer, have indeed helped refine the guidelines. We revised the text as following:

Page 5: *"We fixed the overlap length at 30 bp and varied the GC content. The 40-60% GC content mediates higher PA insertion compared to the other conditions tested (**Extended Data Fig. 7a**). Next, we fixed the GC content at 50-55% and varied the overlap length from 8bp to 96bp. PA efficiency was slightly higher with 30, 45, and 61 bp overlaps than with 16 or 76 bp overlaps (**Extended Data Fig. 7b**). A 96 bp overlap provides efficiency comparable to 30–61 bp overlaps but requires a larger DNA cargo. Nanopore data revealed a slight reduction in insertion accuracy with 8-16bp overlap (relative to the longer lengths) or with 70% GC content, and accuracies were comparable across all other conditions tested (**Extended Data Fig. 7c-d**). For designing split PA donors, we recommend an overlap GC content of ~40-60% to maximize efficiency. An overlap length of ~30 bp represents a practical starting point, as it supports robust PA editing while minimizing the required DNA cargo length."*

Minor

1. Ext. Data Fig. 4b now shows that Mirin (MRN complex inhibitor) has a statistically significant effect on PA efficiency, yet this is not discussed in the main text.

We discussed these results in the revised text. Thank you.

Page 4: *"Among these inhibitors, the DNA-PK inhibitor AZD-7648 increased the insertion efficiency (**Fig. 2a, b**), whereas Mirin decreased PA efficiency (**Extended Data Fig. 4b**)."*

Referee #3:

The revised manuscript has addressed my concerns regarding the existence and efficiency of the prime assembly. I have no further comments and believe the revised manuscript meets the requirements for publication.

We are grateful to the reviewer for the positive assessment of our revised manuscript.