

# Supplementary Text and Figures

## Availability of code and data

All code and configuration files used for the analyses in this manuscript are available at [https://github.com/valenlab/amplican\\_manuscript](https://github.com/valenlab/amplican_manuscript) where they can be used for independent verification. Data analysed is publicly available and accessible at their respective accession numbers. `amplican` is available from Bioconductor as the R package at <http://bioconductor.org/packages/amplican>. To obtain the newest development version visit <https://github.com/valenlab/amplican>.

## Supplementary Note 1: Automatic normalization using control reads

By default `amplican` normalizes through the strict removal of all indel events also found in the control sample above a threshold (default 1%). Due to the stochasticity in sequencing data this approach is better suited to handle more heterogeneous cases than the subtraction method where indel frequency is simply normalized by subtraction of the control indel frequency. In the latter case variation in the levels of indel frequencies in the control versus CRISPR treated samples can lead to partially normalized data. Both normalization methods are outlined in **Supplementary Fig. 18** while **Supplementary Fig. 19** shows examples of highly heterogeneous data where without normalization estimated mutation efficiency would be biased. Normalization becomes even more important when the exact nature of the indel event matters, for instance whether it induces a frameshift or not (**Supplementary Fig. 1**).

All examples in **Supplementary Fig. 1** can be recreated with the use of “`make_comparison_normalized.Rmd`” in the `amplican_manuscript` repository.

## Supplementary Note 2: `amplican` utilizes optimized alignments

CRISPR genome editing events typically result in a single break at a single site and by extension produce a single deletion and/or insertion. Sequence read aligners are generally not optimized for this type of genome editing event which can lead to the aligner fragmenting the indels and creating multiple events (example in **Supplementary Fig. 2**). In the worst case fragmented alignments could shift the indel events resulting in a distortion of the mutation efficiency for those tools that only allow events within a certain distance from the expected site. A more likely outcome however, is the misinterpretation of the nature of the mutation.

Under certain assumptions the theoretically optimal alignment can be obtained by the Needleman-Wunsch algorithm. `amplican` uses this algorithm with optimized parameters to reflect the expectation that a CRISPR experiment should result in one deletion and/or insertion event, of unknown length (match = 5, mismatch = -4, gap opening = 25, gap extension = 0, no end gap penalties). With these parameters the Needleman-Wunsch algorithm performs well over a broad range of test cases (data not shown). `amplican` uses these optimized parameters by default, but also allows for supervision of the alignments through human readable output of individual alignment results (**Supplementary Table 2**).

## Supplementary Note 3: Tools differ in estimating editing efficiency on real data.

We assessed how tools estimate editing efficiency on 263 real CRISPR experiments, of which 151 were previously published by us<sup>1</sup> (datasets from run 1 and run 5 available at BioProject under accession number PRJNA245510), and 112 novel experiments from 5 sets for this study

(datasets from run 6-10 available at ArrayExpress: E-MTAB-6310, E-MTAB-6355, E-MTAB-6356, E-MTAB-6357, E-MTAB-6358). All experiments were conducted by injection into 1 cell zebrafish embryos and sequenced 2 days post-fertilization<sup>1</sup>. Due to the rapid cell division and development these experiments are likely to result in highly heterogeneous mutational efficiencies due to mosaicism. For these experiments the true mutation efficiency is not known and we can therefore not assess how precise the tools are in their estimates. Instead, we quantified how much the tools differ in their estimates (**Supplementary Fig. 3A**) and, to qualitatively assess the underlying reason for their discrepancy, we plotted the estimated mutation efficiency values of the tools relative to the non-normalized ampliCan result (**Supplementary Fig. 3B**). This showed that discrepancies likely originate from different causes. Some, those above the normalized ampliCan estimate, likely stem from a failure to consider control experiments. In our data the experiments impacted by controls is about 5%, but this will depend heavily on the reference genome, heterogeneity of the region and organism under study. Specific examples of the importance of normalization are shown in **Supplementary Fig. 1**. The discrepancies of the other experiments are due to the steps in the processing pipeline, e.g. off-target detection, primer dimer filtering, alignment strategy and read merging. To investigate the specific sources of these discrepancies and quantitatively assess the performance of the tools we created several synthetic benchmark datasets.

#### **Supplementary Note 4: Synthetic data set evaluation**

The latest available versions was used for all tools and packages. The assessment set from Lindsay et al.<sup>2</sup> paper (Synthetic Dataset 2, Supplementary 4) was replicated with the same settings and seed values as described (**Supplementary Fig. 4**). The script from Lindsay et al. was used for parsing, but a bug in the code was fixed for the CRISPResso output. In Lindsay et al. only NHEJ estimation of mutation efficiency was considered for CRISPResso, skipping HDR and “mixed” mutation frequencies. However, fixing this error did not influence CRISPResso’s overall performance in any significant way. Versions of tools, scripts and details needed for replication are available in the [https://github.com/valenlab/amplican\\_manuscript](https://github.com/valenlab/amplican_manuscript) repository. ampliCan used the same amplicon sequences as CRISPResso.

It should be noted that Synthetic Dataset 2 from Lindsay et al. (used for **Supplementary Fig. 4**) is not a good approximation of a real life situation. First, the sequence matching the primers can not be very divergent as they would then fail to amplify. Second, several experiments are badly designed in that the target sites are very close to the sequencing end of the reads. This makes it difficult to correctly call indels with support from both paired reads. Third, paired-end sequencing of 200bp or longer is somewhat expensive and error-prone and most labs would seek to restrict this to shorter reads. To account for this we created an additional set, Synthetic Dataset 3, in a similar fashion to Synthetic Dataset 2, but with the following minor modifications. First, the length of amplicons and reads (150 bp) were adjusted. Second, gRNA target sites were designed to be covered by both reads. Third, PCR off-target reads were created without mutating the primer sequences. Finally, mutation efficiency was tested across a range of mismatch rates, 10%, 20% and 30% (**Fig. 2, Supplementary Fig. 5**), to reflect different levels of similarity to the contaminant reads.

For Synthetic Dataset 2 ampliCan matches the perfect score of CrispRVariants and AmpliconDivider. However, on Synthetic Dataset Dataset 3 ampliCan is more consistent at

estimating the known mutation efficiencies within the dataset (**Fig. 2**). AmpliconDIVider has no filtering step and is confused by the contaminating reads. CrispRVariants has a filtering step, but is unable to discern divergent off-target sequences (e.g. homologous regions) that are still able to align to the correct target site. As in the benchmark from Lindsay et al. CRISPResso performs poorly on all benchmarks. When increasing mismatch rate (from 10% of all bases to 20% and 30%), AmpliconDIVider and CrispRVariants get closer to the correct estimated indel rate, but in all cases ampliCan obtains the highest precision and shows the most robust performance (**Fig. 2, Supplementary Fig. 5**).

### **Supplementary Note 5: ampliCan is able to correctly call longer indels**

We have found that even without targeted insertion CRISPR mutagenesis can frequently result in some proportion of longer indels (**Supplementary Fig. 20**). In particular, we have observed unintended insertions from lentiviral vectors used to introduce the guides and Cas9 (**Supplementary Fig. 21**)<sup>3</sup>.

Current tools primarily rely on either global mapping (CrispRVariants, AmpliconDIVider) (**Supplementary Tab 1**) that can have problems identifying the correct loci in the presence of a larger insertion or have certain processing steps that are incompatible with longer events (see below for CrispRVariants). This mitigates primer dimer contamination problems (which can be identified by too large deletion gaps after alignments), but ignores bona fide large indels. These tools are therefore often unable to handle longer indels whether unintended or targeted. Long deletions are also a problem for some tools. For instance, CrispRVariants filters out any deletion that does not start or end within the gRNA complementary sequence plus a buffer of 5 bp. Any deletion spanning this region is ignored. This can be used as a strategy to filter primer-dimers, but also has the side-effect of ignoring any bona fide longer deletions. ampliCan uses a local alignment strategy that can detect these longer indels and a more realistic model of primer-dimer artifacts (**Supplementary Note 8**).

We noticed that in the Synthetic Dataset 2 from Lindsay et al. (CrispRVariants benchmark dataset) large indels (>10 bp) were disabled. To assess the capabilities of leading tools in handling longer indels we created Synthetic Dataset 4. We made three subsets: 1) with no indels > 10bp, 2) with a mix of indels by simply removing the line disabling longer reads in the Lindsay et al. script. 3) To check explicitly how tools handle experiments with planned shorter insertion of donor sequence we created a third scenario described as “insertions > 10bp” on the figure. 4) For completeness we also created a set of large deletions. ampliCan match the best competitors on the the set with no long indels and consistently outperform the other tools on the mixed set and the set only containing long indels (**Fig. 2, Supplementary Fig. 6**).

### **Supplementary Note 6: ampliCan consistently recovers the true HDR efficiency when faced with diverse donor templates**

ampliCan takes into account the donor template and the original genomic sequence to define the set of events that corresponds to a correct HDR editing experiment, but allowing for some background sequencing noise (currently 3 mismatches by default). This is unlike CRISPResso, the other CRISPR tool that can handle HDR events, which do not model events but simply align reads against donor and original sequence picking the best-scoring instance. The advantage of ampliCan’s approach is that it accounts for alignment imperfections in a more robust fashion, allowing for complex donor-amplicon relations and sequencing errors.

We designed a dataset for benchmarking the HDR calling capabilities of the most popular tools. Using the same loci as in **Supplementary Note 3** we tested 20 different donor templates for each of three kinds of donor types: with point mutations, insertions or deletions of variable length from 5bp to 70bp introduced into the amplicon sequences. We simulated 2000 reads with different levels of HDR efficiency rate (0, 33, 66, 90). In this benchmark set only ampliCan makes no errors (**Supplementary Fig. 7**).

### **Supplementary Note 7: Visualization and aggregation of the complete activity of gRNAs**

While the default alignment plot shows the most abundant reads across the expected cut site it doesn't provide an overview over all editing events. ampliCan therefore also produces multiple plots that aggregate and visualize editing events (**Supplementary Fig. 8**). Unlike the alignment plots these show the complete activity of the gRNA allowing for comparison of gRNAs by manual inspection. The pipeline in ampliCan treats forward and reverse reads separately which, after visualization, makes it possible to spot read-related problems immediately (**Supplementary Figs. 15, 16, 23**). In addition, ampliCan provides meta plots that aggregate information across groups of experiments allowing for visualization of deletions, mismatches or insertions across groups of gRNAs, amplicons or any other set. This can for instance show the combined activity of a single guide across multiple experiments.

ampliCan builds on top of ggbio<sup>4</sup> and ggplot2<sup>5</sup> packages and provides higher level functions that automatically group event data (eg. collapse on start and end of deletion) and plot results. Users can extend those plot objects and treat them like any other ggplot2 object. ampliCan supports multiple types of meta plots to facilitate comparison of not only the gRNAs, but also any group that a user wants e.g. barcode, amplicon, type of treatment (**Supplementary Figs. 11, 19, 22**).

### **Supplementary Note 8: Filtering of noise**

Multiple sources of noise can confound the estimation of cut rates, low quality reads, primer-dimers, PCR off-target amplification and sequencing artifacts. ampliCan has three filters to remove noise from different sources: 1) low quality reads, 2) primer-dimers, and 3) erroneously assigned reads and sequencing artifacts.

#### **1. Low quality reads**

ampliCan offers basic read quality overview with the use of ShortRead<sup>6</sup> package and filters for minimum base quality (default: 0) in a read, average base minimum quality (default avg min: 30) and the presence of ambiguous (N) letters.

#### **2. Primer-dimers**

Filtering primer-dimers is a balance between getting rid of erroneous reads and allowing for longer deletions. For instance, CrispRVariants ignores all alignments with deletions larger than 33 bp (the guide plus a buffer of 5 bp) and is frequently unable to map long insertions (**Fig. 2B, Supplementary Fig. 6**). This effectively removes all primer dimers, but also ignores any bona fide longer indels. ampliCan instead tries to estimate the likely length of a primer dimer deletion by taking the length of the amplicon, subtracting the primer lengths with a small buffer (30 bp) to arrive at maximally allowed deletion length. This results in a more realistic estimate of the

length of artificial deletions that would result from primer-dimers. As an example, for an amplicon of size 150 with primers of length 20, the maximum deletion length would be 80 bp ( $150 - (2 \times 20 + 30)$ ).

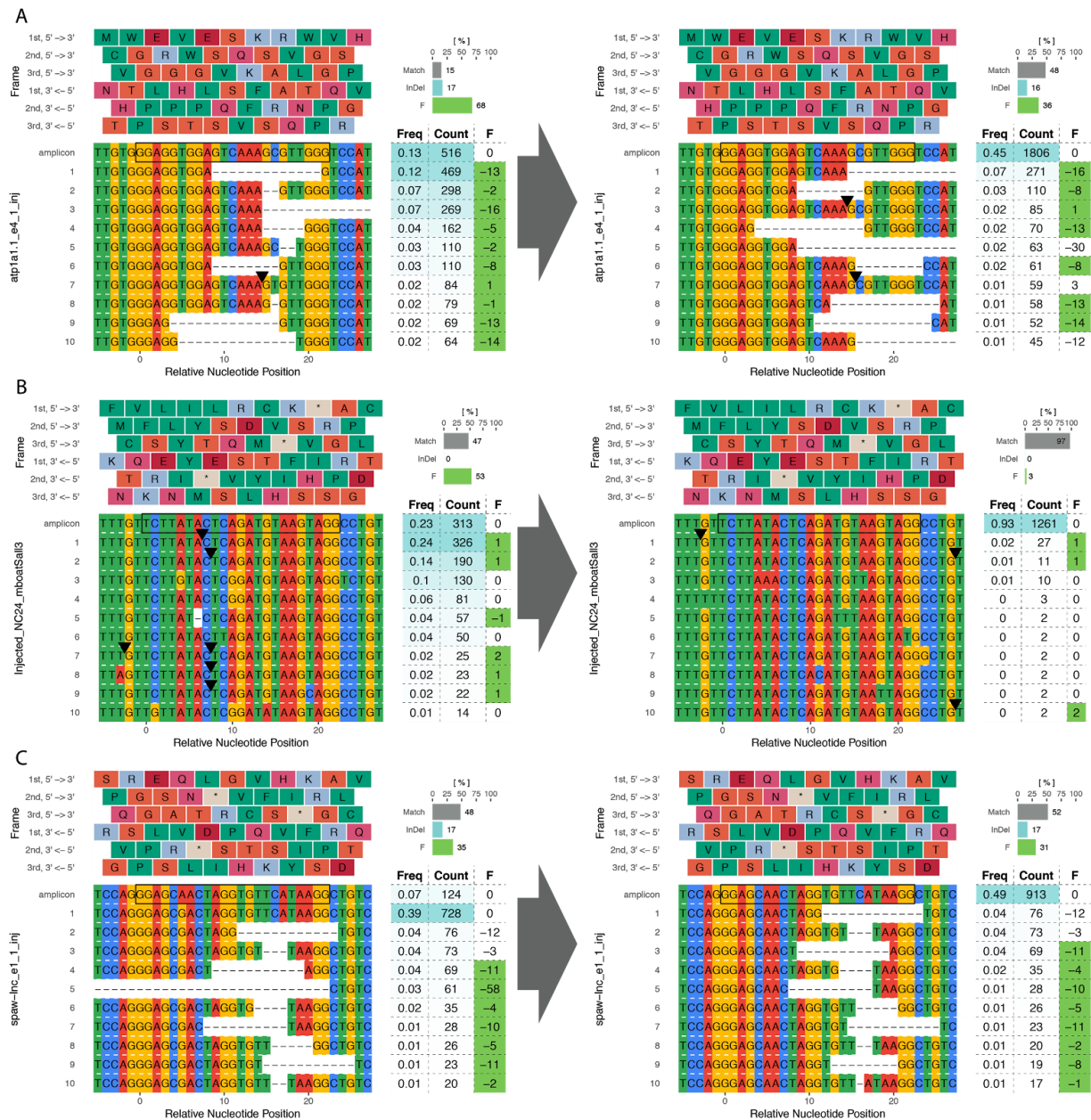
### 3. Erroneously assigned reads and sequencing artifacts

An assumption of ampliCan is that a CRISPR editing event will result in a low number of indel events resulting in a good alignment with few discrepancies to the reference sequence or (if available) the control experiment. To accomplish this ampliCan uses a two dimensional clustering method based on sequence alignment score and sequence alignment indel events to filter out erroneous reads and sequencing artifacts. This takes all alignments and performs k-means clustering with different 1-3 clusters. It then uses the silhouette criterion to determine the optimal number of clusters. In the case of 1 cluster, all reads are either edited or perfectly matching the reference/control. In the case of 2 you have both edited and unedited reads. In the case of 3 clusters, cluster with center that has the biggest number of events and lowest alignment score (in normalized relation on 0-1 scale) means that you in addition have a group of sequencing artifacts or reads that poorly align to the loci (example in **Supplementary Fig. 13**).

ampliCan provides plots that shows the impact of each of the filtering steps, across the whole library for read quality (**Supplementary Fig. 24**) and for each experiments for primer-dimer and assignment/artifacts issues (**Supplementary Fig. 10**).

### **Supplementary Note 9: Read assignment**

ampliCan assigns reads to the respective experiment by matching primers used in the amplification of the loci. These region should be immutable and match the reads since an indel spanning a primer would either result in failure to amplify the locus or be “corrected” by the primer when it amplifies the target site. However, since small sequencing and primer synthesis errors could potentially occur in the primer part ampliCan allows for up to 2 mismatches (user customizable) between the primers and reads. During this process it is possible that some reads will be unassigned and not match any of the experiments. While these reads are typically noise from the high-throughput nature of the sequencing experiment, they could in some cases be helpful in troubleshooting failed experiments. ampliCan therefore provides human readable alignments of the top 5 most abundant forward and reverse read pairs aligned to each other (**Supplementary Fig. 17**). In some cases these correspond to off-target PCR amplicons and close homologous regions as well as errors in the specification of the experiment.



**Supplementary Fig 1.** Examples of normalization with controls on 3 real experiments. In heterogeneous data it can be challenging to derive the true mutation profile. **A.** ampliCan automatically removes wild type mutations and reduce the number of frameshift inducing mutations from 68% to 36%. **B.** A large number of insertions could be mistaken for CRISPR activity reducing the total indel rate from 53 % to 3 %. **C.** A large fraction of reads (39%) carry a G instead of an A. ampliCan automatically adjusts reads in relation to the control to merge unedited reads and obtain a more accurate count.

```

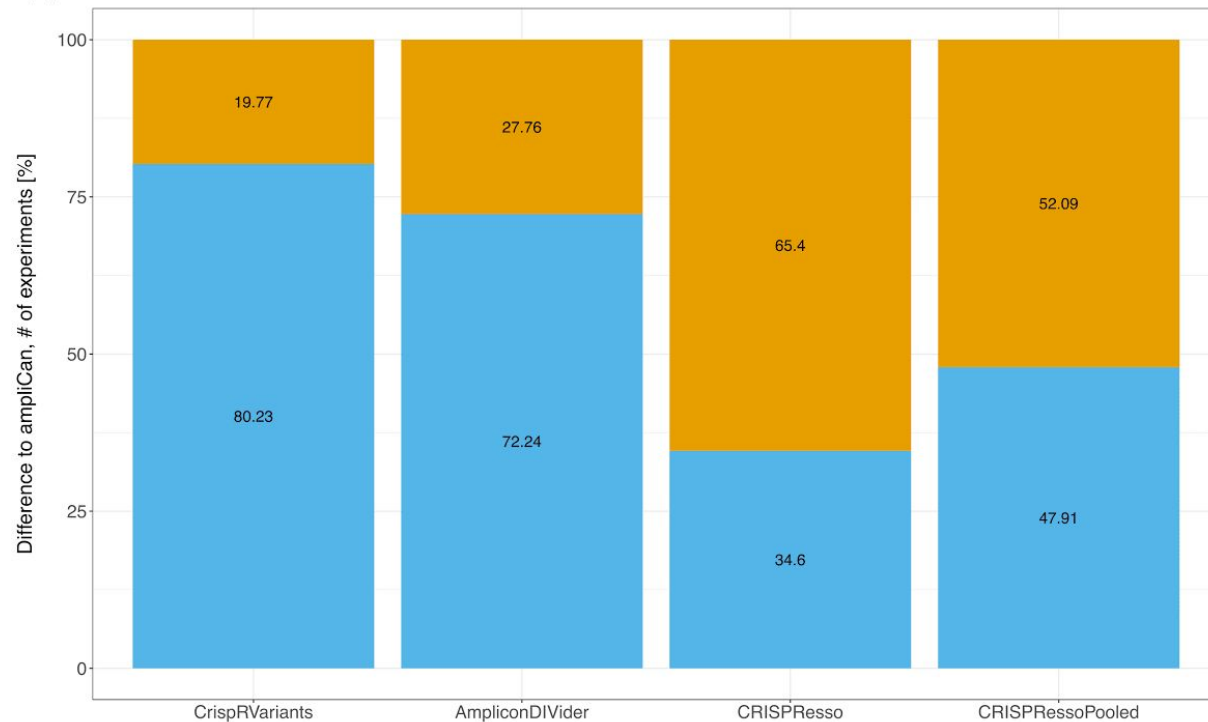
      G T G G T C A G G G G G C G T T T A T T C T G C C G G - - A C T - - - A T A C C C T
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      G T G G T C A G G G A A C - - - - - T G G T G G A G G T C A C T G G G A T A C C C T

      G T G G T C A G G G G G C G T T T A T T C T G C C G G A C T - - - - - - - - - - - - - - - A T A C C C T
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
      G T G G T C A G G G A - - - - - - - - - - - - - - - A C T G G T G G A G G T C A C T G G G A T A C C C T

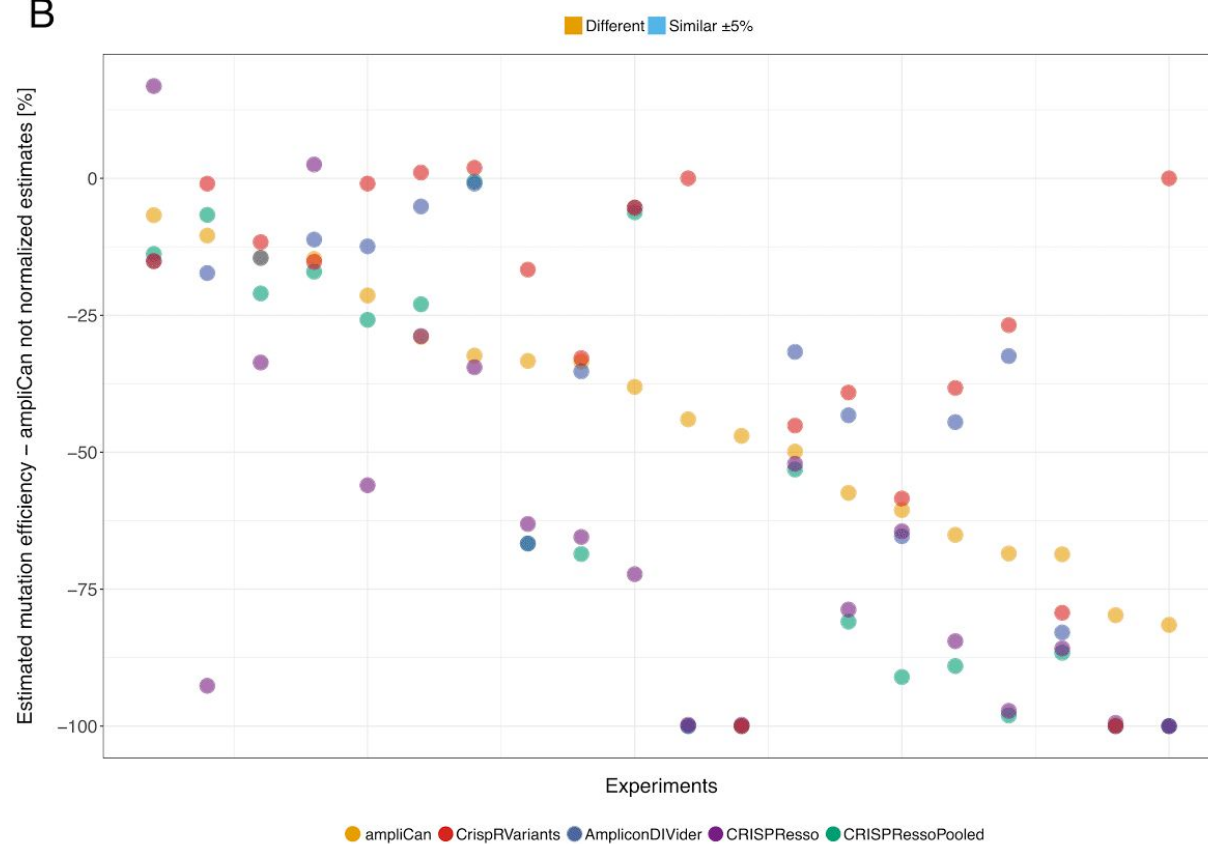
```

**Supplementary Fig 2.** Alignments dependent on multiple parameters and are often a tradeoff between the cost of gaps and the cost of mismatches. The top alignment shows a typical alignment for many aligners that are not optimized for genome editing (gap opening 10, gap extension 2, match 5, mismatch -4 and no end gap penalty). The bottom alignment, illustrates the result with ampliCan parameters (gap opening -25, gap extension 0, match 5, mismatch -4 and no end gap penalty) that are optimized for few events as is expected for CRISPR activity.

A

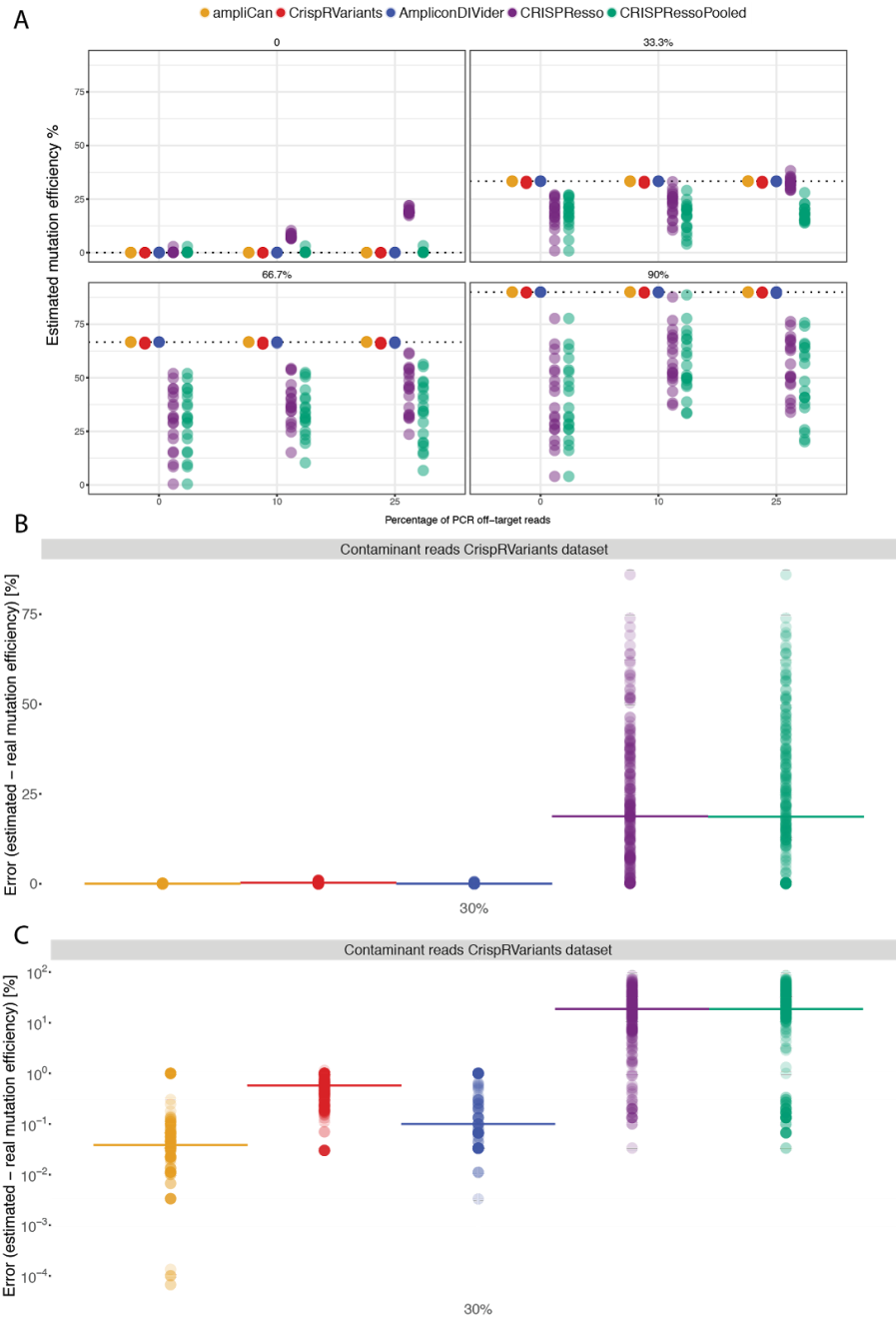


B



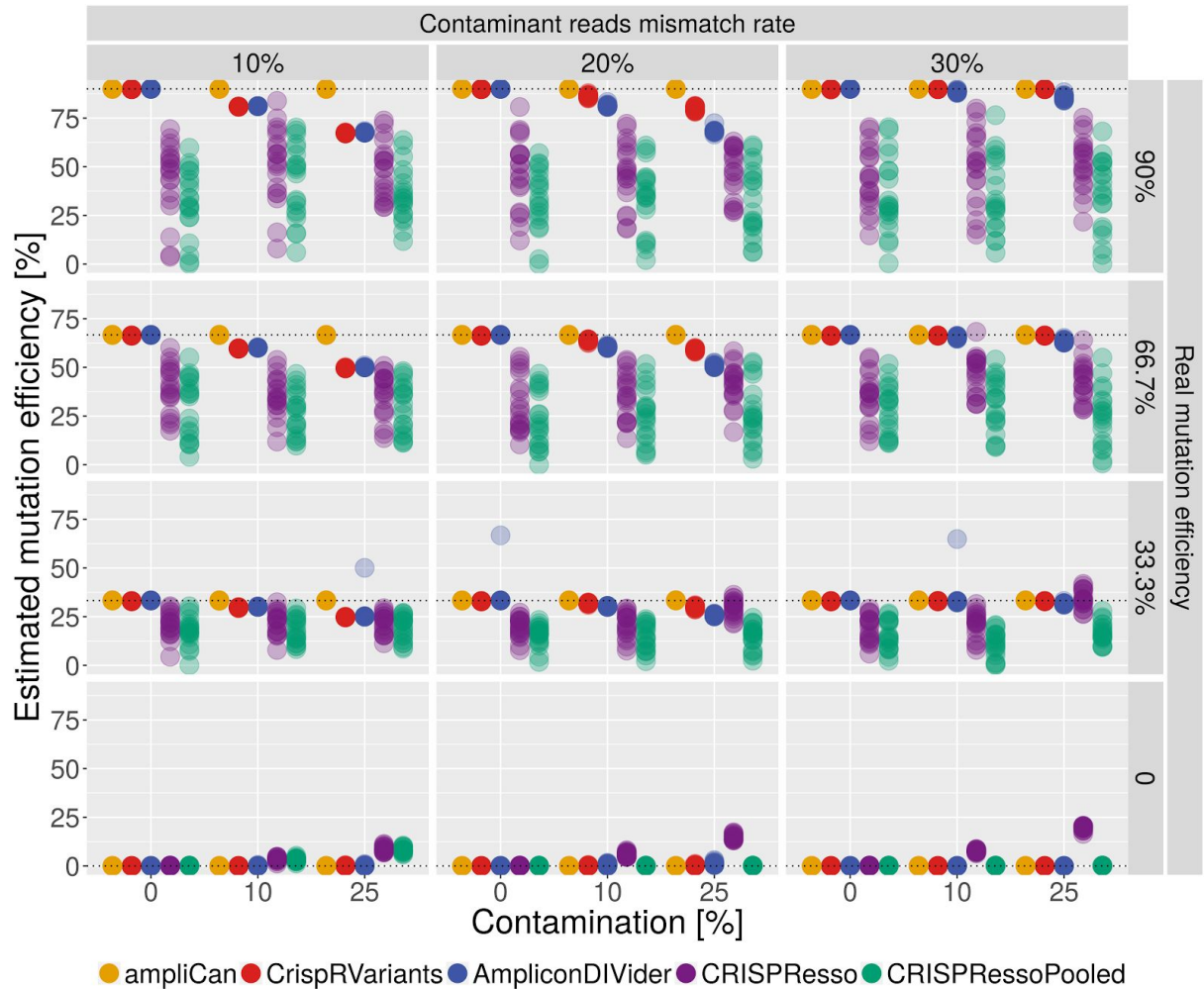


**Supplementary Fig 3.** Comparison of leading tools on real CRISPR experiments. **A.** Summary of differences between ampliCan and other tools. CrispRVariants reports similar editing efficiencies to ampliCan in ~80% out of 263 experiments. The remaining experiments are due to controls (~5%) and processing (~15%) **B.** Experiments (x axis, sorted) where estimated mutation efficiency differs by at least 5% from non-normalized data. y-axis shows differences in relation to non-normalized ampliCan estimates. Differences between tools predictions staying above line created by ampliCan prediction are likely to be due to lack of normalization, while the predictions below the ampliCan are likely due to the alignments, processing and filtering of data.

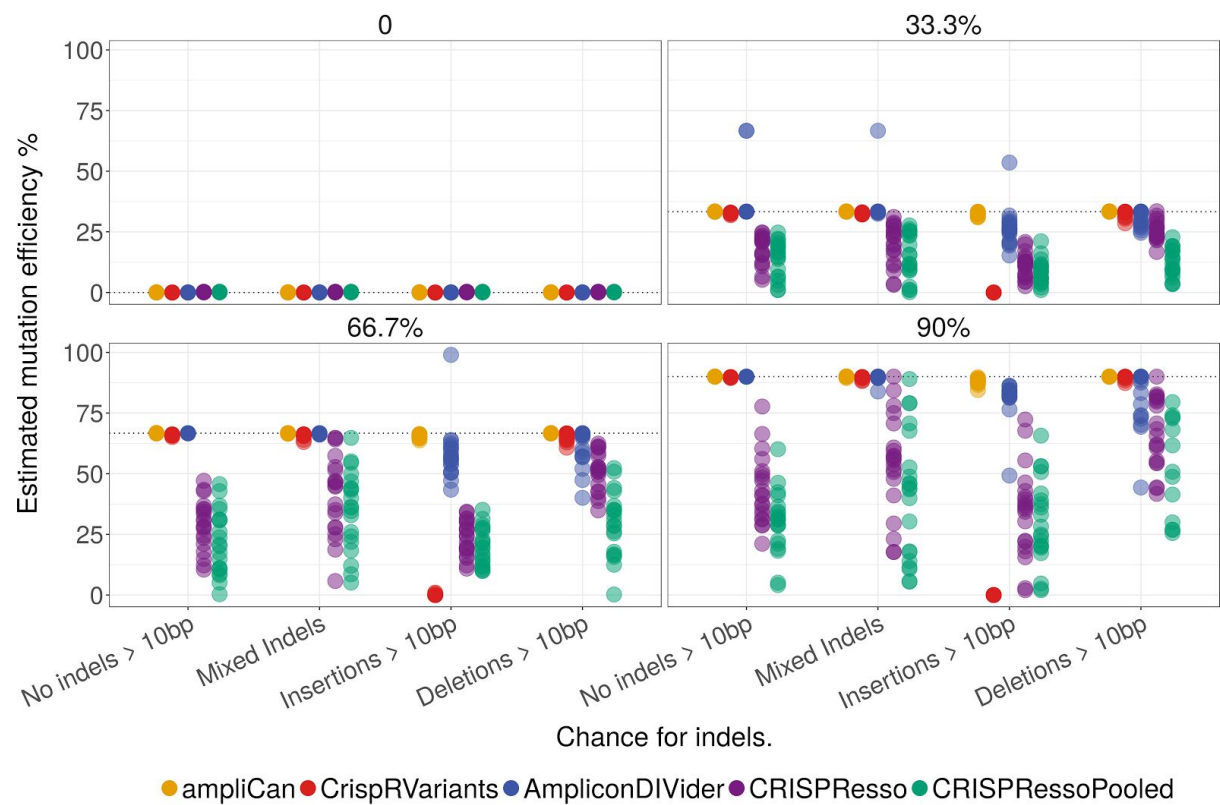


**Supplementary Fig 4. A.** Performance of leading tools when facing different mutation efficiencies and fractions of contaminating reads. These sets are obtained from the benchmark data in Lindsay et al. (CrispRVariants)<sup>2</sup>, Synthetic Dataset 2 (Supplementary Fig. 10 in Lindsay et al.<sup>2</sup>). Each dot in the plot correspond to the estimated mutation efficiency calculated by a single tool for a single experiment, while the dotted line shows the true mutation efficiency. The fraction of contaminant reads

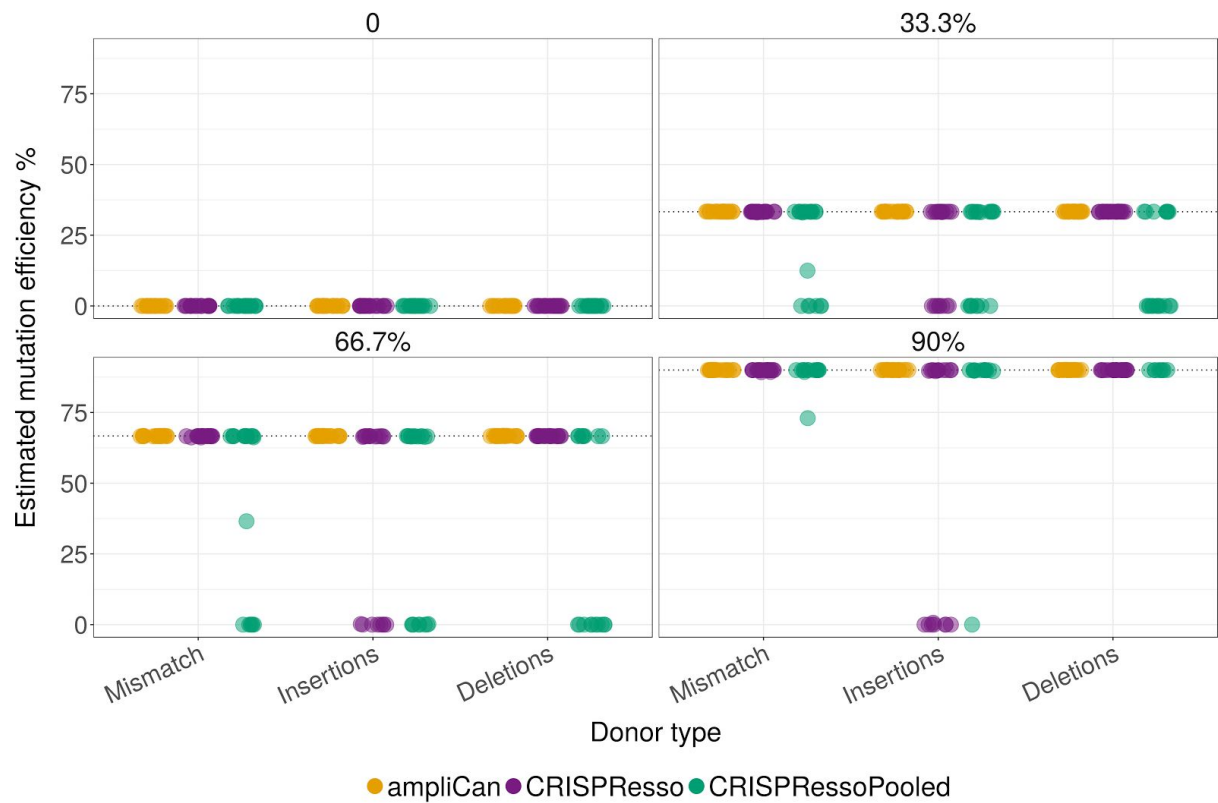
varies on the x-axis. **B.** Error rates (**C.** log10 scaled) of the same experiments. The median error is indicated by the horizontal line.



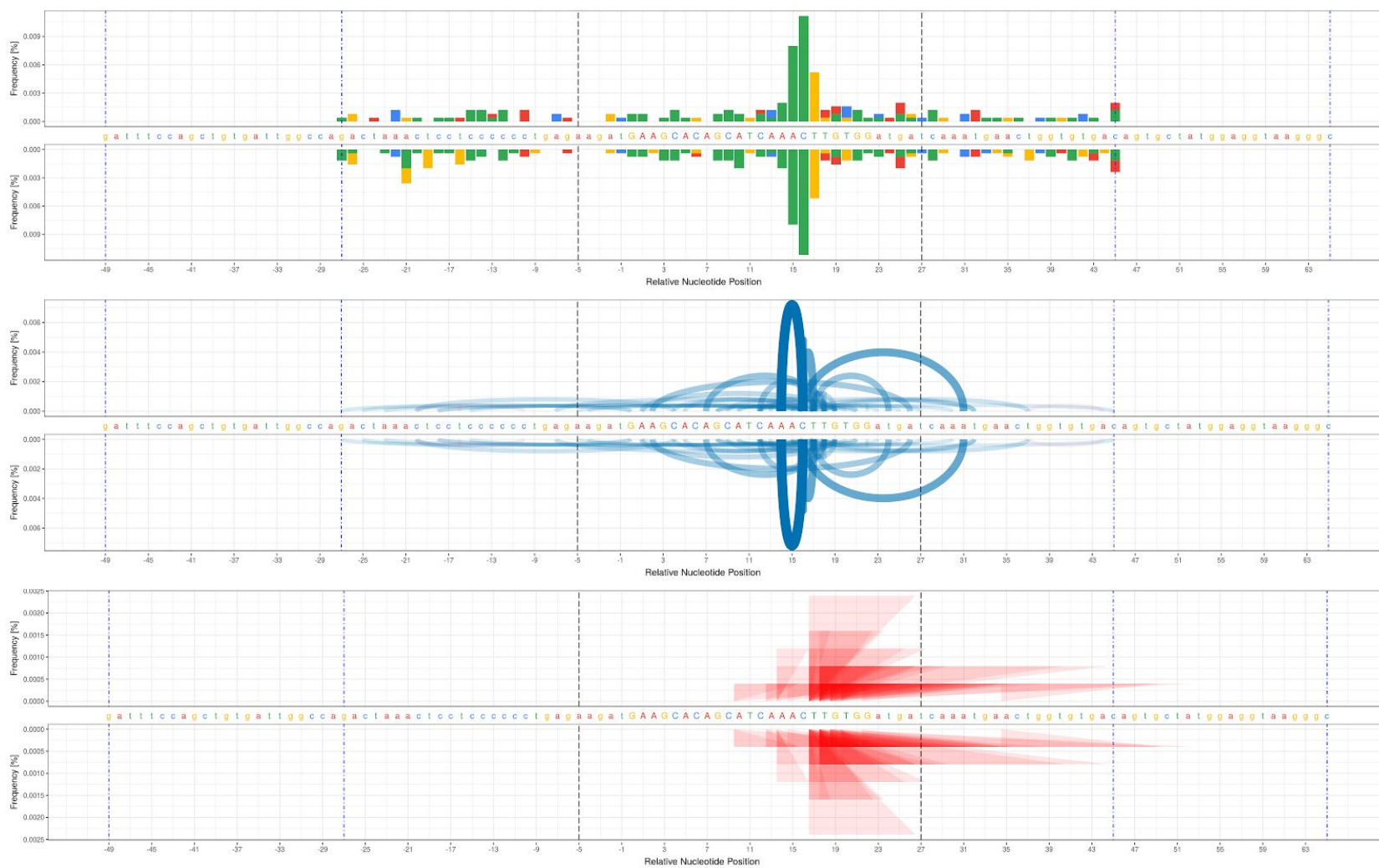
**Supplementary Fig 5.** The data from left panel of Fig. 2 split by the true mutation efficiency and with a baseline of 0% true mutation efficiency added for comparison. The dots shows estimated mutation efficiency by leading tools on reads with increasing contamination (as 0, 10 and 25 percentage of all reads) with different mismatch rate of the off-target reads (10%, 20%, 30%). Each point corresponds to one experiment. True mutation efficiency is indicated with dotted lines, and labelled to the right of the charts. Contamination is simulated by introducing random mismatches (Contaminant read mismatch rate) in reads mapping to the loci, similar to the benchmark in Lindsay et al.<sup>2</sup>. The mismatch rate is indicated at the top. Only ampliCan shows robustness to the whole range of different mismatch rates and mutational efficiencies.



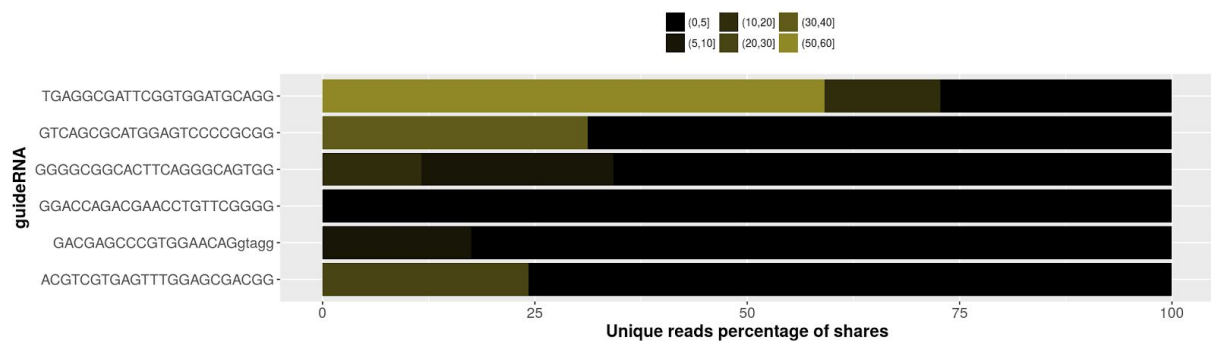
**Supplementary Fig 6.** Performance of leading tools grouped on simulated data with large indels. Some tools find larger indels challenging. ampliCan consistently returns values closest to the real mutation efficiency.



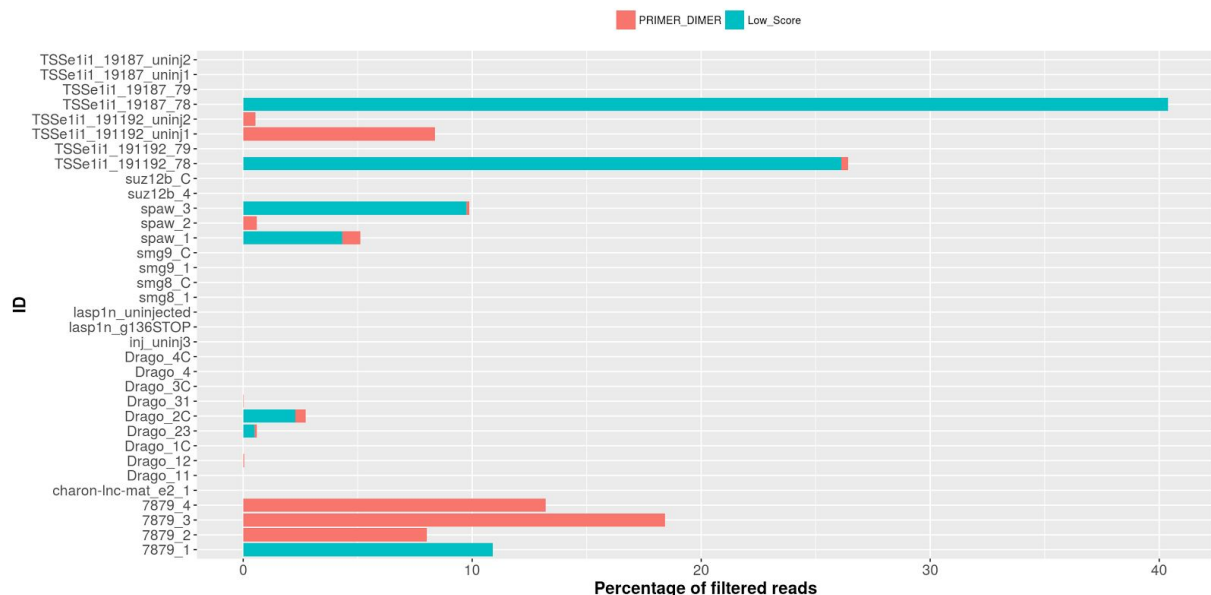
**Supplementary Fig 7.** Performance of ampliCan, CRISPResso and CRISPResso Pooled on simulated data with variable donor templates. Dotted line represents real HDR efficiency. Mismatch, insertions and deletions represent different donor types of variable length (20 donors from ranges 5-70bp). ampliCan manages to handle all kinds of donor templates. CRISPResso in situations where alignment is imperfect may have trouble with correct estimation. No noise of NHEJ was added in this simulation.



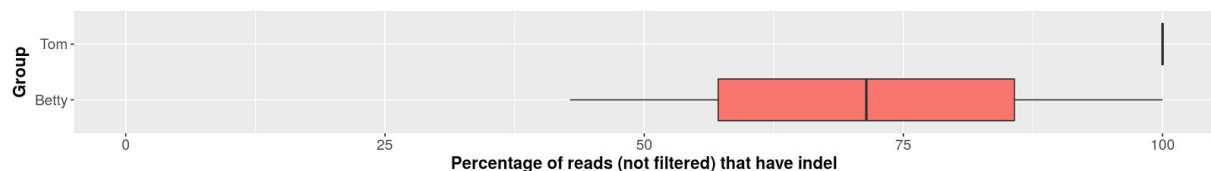
**Supplementary Fig 8.** A number of plots are produced by ampliCan to investigate editing events: mismatches to the reference (**top**), aggregated deletions (**middle**) and aggregated insertions (**bottom**). Dashed blue vertical lines indicate where the primers start and end. The black dashed lines mark the region around the cut site where we require a mutation event to overlap to be considered a CRISPR event. The top half of the plots shows the forward reads, while the bottom shows the reverse reads. The amplicon sequence is shown in the middle, with uppercase letters indicating the gRNA. The y axis shows the efficiency of the events summed over all reads. This allows for immediately discerning which bases are edited above the background noise from sequencing.



**Supplementary Fig 9.** Example of a heterogeneity plot produced by ampliCan. In this, identical reads are collapsed together and grouped by gRNA. A stronger shade of yellow indicates a large group of homogeneous reads. This plot can give insight into the heterogeneity of the outcome. A high level of heterogeneity can indicate sequencing problems or mosaicism. Reads can also be aggregated on experiments or any other user selected grouping rather than guides.



**Supplementary Fig 10.** Example of bar plot showing fraction of reads that were filtered out of the experiments. Red bars correspond to a primer-dimer filter, and blue bars indicate low quality reads.



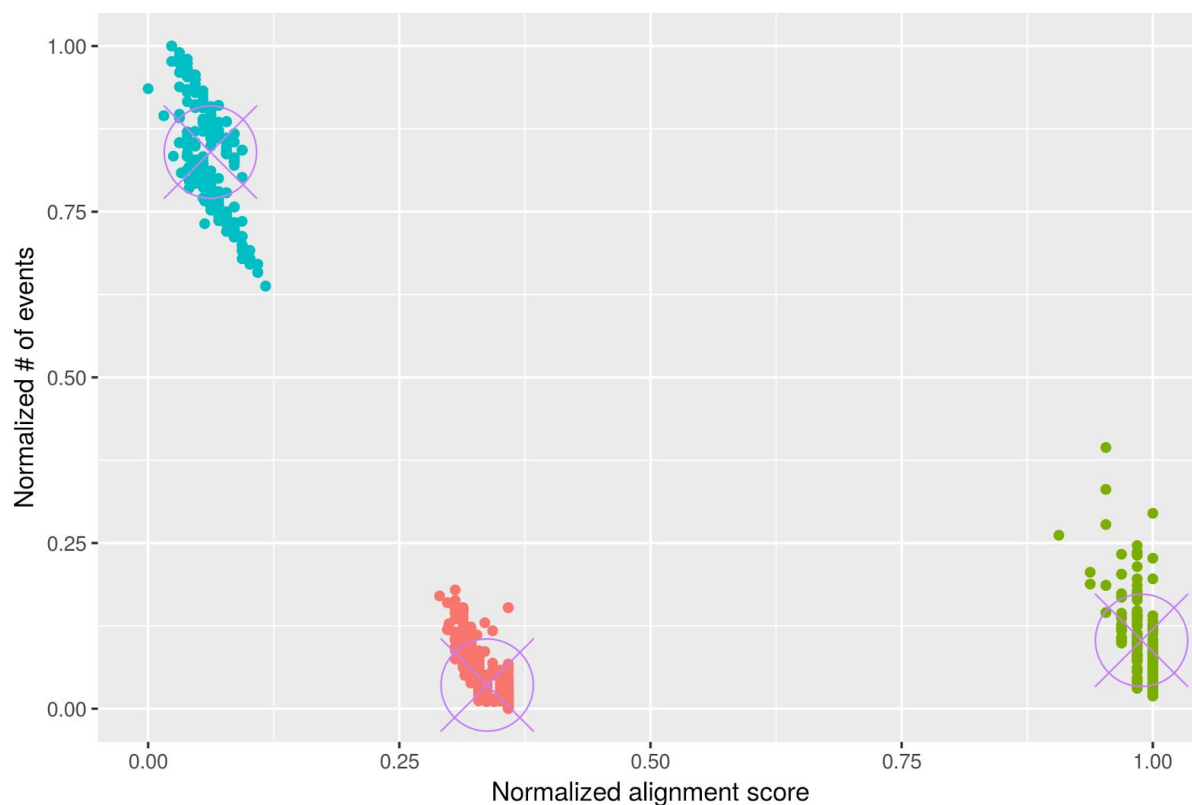
**Supplementary Fig 11.** Example of comparison plots in ampliCan test data where Indel rates are grouped by the researcher performing the experiments. These can be grouped on any user specified criteria.



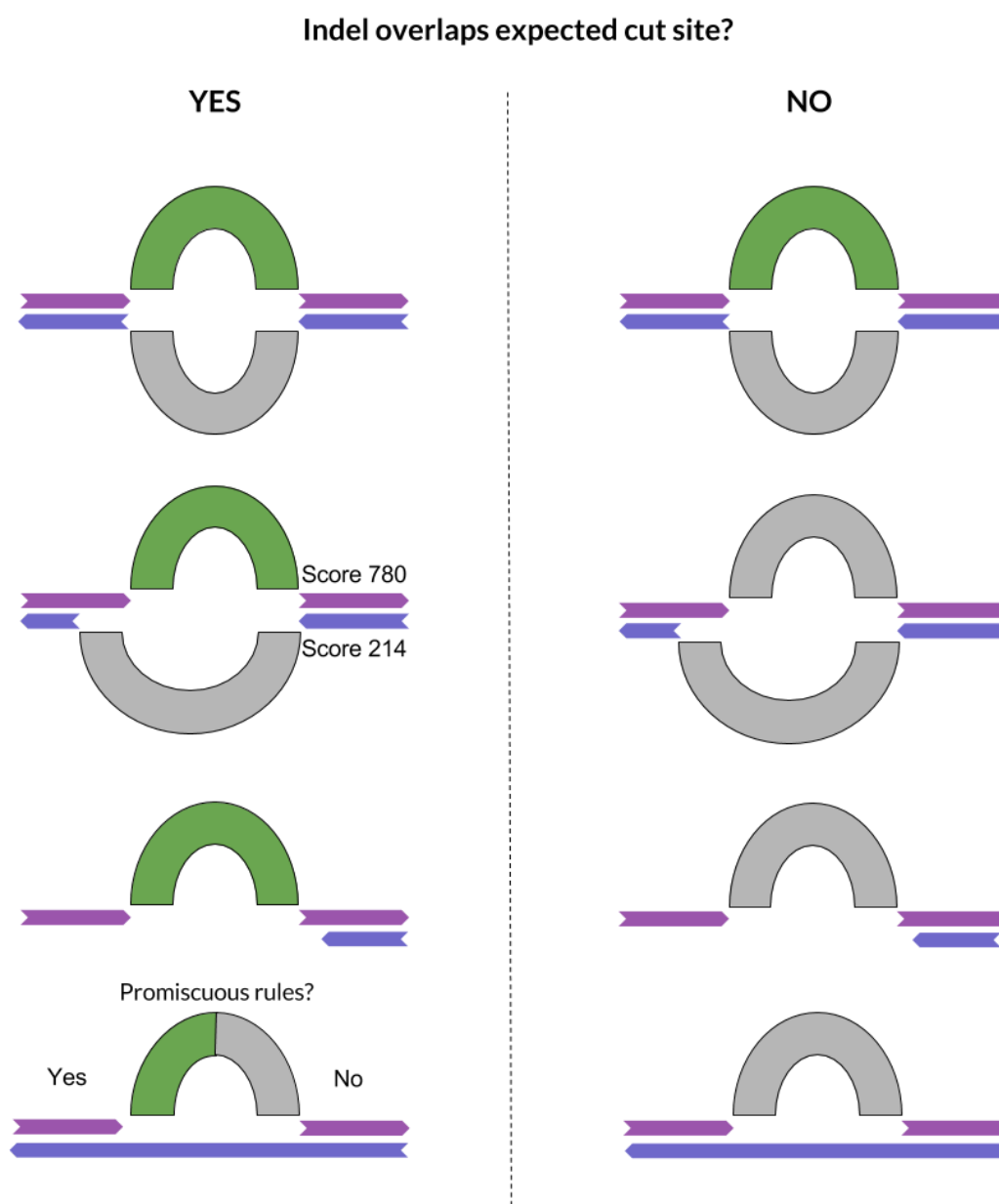


**Supplementary Fig 12.**

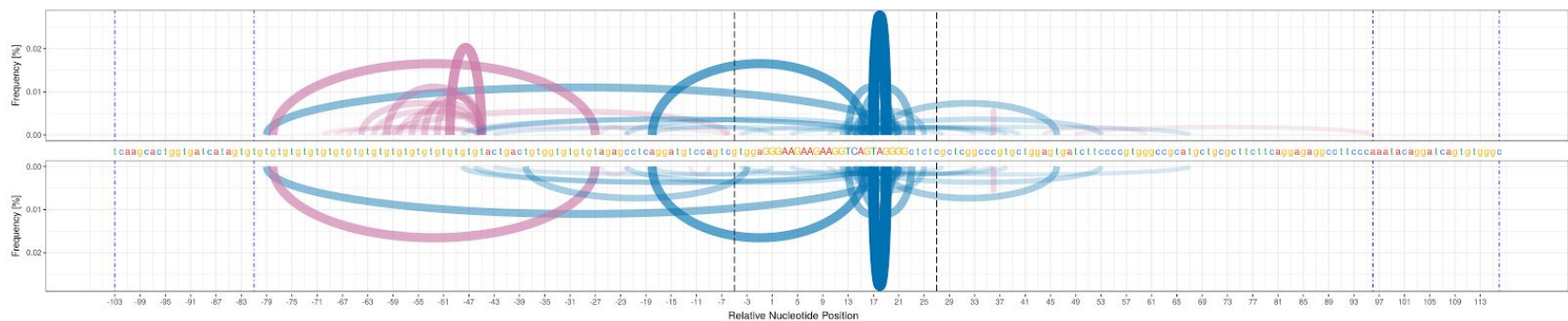
Overview of the ampliCan pipeline



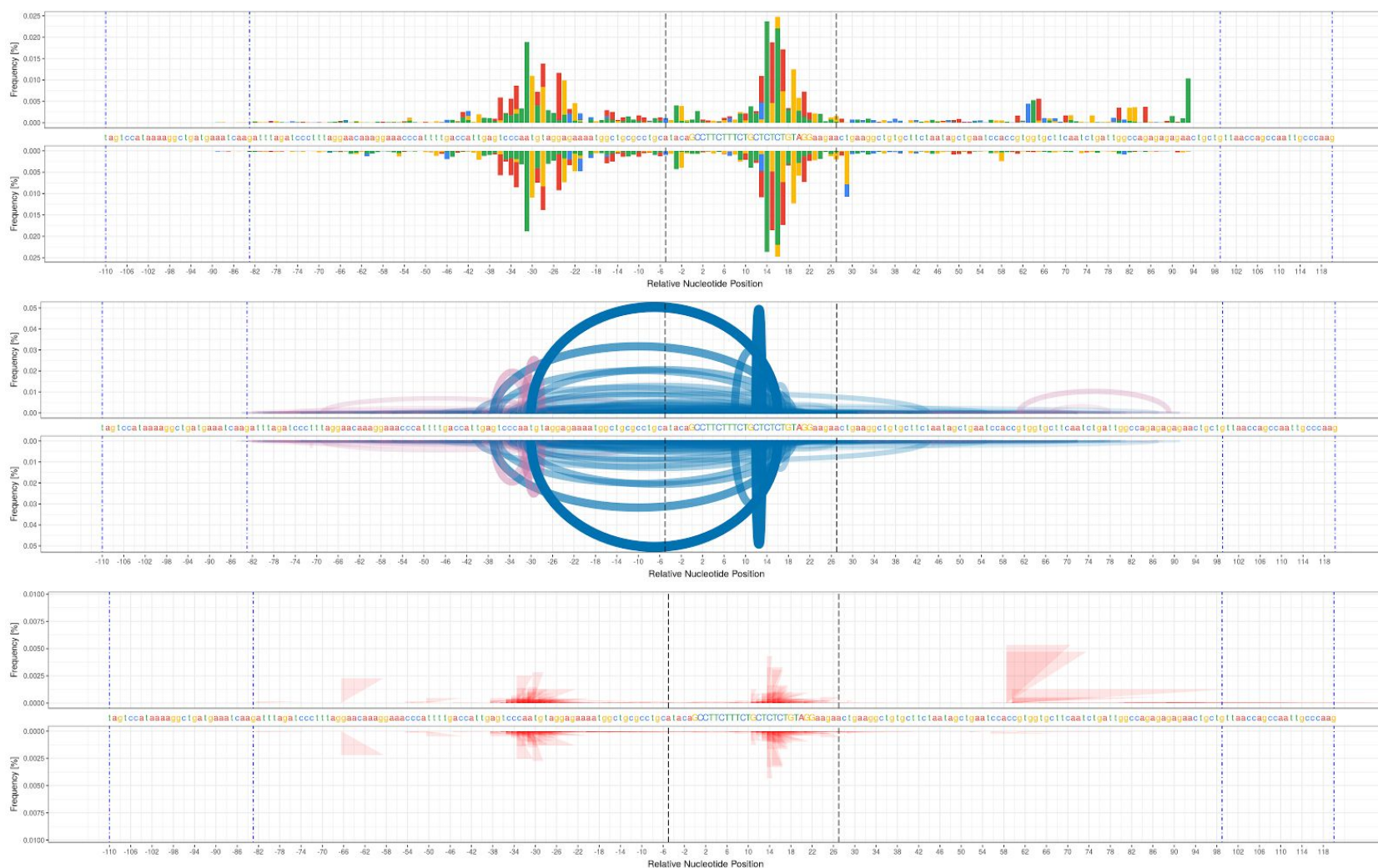
**Supplementary Fig 13.** Example of k-means clustering of reads during filtering of contaminant reads. A low alignment score (x axis) combined with a high number of events (y axis) indicate erroneous reads. Silhouette criterion is used to determine whether data should be clustered into two (read with no edits and reads with editing events) or three clusters (a noise cluster). In the case of three clusters, the cluster with its center (purple) closest to the upper left corner is filtered.



**Supplementary Fig 14.** The paired-end read consensus rules for ampliCan. Events marked in green are considered real cut sites while those in gray are not. When reads from forward (purple) and reverse (blue) reads are in agreement there is a consensus (top row). In situations where an event is only covered by one read, that read is preferred. When events from the two reads are in disagreement, but overlap each other, the event from the strand with a higher alignment score will be used. In cases where one of the strands ends prematurely we treat the situation as if it only has one strand. In rare case where there are events on one strand and the other has continuous alignment ampliCan allows users to define the behaviour (default is promiscuous rule enabled).



**Supplementary Fig 15.** ampliCan evaluates forward and reverse reads separately which allows users to spot potential problems. Here, purple colour indicates cuts outside of the gRNA expected cut site, a large majority of those reads comes from problematic alignments, where forward read and reverse read are in disagreement.



**Supplementary Fig 16.** Mismatch plot (**top**), deletion plot (**middle**) and insertion plot (**bottom**). This presents a case where the user might have mischaracterized their own experiment which can be spotted with these plots. Here nickases were used, but only one of the gRNAs was specified in the configuration file. A bimodal distribution of mismatch events and

insertions is the result. A quick examination allows the user to realize the expected cut site should be extended to include the second gRNA, enabling more precise mutation efficiency estimation.

|                        |
|------------------------|
| 1 Description          |
| 2 Barcode Summary      |
| 3 Top unassigned reads |
| 3.1 barcode_1          |

## 3 Top unassigned reads

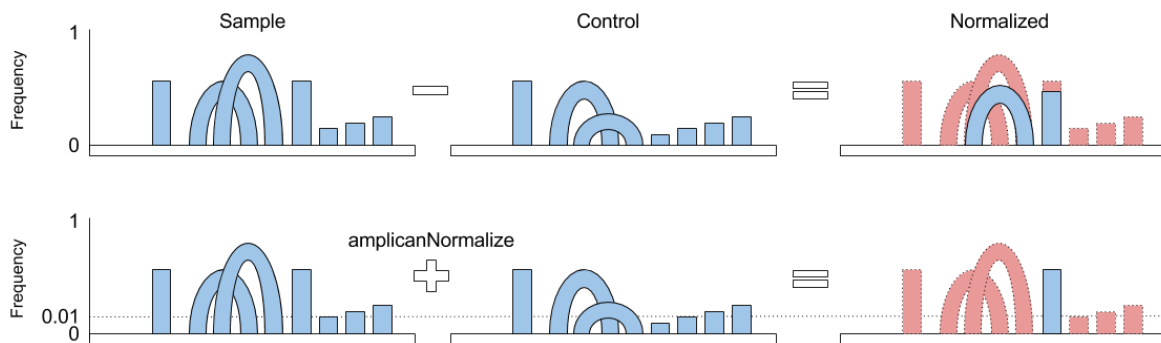
### 3.1 barcode\_1

| Forward | Reverse | Counts | Frequency |
|---------|---------|--------|-----------|
| P1      | S1      | 1      | 0.0714286 |

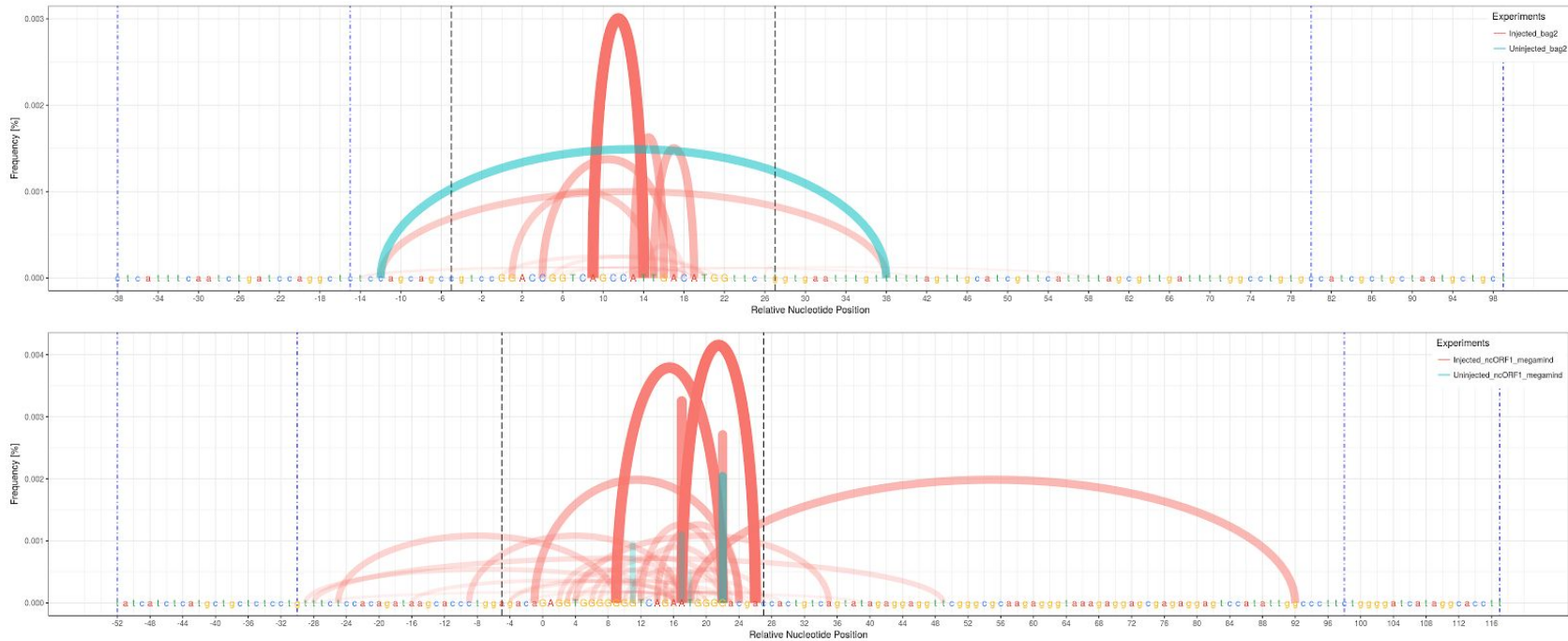
  

|    |     |  |     |
|----|-----|--|-----|
| P1 | 1   | AAAT - - ACTGTCTTGTGACCAAACTTCTTAAGGTGCTGTTTT - GATGAT | 47  |
|    |     |  |     |
| S1 | 1   | AAGCTGACGGCTAAATGA - - AAAATATCTGAAACATCTGTTCCAGGTGCT  | 48  |
|    |     |  |     |
| P1 | 48  | AAACTTTATTGTGCTTTTGTAGTTGTGCCCTTGTGTTGGCAGAGGGTCA      | 97  |
|    |     |  |     |
| S1 | 49  | GCGTATGCCAGGGCAGA - GAAGAAG - GTCAGGGAAGTCACTGGAGGTCA  | 96  |
|    |     |  |     |
| P1 | 98  | - - - GCAGACCAGTAAGTCTTCTCAATTTCTTTTATTATGTATGTAGT     | 144 |
|    |     |  |     |
| S1 | 97  | CTGGGATACCCTT - - - TCTTCCACACCAATGGGAAAGGAGTCTTGCCA   | 143 |
|    |     |  |     |
| P1 | 145 | GATAAA - A   | 151 |
|    |     |  |     |
| S1 | 144 | GATGACCA   | 151 |
|    |     |  |     |

**Supplementary Fig 17.** Screenshot of the example barcode report, top unassigned read section. Human readable alignment of forward and reverse reads of the top most frequent unassigned reads is presented. Huge fragmentation and poor alignment suggest contamination, while low frequency indicate proper specification of the primers in the config file.

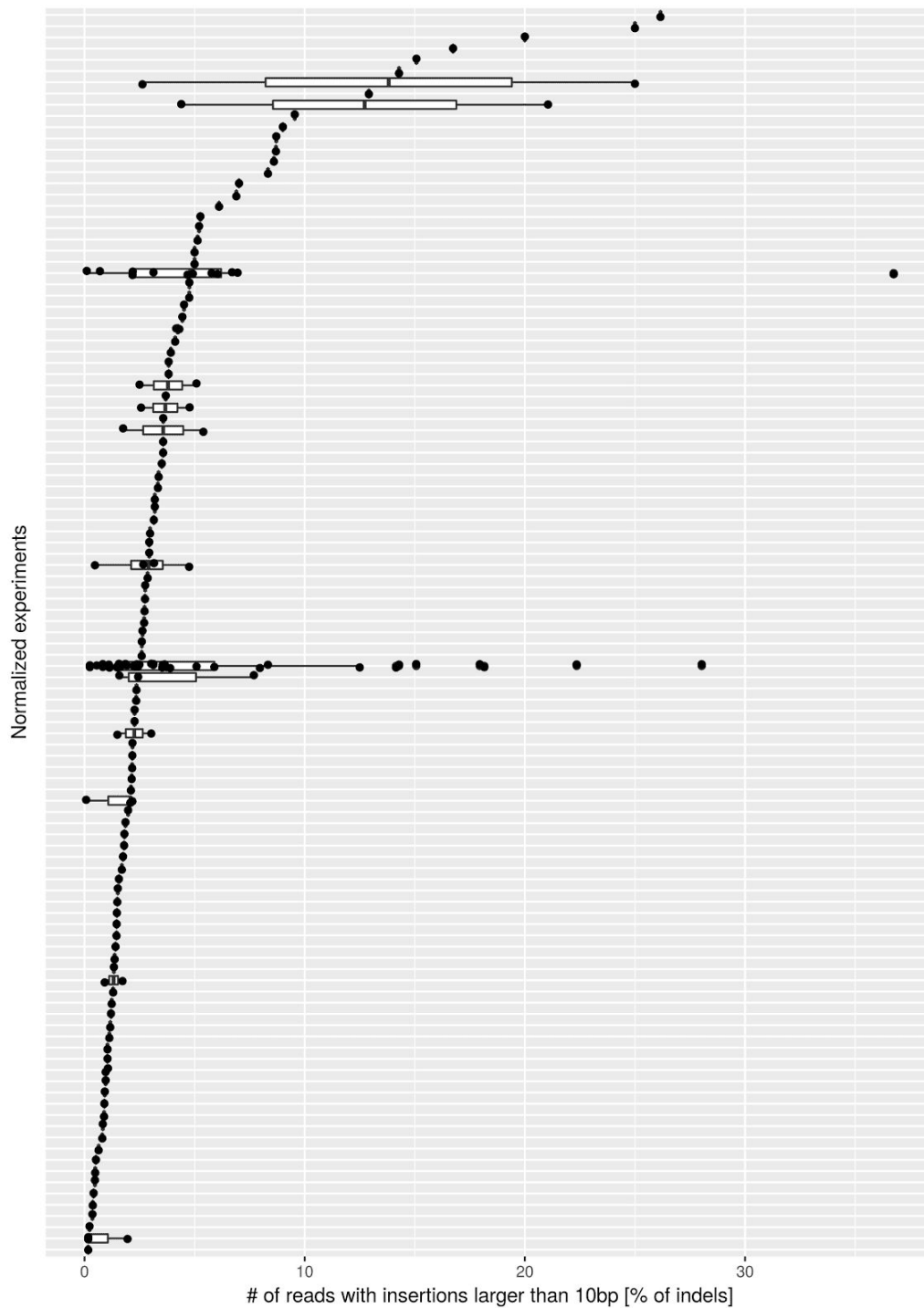


**Supplementary Fig 18.** Two common methods for normalization using controls. In the first, the frequencies from events in the control sample are subtracted from the frequencies in the treated sample (subtraction method). In the second method, all events occurring in the control above a frequency threshold (ampliCan default: 0.01) are removed from the treated sample. The latter is more robust to stochastic differences.

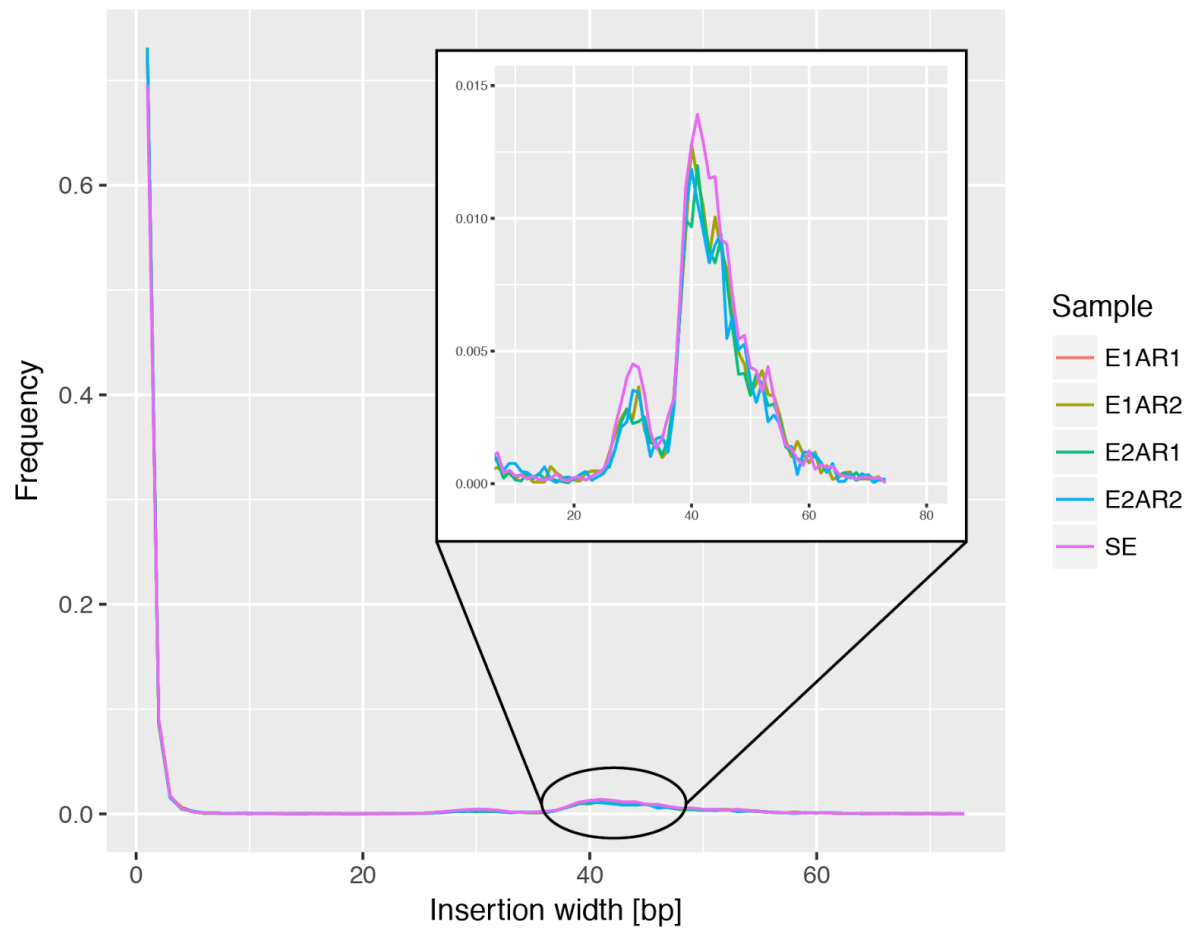


**Supplementary Fig 19.** Deletion plots produced by ampliCan for two experiments showing deletions in Cas9 injected (red) versus control (cyan) samples. The archs indicate deletions (x-axis, start to end of arch) present in the samples at a frequency indicated by the y-axis and transparency. The blue, vertical dotted lines shows the start and end of the primers. In the top panel experiment normalization using the subtraction method would filter the big deletion also present in the control, but it would not completely get rid of the 1bp long deletion in the lower panel experiment. Both cases would get expunged with the default ampliCan normalization.

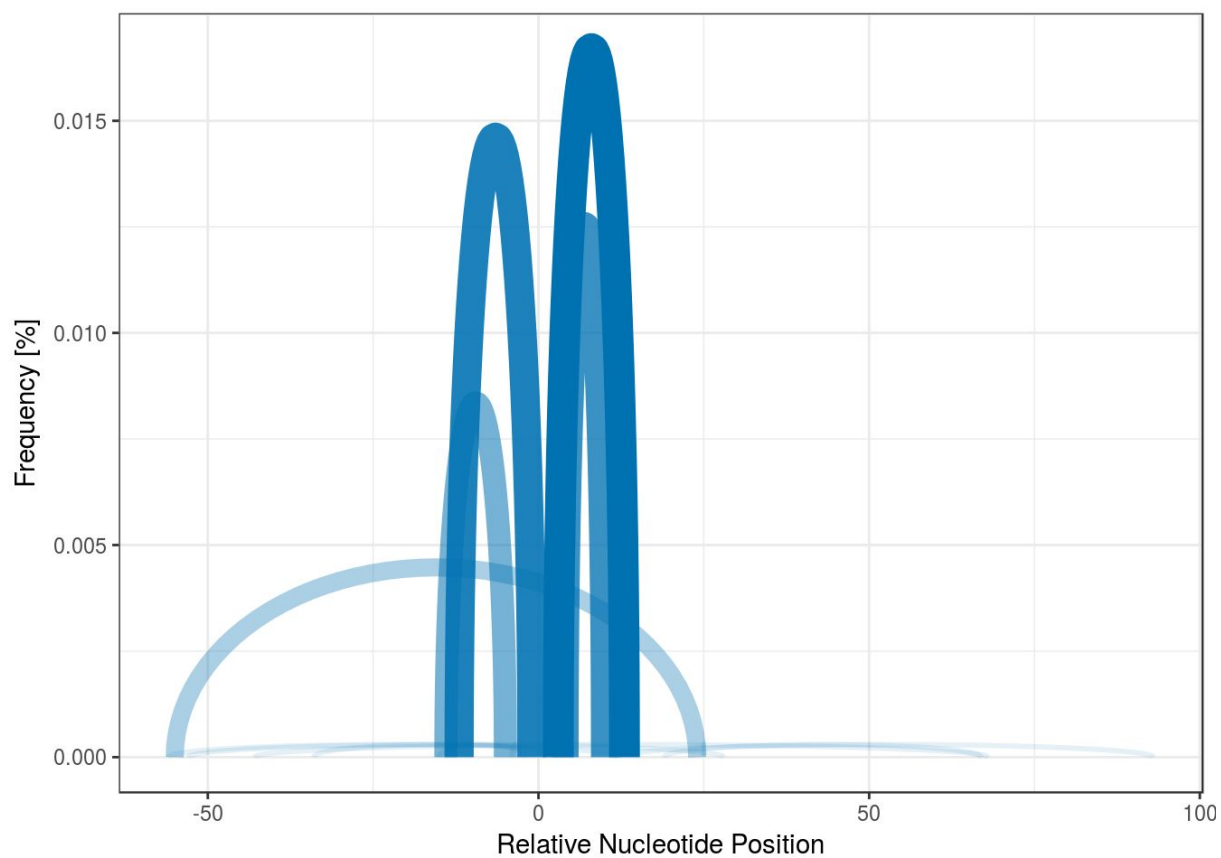




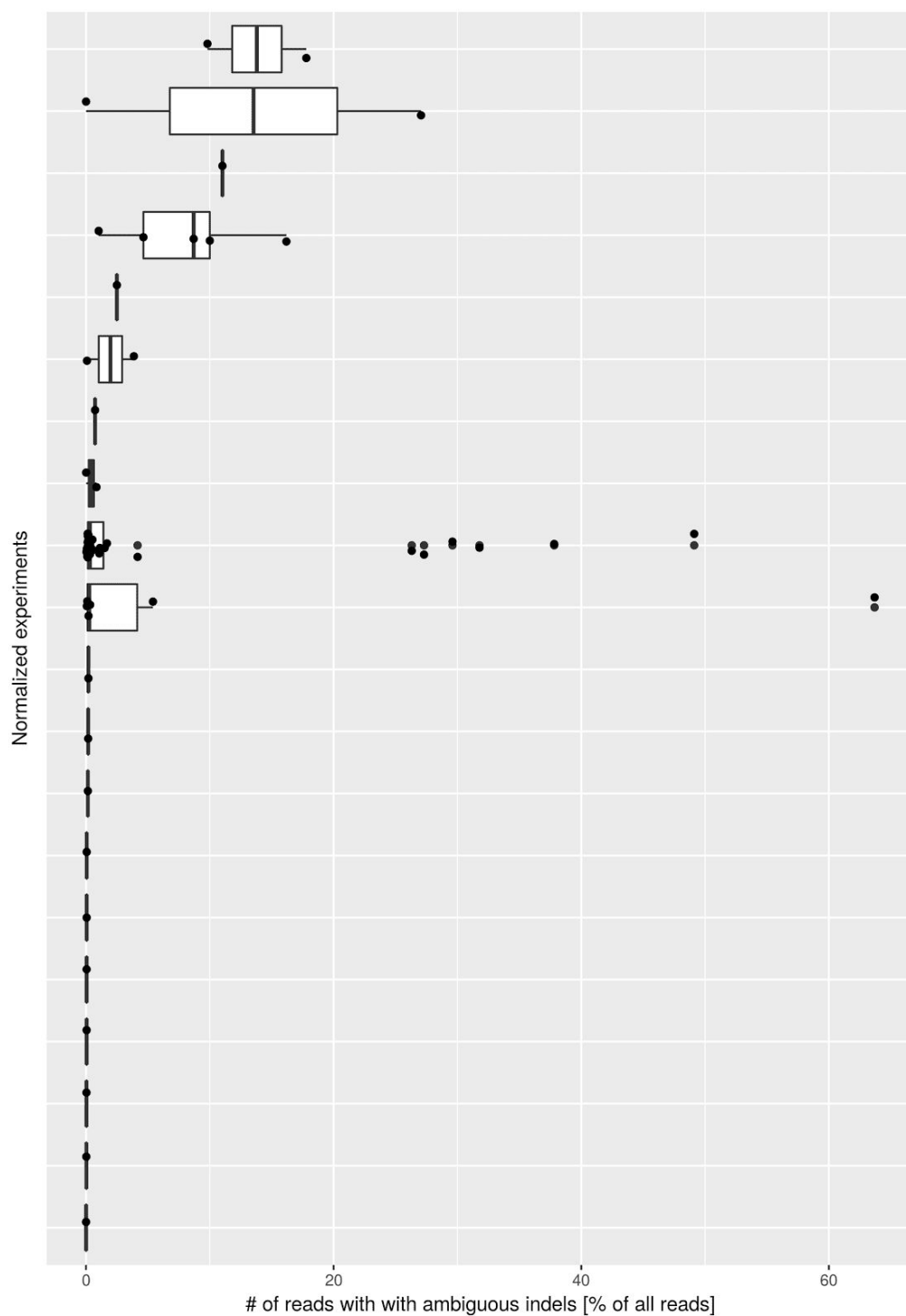
**Supplementary Fig 20.** Fraction of reads having indels greater than 10 bp across 176 experiments. Each dot represents one experiment and are grouped in rows by having the same gRNA (replicates). All experiments are normalized using wild type controls ensuring that these are real indel events. The higher mean for some of the replicated experiments suggests that some gRNAs have a higher chance of resulting in long indels.



**Supplementary Fig 21.** Distribution of insertions width for lentiviral samples on Chari et al. datasets. Shows the proportion (y-axis) of reads with a given insert length (x-axis). The insert shows a population of unintended larger insertions. Around 90% of these originate from the lentiviral vector used to transfect the cells.



**Supplementary Fig 22.** Example of a deletion metaplot produced by ampliCan summarizing multiple experiments. Here, an aggregation of editing events from many experiments (many targets) using the same gRNA is presented giving an overall gRNA cut profile. Position 0 is relative to the first 5' base of gRNA.



**Supplementary Fig 23.** Percentage of reads with ambiguous indels caused by disagreement of forward and reverse reads. ampliCan consensus rules help to mitigate mis-estimation that could arise from these events.

Quality of all reads



**Supplementary Fig 24.** Waffle plot of the quality of reads across all experiments.

## Supplementary Tables

| Tool   | Aligner  | Notes   |
|--|--|---|
| ampliCan   | Needleman-Wunsch from Biostrings <sup>7</sup> as local alignment |   |
| CrispRVariant <sup>2</sup>                               | BWA-MEM <sup>8</sup> as global alignment                         | Filters primer dimers by restricting start/end of indel, but may result in missing larger deletions. Larger insertions can be missed through mapping.                           |
| ampliconDIVider <sup>9</sup><br>(ampliconDIV_minimal.sh) | BWA-MEM as global alignment                                      | Sometimes returns estimates above 100%, these values were filtered. We run only the variant counting step of the pipeline. The full pipeline requires the commercial novoAlign. |
| CRISPResso & CRISPRessoPooled <sup>10</sup>              | BWA-MEM + FLASH (in Pooled) as local alignment                   | Poor performance due to unknown issues. It was installed and run in the same fashion as in Lindsay et al. analysis. CRISPRessoPooled uses local-global alignment strategy.      |

**Supplementary Tab 1.** Table of compared tools, with align strategy.

```

ID: ID_1 read_id: 1 Count: 3
AAGCTGACGGCTAAATGAAAAATGTCAAACGTCTGTTCAG-----AAAAAAAAAAAAAAAAAAATCCACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCAGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT
AAGCTGACGGCTAAATGAAAAATGTCAAACATCTGTTCCAGGTGCTCGGTATGCCAGGGCAGAGGAGTGGTCAGGAACTGGTGGAGGTCACTGGGATACCCCTTC-----TTCCACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCTGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT

AAGCTGACGGCTAAATGAAAAATGTCAAACGTCTGTTCAG-----AAAAAAAAAAAAAAAAAAATCCACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCAGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT
AAGCTGACGGCTAAATGAAAAATGTCAAACATCTGTTCCAGGTGCTCGGTATGCCAGGGCAGAGGAGTGGTCAGGAACTGGTGGAGGTCACTGGGATACCCCTTC-----TTCCACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCTGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT

ID: ID_1 read_id: 2 Count: 2
AAGCTGACGGCTAAATGAAAAATGTCAAACGTCTGTTCAG-----AAAAAAAAAAAAAAAAAAACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCAGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT
AAGCTGACGGCTAAATGAAAAATGTCAAACATCTGTTCCAGGTGCTCGGTATGCCAGGGCAGAGGAGTGGTCAGGAACTGGTGGAGGTCACTGGGATACCCCTTCCTTC-----CACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCTGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT

AAGCTGACGGCTAAATGAAAAATGTCAAACGTCTGTTCAG-----AAAAAAAAAAAAAAAAAAACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCAGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT
AAGCTGACGGCTAAATGAAAAATGTCAAACATCTGTTCCAGGTGCTCGGTATGCCAGGGCAGAGGAGTGGTCAGGAACTGGTGGAGGTCACTGGGATACCCCTTCCTTC-----CACACCAATGGGAAAGGAGTCCTGCCAGATGACCATCC
CAACTGTGTTGCTGCAGCCAGATCCAGGTGTGTTGGCCTTGTGTAATT

```

**Supplementary Tab 2.** Example of human readable output. Aligned reads are assigned to the experiment (ID, read\_id) and sorted based on count (Count). For each pair alignment is presented with top part representing forward read aligned to amplicon and bottom presenting reverse read aligned to amplicon.

| seqnames | start | end | width | strand | originally | replacement           | type      | read_id | score | counts |
|----------|-------|-----|-------|--------|------------|-----------------------|-----------|---------|-------|--------|
| ID_1     | 108   | 127 | 20    | +      |            | AAAAAAAAAAAAAAAAAAAAA | insertion | 1       | 597   | 3      |
| ID_1     | 112   | 131 | 20    | +      |            | AAAAAAAAAAAAAAAAAAAAA | insertion | 2       | 557   | 2      |
| ID_1     | 42    | 107 | 66    | +      |            |                       | deletion  | 1       | 597   | 3      |
| ID_1     | 38    | 111 | 74    | +      |            |                       | deletion  | 2       | 557   | 2      |
| ID_1     | 24    | 173 | 150   | +      |            |                       | deletion  | 3       | 193   | 1      |
| ID_1     | 34    | 117 | 84    | +      |            |                       | deletion  | 4       | 532   | 1      |
| ID_1     | 31    | 31  | 1     | +      | A          | G                     | mismatch  | 1       | 597   | 3      |
| ID_1     | 163   | 163 | 1     | +      | T          | A                     | mismatch  | 1       | 597   | 3      |
| ID_1     | 31    | 31  | 1     | +      | A          | G                     | mismatch  | 2       | 557   | 2      |
| ID_1     | 163   | 163 | 1     | +      | T          | A                     | mismatch  | 2       | 557   | 2      |
| ID_1     | 23    | 23  | 1     | +      | T          | G                     | mismatch  | 3       | 193   | 1      |
| ID_1     | 176   | 176 | 1     | +      | A          | T                     | mismatch  | 3       | 193   | 1      |
| ID_1     | 177   | 177 | 1     | +      | G          | C                     | mismatch  | 3       | 193   | 1      |
| ID_1     | 31    | 31  | 1     | +      | A          | G                     | mismatch  | 4       | 532   | 1      |
| ID_1     | 171   | 171 | 1     | +      | G          | A                     | mismatch  | 4       | 532   | 1      |
| ID_1     | 108   | 127 | 20    | -      |            | AAAAAAAAAAAAAAAAAAAAA | insertion | 1       | 597   | 3      |
| ID_1     | 112   | 131 | 20    | -      |            | AAAAAAAAAAAAAAAAAAAAA | insertion | 2       | 557   | 2      |
| ID_1     | 42    | 107 | 66    | -      |            |                       | deletion  | 1       | 597   | 3      |
| ID_1     | 38    | 111 | 74    | -      |            |                       | deletion  | 2       | 557   | 2      |
| ID_1     | 24    | 173 | 150   | -      |            |                       | deletion  | 3       | 193   | 1      |
| ID_1     | 34    | 117 | 84    | -      |            |                       | deletion  | 4       | 532   | 1      |
| ID_1     | 31    | 31  | 1     | -      | A          | G                     | mismatch  | 1       | 597   | 3      |
| ID_1     | 163   | 163 | 1     | -      | T          | A                     | mismatch  | 1       | 597   | 3      |
| ID_1     | 31    | 31  | 1     | -      | A          | G                     | mismatch  | 2       | 557   | 2      |
| ID_1     | 163   | 163 | 1     | -      | T          | A                     | mismatch  | 2       | 557   | 2      |
| ID_1     | 23    | 23  | 1     | -      | T          | G                     | mismatch  | 3       | 193   | 1      |
| ID_1     | 176   | 176 | 1     | -      | A          | T                     | mismatch  | 3       | 193   | 1      |
| ID_1     | 177   | 177 | 1     | -      | G          | C                     | mismatch  | 3       | 193   | 1      |
| ID_1     | 31    | 31  | 1     | -      | A          | G                     | mismatch  | 4       | 532   | 1      |
| ID_1     | 171   | 171 | 1     | -      | G          | A                     | mismatch  | 4       | 532   | 1      |
| ID_2     | 115   | 127 | 13    | -      |            |                       | deletion  | 1       | 845   | 3      |
| ID_2     | 106   | 114 | 9     | -      |            |                       | deletion  | 2       | 865   | 2      |
| ID_2     | 101   | 105 | 5     | -      |            |                       | deletion  | 3       | 885   | 1      |
| ID_2     | 115   | 127 | 13    | +      |            |                       | deletion  | 1       | 845   | 3      |
| ID_2     | 106   | 114 | 9     | +      |            |                       | deletion  | 2       | 865   | 2      |
| ID_2     | 101   | 105 | 5     | +      |            |                       | deletion  | 3       | 885   | 1      |
| ID_3     | 171   | 171 | 1     | +      |            | T                     | insertion | 1       | 819   | 5      |
| ID_3     | 74    | 89  | 16    | +      |            | CCCCCCCCCCCCCCCCC     | insertion | 3       | 860   | 1      |
| ID_3     | 66    | 83  | 18    | +      |            |                       | deletion  | 1       | 819   | 5      |

**Supplementary Tab 3.** Example GenomicRanges table output with additional meta-columns. This representation of alignments allows for efficient manipulation and processing of the data.

| ID   | Barcode   | Forward_Reads | Reverse_Reads | Group | Control | guideRNA                 | Forward_Primer        | Reverse_Primer        | Direction | Amplicon                              |
|------|-----------|---------------|---------------|-------|---------|--------------------------|-----------------------|-----------------------|-----------|---------------------------------------|
| ID_1 | barcode_1 | R1_001.fastq  | R2_001.fastq  | Betty | 0       | AGGTGGTCAGGGAAGTGG       | AAGCTGACGGCTAAATGA    | AATTACACAAGCGCAACACAC | 0         | ...cagaggAGGTGGTCAGGGAAGTGGtga...     |
| ID_2 | barcode_1 | R1_001.fastq  | R2_001.fastq  | Tom   | 0       | TGACCCCTCTGCCAACACAAGGGG | TGACCAACCTTCTTAAGGTGC | CTCTGCTGCAAAATGCAAGG  | 1         | ...agttgtgCCCCTTGTGTTGGCAGAGGGTCag... |
| ID_3 | barcode_2 | R1_002.fastq  | R2_002.fastq  | Tom   | 0       | AGGTGGTCAGGGAAGTGG       | AAGCTGACGGCTAAATGA    | AATTACACAAGCGCAACACAC | 0         | ...ggAGGTGGTCAGGGAAGTGGtga...         |
| ID_4 | barcode_2 | R1_002.fastq  | R2_002.fastq  | Betty | 0       | GTCCCTGCAACATTAAAGGCCGG  | GCTGGCAACATTCTACCAGT  | GAGCGCTGAGGCAGGATTAT  | 0         | ...ccaGTCCCTGCAACATTAAAGGCCGgaag...   |

**Supplementary Tab 4.** Example ampliCan config file. ampliCan requires this file as minimal input, together with relevant fastq files. More precise, up to date description of the file can be found in the ampliCan vignettes.

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