Inference of CRISPR Edits from Sanger Trace Data

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Abstract

Efficient precision genome editing requires a quick, quantitative, and inexpensive assay of editing outcomes. Here we present ICE (Inference of CRISPR Edits), which enables robust batch analysis of CRISPR edits using Sanger data. ICE proposes potential editing outcomes for single guide, multiplex editing, base editing, and homology-directed repair experiments and then determines which are supported by the data via regression. Additionally, we develop a score called ICE-D (Discordance) that can provide information on large or unexpected edits. We empirically confirm through over 1,800 edits that the ICE algorithm is robust, reproducible, and can analyze CRISPR experiments within days after transfection. We also confirm that ICE strongly correlates with NGS analysis (Amp-Seq). ICE is an improvement over current analysis tools in that it provides batch analysis, is free to use, and can detect a wider variety of edits. It provides investigators with a reliable editing tool that can significantly expedite CRISPR editing workflows. Our ICE tool is available online at ice.synthego.com and the source code is at github.com/synthego-open/ice

Introduction

CRISPR is a precise and programmable tool used for genome editing. Because of its experimental ease, CRISPR technology has increased in popularity in recent years. CRISPR creates a double-stranded break (DSB), and repair can produce a mutated sequence. However, the outcomes of the repair process are unpredictable, resulting in a heterogeneous population. Additionally, it is not readily apparent to the researcher if an edit has occurred and whether or not to continue culturing the edited cells. Various methods have been developed to address the problem of identifying the sequences present in an edited population at the targeted locus.

Previous methods to infer the composition of an unknown mixture of sequences have included Tracking Indels by Decomposition (TIDE) [1], compressed sensing [2], and next-generation sequencing (NGS) of amplicons (Amp-Seq). A major benefit of the TIDE approach is that only Sanger data are required for analyzing mixed populations. However, these software tools are not easy to use and also do not scale well to many samples. Amp-Seq offers sequence-level resolution and more sensitivity, but it is less widely available, has a longer turnaround time, and comes at a higher cost per sample unless a very large number of samples are batched.

In order to develop a quick and robust method for verifying CRISPR edits, we focused on improving the method set forth in TIDE. In the TIDE method, the edited locus is amplified and sequenced using the Sanger method for both a control and edited sample. Ideally, the edit site is within 200-300 bp of the sequencing primer, resulting in a read where the first hundred base pairs are of high quality and the following bases are potentially mixed (indicating an edited population with heterogeneous outcomes). Computationally, different editing proposals are

generated using the control sample and regression is used to compute how much of each editing outcome is observed in the mixed Sanger read.

While TIDE has been successful in reducing the barriers to entry for CRISPR, several characteristics of the program hinder its ease of use and automatability. For instance, users have to manually tune algorithm parameters and process each sample (a batch processing version of the algorithm is not readily available). Both of these aspects make TIDE a time-consuming analysis. Additionally, TIDE is limited to processing single guide experiments indels and cannot handle experiments where multiple guides are use simultaneously. Here we present an improved algorithm, called ICE (Inference of CRISPR Edits), that addresses the issues stated above and makes the editing analysis process more robust and automated.

Methods

ICE Algorithm

We re-implemented the TIDE [1] algorithm in Python and then added improvements to support more analysis cases and to make the algorithm more robust. Figure 1 shows the algorithm flowchart that corresponds to the steps below.

Step 1: We create an alignment for the two trace files by finding a high-quality window of the control trace upstream of the cut site and trimming it to end 15 bp upstream of the cut site. This alignment window is defined as a region of the Sanger trace that has a windowed average with Phred quality scores of greater than 30. The alignment window in the control is then aligned against the edited sample. By ignoring the poor quality bases often found at the very beginning of a Sanger trace, we found that this alignment method is robust and scales well for reliably processing many ab1 files.

Step 2: We defined the inference window to be the segment of the trace data used for the regression. The inference window starts 25 bp upstream of the cut site and extends up to 100 bp downstream of the cut site. The algorithm trims the inference window based on the quality score of the control sample. We limit the inference window length as adding extra bases has diminishing returns and can hurt the regression as Sanger sequencing quality decreases over the length of the read.

Step 3: We also made improvements to the edit proposal process, a step in which the algorithm generates potential post-editing genome sequences for use in the analysis. We aimed to support the analysis of many use cases for precision genome engineering, including the following:

1. Editing with a single guide: the algorithm uses a default indel range of deletions of up to 30 bp and insertions of up to 14 bp to generate a list of potential edits (sequences and traces). For deletions, the associated trace data for that base is simply deleted, while for insertions a uniform distribution of 25% for each base is inserted. The trace data for other bases is copied from the wild-type. These simulated traces are then used in the non-negative least squares regression.

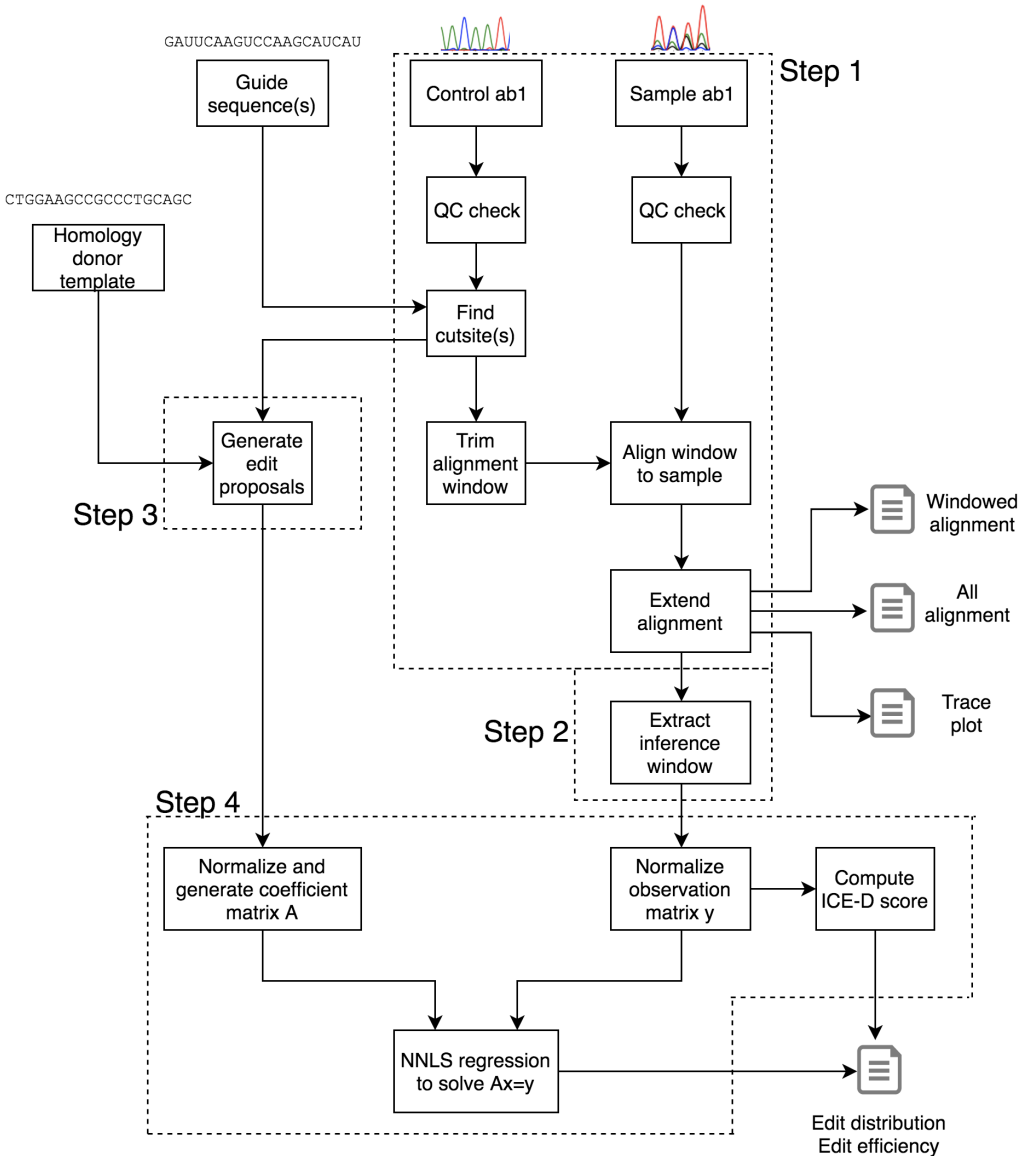


Figure 1. Algorithm flowchart. The inputs to the algorithm (top) are the control ab1 file, the sample ab1 file, the guide sequence(s), and optionally the homology donor or base editor template. The algorithm checks the data quality, generates edit proposals, and then runs a non-negative least squares regression to identify which edit proposal sequences are most likely present in the sample. The program then outputs various plots and data files that the investigator can use to determine the quality of the predictions, what percentage of the sample population has been edited, and what the sequences are.

2. Editing with multiple guides simultaneously: the algorithm first generates edit proposals for each guide as above; then, for every pair of guides, the algorithm generates multiplex edit proposals which cover the following two classes: a) both guides cut independently with indel formation or b) both guides cut “in tandem” and the intervening sequence is dropped out.
3. Homology-directed repair: the algorithm aligns the input template with the control sequence and then for any bases that differ in the alignment are simulated by a

score of 97% for the desired base and 1% for each of the three other bases. The alignment expects at least 15 bp of sequence on both ends of the template to match the genomic target.

4. Base editing: the same method as in the homology-directed repair case can be used to analyze samples. It would be necessary to input a template sequence that mimics the expected base editing outcomes.

Step 4: After the edit proposal stage, a regression is performed to infer the abundances of each proposal sequence. In the regression, x is solved for in the equation Ax=y, where A is a matrix composed of the simulated traces and y is the observed outcomes vector (the edited sequencing trace). Non-negative least squares regression finds a linear combination of the edit proposals that best explains the observed edited sample trace.

ICE-D Algorithm

We next wanted to address the issue of complex edits that result in large insertions or deletions that are not expected and would not be accounted for by any edit proposal. In our case, we noticed that multiplex edited samples when analyzed with only a single guide have a low ICE r^2 and low reported editing, but upon manual inspection of the Sanger trace reveals that much of the trace is discordant with the control.

To handle unexpected edits, we created a new, ICE-D (ICE-Discordance) that looks at the average discordant signal measure called ICE-D (ICE-Discordance) that looks at the average discordant signal. ICE-D is motivated by the observation that given a high quality alignment upstream of the cut site, we could assume that any signal downstream of the cut site discordant with the control trace should be evidence of editing. The ICE-D score is proportional to the discordance score in the inference window. We calibrated the ICE-D correction factor by finding the ICE score and the average discordant signal for 1805 edits and then performing a least squares linear regression between the two metrics.

Program Outputs

For interpreting results and checking algorithm settings, ICE outputs summary json and xlsx spreadsheets, plots, and alignments.

Source Code

The source code is available at github.com/synthego-open/ice, a docker container is on the docker hub at synthego/ice and a publicly accessible webtool can be found at http://ice.synthego.com. Currently the variant and multiplex editing modes are only available with the command line version but this functionality will be added to the webtool soon.

Editing of Cell Cultures

Editing was performed with Synthego synthetic single guide RNAs (sgRNAs) on a variety of cell lines. In general, sgRNAs were synthesized with or without modifications, and the

sgRNAs were complexed with Cas9 at a molar ratio of 9:1 (sgRNA:Cas9) to form Ribonucleoproteins (RNPs). The resulting RNPs were transfected into the respective cell line using a Nucleofector (Lonza; Basel, Switzerland). Transfected cells were recovered in normal growth medium, plated into 96-well plates, and incubated in humidified 37°C/5% CO_2. After 48h, cells were lysed and genomic DNA was extracted from the cells using QuickExtract™ DNA Extraction Solution (Lucigen; Middleton, WI) to each well of the plate.

Sanger Sequencing

For each target, we designed PCR primers to amplify a 500-800 bp segment containing the cut site. PCR was performed on lysed genomic samples using Taq polymerase. Sequencing was then performed through a commercial vendor (Sequetech; Mountain View, CA) with one of the two primers used for amplification.

Next Generation Sequencing of Amplicons

We targeted 32 genes with three single guide edits and one multiplex guide edit (co-transfection of all three guides) per gene for a total of 96 samples. For Sanger sequencing, three to four replicate edits were performed and sequenced. One of the replicate samples was amplified for Amp-Seq. One gene, comprising four samples, repeatedly failed Sanger sequencing and was dropped from subsequent analysis.

For Amp-Seq, a 200-300 bp segment containing the cut site was amplified from each sample. The amplicons were purified, quantified using a Nanodrop, and sent to the MGH DNA core facility for their CRISPR sequencing service. A summarization analysis was performed using the MGH NGS data pipeline, which reported sequences, and their abundances, present in the sequenced samples.

In parallel, 500-800 bp amplicons were PCRed from three to four replicates for Sanger sequencing. Four of the samples belonging to the same gene failed Sanger sequencing and were excluded from subsequent analysis.

For comparing between ICE and Amp-Seq, the ICE replicates were averaged together to compare with the Amp-Seq findings. Indels were summarized by length and then compared; ICE predicted sequences were also compared with contigs assembled from Amp-Seq data.

Comparison of ICE and TIDE

Thirty-seven samples with a high R-squared value (>0.95) where chosen from our ICE analysis of 1805 samples. All edits were conducted using a single guide and samples were chosen to span a range of indel percentages. These samples were then manually analyzed using the TIDE website [4] on December 15, 2017. Default parameters for the TIDE website were first tried; subsequent parameter tuning was then performed if the initial default parameters resulted in a failed analysis. The overall indel percentage (editing efficiency) was then compared between TIDE and ICE.

Simulated Homology Directed Repair and Base Editing

SNP rs2072579 was amplified from PGP1 (George Church's induced pluripotent stem cell line) and HEK293 genomic lysate. The amplicons were first sequenced to verify that PGP1 was homozygous G/G and HEK293 was homozygous C/C. Amplicon masses were quantified

using a Fragment Analyzer (AATI; Ankeny, Iowa) and then hand-pipetted to generate mixes to simulate different editing outcomes ranging from 5% to 95% single base editing.

Results

The ICE software is easy to use with no parameter tweaking required. The default indel limits are set for single cuts at -30, +14, which should cover most use cases. The software also outputs files that aid the user in interpreting and quality checking edits.

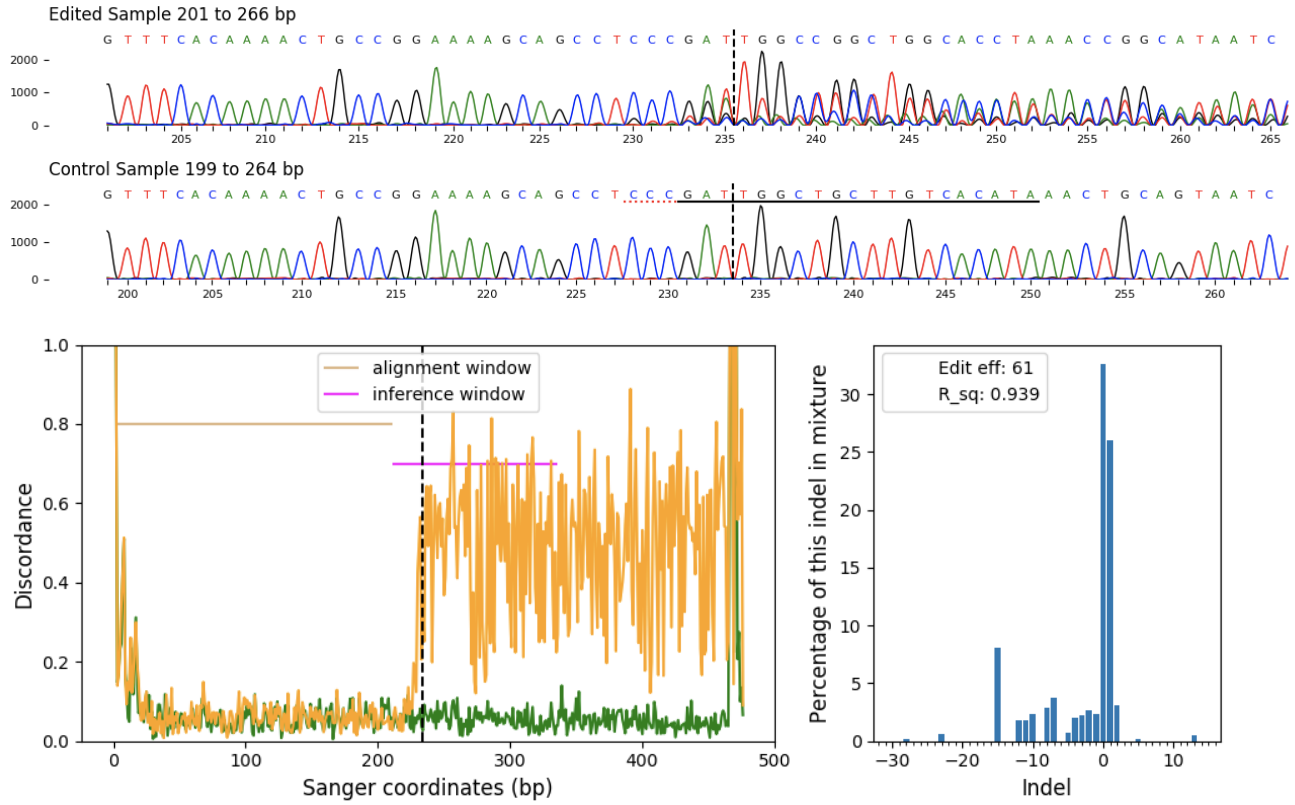


Figure 2. An example of the outputs from the ICE software for a guide targeting the human gene GRK5. Trace file segments spanning the cut site from the control and the edited samples are generated for every analysis. The guide sequence is underlined in the the control trace on the bottom, while the PAM sequence is denoted by a dotted red underline. Vertical dotted lines denote the expected cut site. For ICE variant analyses, the bases expected to be changed are underlined in both the control and edited traces.

We confirmed that our ICE tool was robust by performing an analysis on a batch of 1805 edits performed over multiple experiments. Our ICE tool takes on average four seconds to process each sample on a laptop in single threaded mode (MacBook Pro 2017). We also used this batch of edits to calibrate our ICE-D correction factor. The ICE-D correction factor is multiplied by the average discordant signal downstream of the cut site in the inference window to yield a proposal-agnostic guess of the indel percentage present in the cell.

We then tested the ability of ICE-D to detect unexpected edits by re-running the analysis and only supplying one guide sequence for multiplex samples. When only one guide is provided, the edit proposal stage does not generate all edit outcomes possible for a multiplex guide experiment. The lack of all possible edit outcomes then becomes an issue if those sequences are actually present and contribute to the Sanger signal. In those cases, both ICE and TIDE will give a low goodness-of-fit metric and may underestimate the indel percentage present. However, ICE-D is still able to detect editing as can be seen by the gray dots in the right panel of Fig 3.

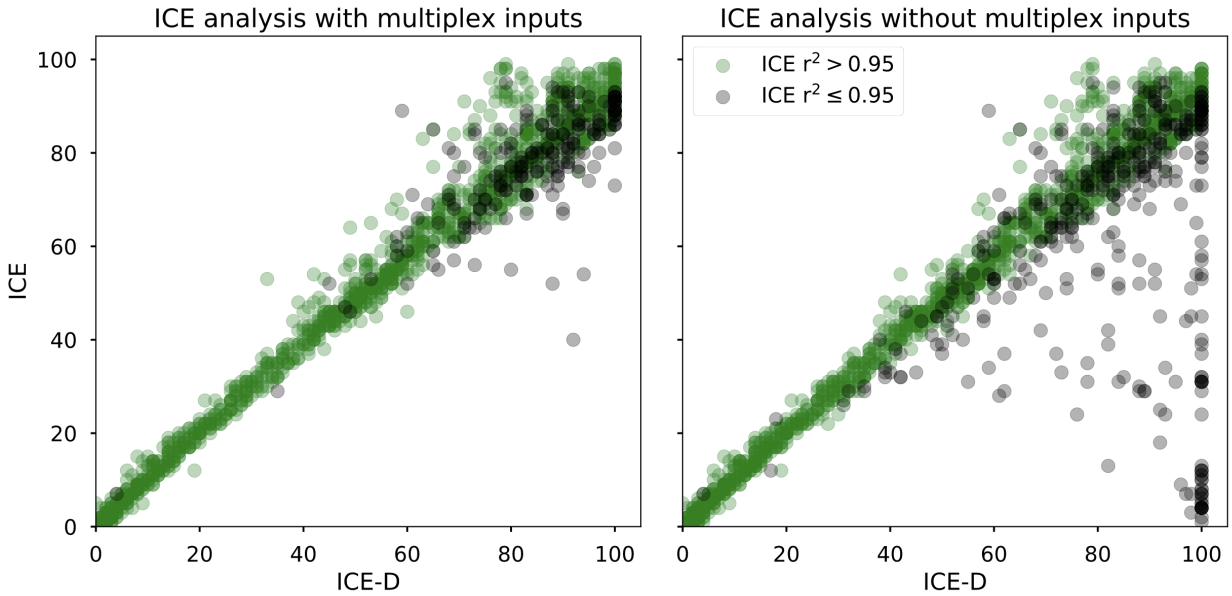


Figure 3. The ICE-D empirical correction factor was calibrated using ICE results from 1805 edits. The left figure shows analysis being performed with multiplex guide sequence inputs, while the right figure shows the results if multiplex guide inputs are not provided. For the analysis with multiplex guide information, 78% of the samples have high (>0.95) ICE r^2, and that number drops to 71% for the analysis without the multiplex guide information. In the right plot, ICE-D is able to detect unexpected edits when the ICE goodness of fit is low (<0.95) and ICE-D score is high (gray points in the bottom right of the plot).

To validate our algorithm, we compared ICE results with results from TIDE and Next Gen Sequencing of amplicons (Amp-Seq). First, we analyzed 37 samples through both TIDE and ICE using default settings. These samples were chosen to span a range of 0-95% editing efficiencies and for having a high r^2 in ICE. Figure 2 shows that ICE and TIDE correlation is high with a r^2 of 0.95, and ICE-D and TIDE have an r^2 of 0.85. However, when using TIDE, users frequently need to tune the parameters for alignment to be able to get an interpretable result.

UCK2 multiplex deletion: comparison of sequences from ICE (top) and Amp-Seq (bottom)

```
9.8%   -30:md-0[g1],-0[g2]      CATTATCTGCTCTGTGCTTTGCAGGA|-----------------
-------------|ACGTGGTGCTCTTTGAAGGG

>G11_CG11_CONTIG_240_p2    5977 pairs of Amp-Seq reads, 9.99%
GGCTCTGTAAGACCATATTAGCCAAGTCTTTAGCCCCCGCTTGAAGTCTGTGGGGGCAAGGAACCCAGAACCCAG
CCCAGCCAGATGTTCTGGCCCttgttctctgtcccttgctagcccctgcctggcttggccCATTATCTGCTCTGT
GCTTTGCAGGAACGTGGTGCTCTTTGAAGGGATCCTGGCCTTCTACTCCCAGGAGGTACGAGACCTGTTCCAGAT
GAAGCTTTTTGTGGA
```

Fig 4. An example of a large, multiplex edit. Three guide sequences targeting the UCK2 gene were transfected into an HEK293 cell culture. The ICE software predicts a 9.8% -30 deletion involving two guides. Separately, we performed Amp-Seq on the sample which shows the exact same sequence and estimates similar proportion of the sample (9.99%).

Having shown that ICE correlates well with both TIDE, we next sought to validate our ICE-D measure. We checked by running both ICE and ICE-D on over 2000 samples to derive the empirical correction factor of 1.6. We then saw a correlation with r^2=0.86 as shown in Figure 4.

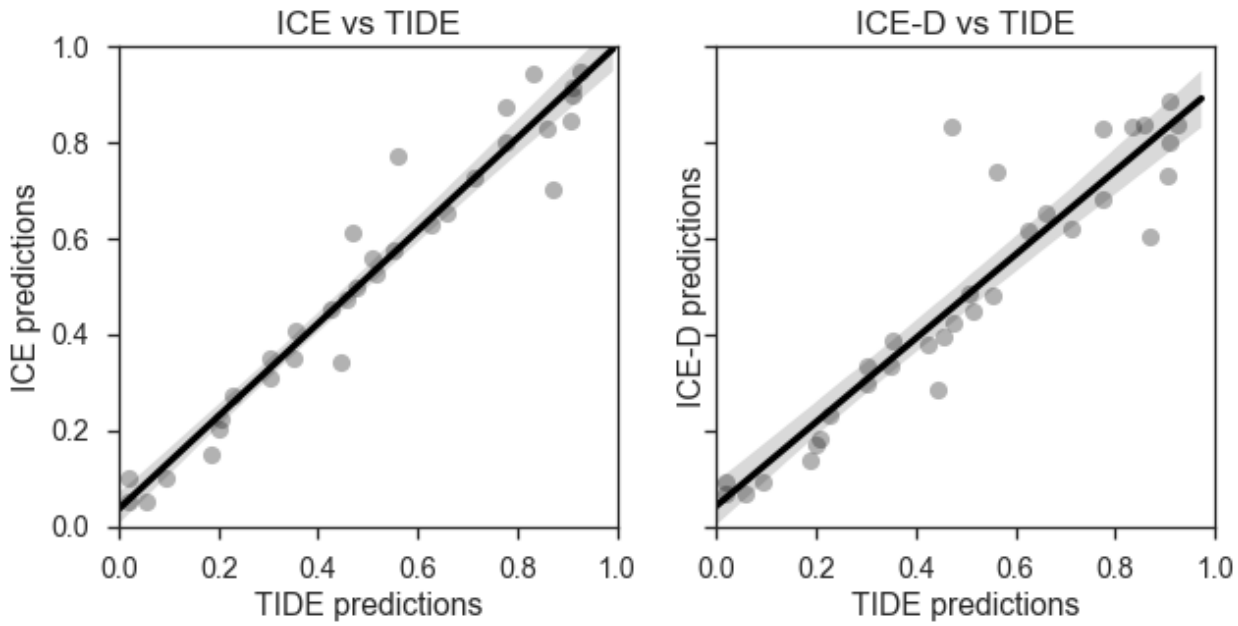
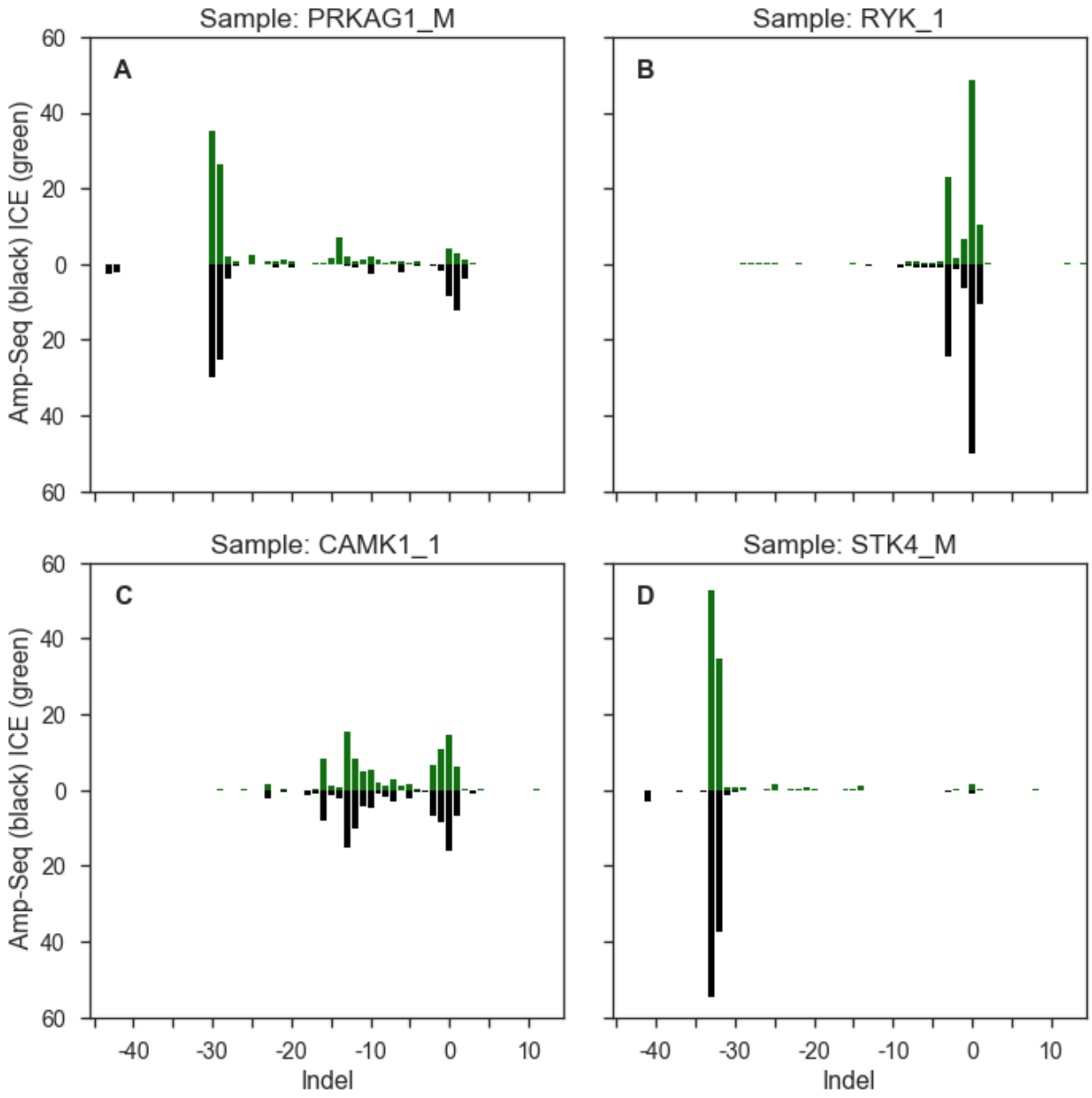


Figure 3. ICE and ICE-D agree well with TIDE across 37 samples. ICE-D relies on more assumptions and has a poorer correlation with TIDE. Pearson r^2=0.95 for TIDE vs ICE and r^2=0.85 for TIDE vs ICE-D.

We next sought to show that ICE correlates well with the current gold standard of Amp-Seq. We performed amplicon sequencing on 92 samples using MGH CRISPR sequencing service. We correlated the ICE predictions with the Amp-Seq results for each indel size in all samples. We found a high correlation, with an overall r^2 = 0.93. To show that the ICE predictions are correct at the sequence level, instead of a summarized indel size, we also

manually inspected the sequences from Amp-Seq and ICE. In Appendix A, we have some examples of sequences predicted by ICE matching Amp-Seq results.



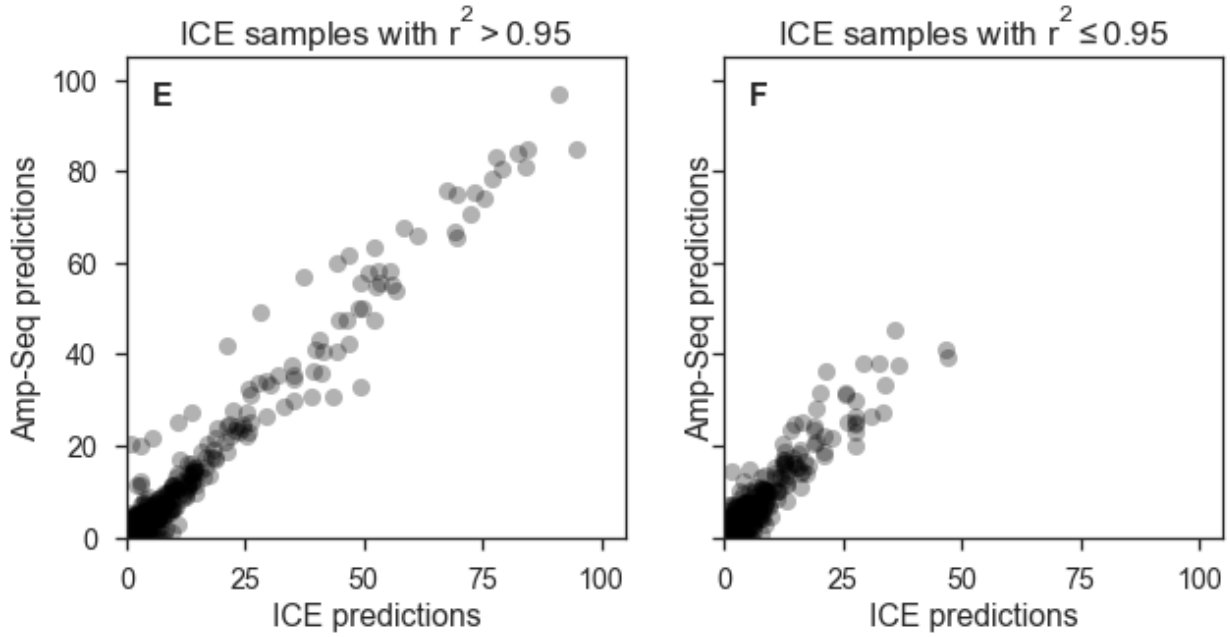


Figure 4. Amp-Seq results were compared with ICE results for 92 samples. The top four panels show the amplicon sequencing and ICE predictions for indel distributions for four samples. The scatterplots at the bottom compare all of the pairwise points from the indel distributions. The correlation of Amp-Seq with ICE is r^2=0.96 for ICE samples with a high quality analysis (rsq > 0.95). ICE results a lower quality score (rsq $\leq$ 0.95) are less correlated with pearson r^2=0.88, but still informative. For each sample, the averages of up to four ICE replicates are compared to the results from the Amp-Seq run.

To validate ICE variant analysis performance, we sequenced mixes of DNA that simulate a range of variant outcomes. We amplified the locus surround SNP rs2072579 from the HEK293 cell line and George Church's PGP1 iPSC line. Sanger sequencing confirmed the samples are homozygous and different at that position. We then quantified the amplicons with a Fragment Analyzer, mixed them in different ratios (5%,10%, 20%, 40%, 60%, 8%, 90%, 95% of PGP1 in the mixture), and sequenced the mixed samples. The sequencing data were then analyzed with ICE simulating an experiment in which the HEK293 cell line (C/C) is edited to have a homozygous G/G at SNP rs2072579. The predictions are highly correlated with the expected percentages (Fig. 5).

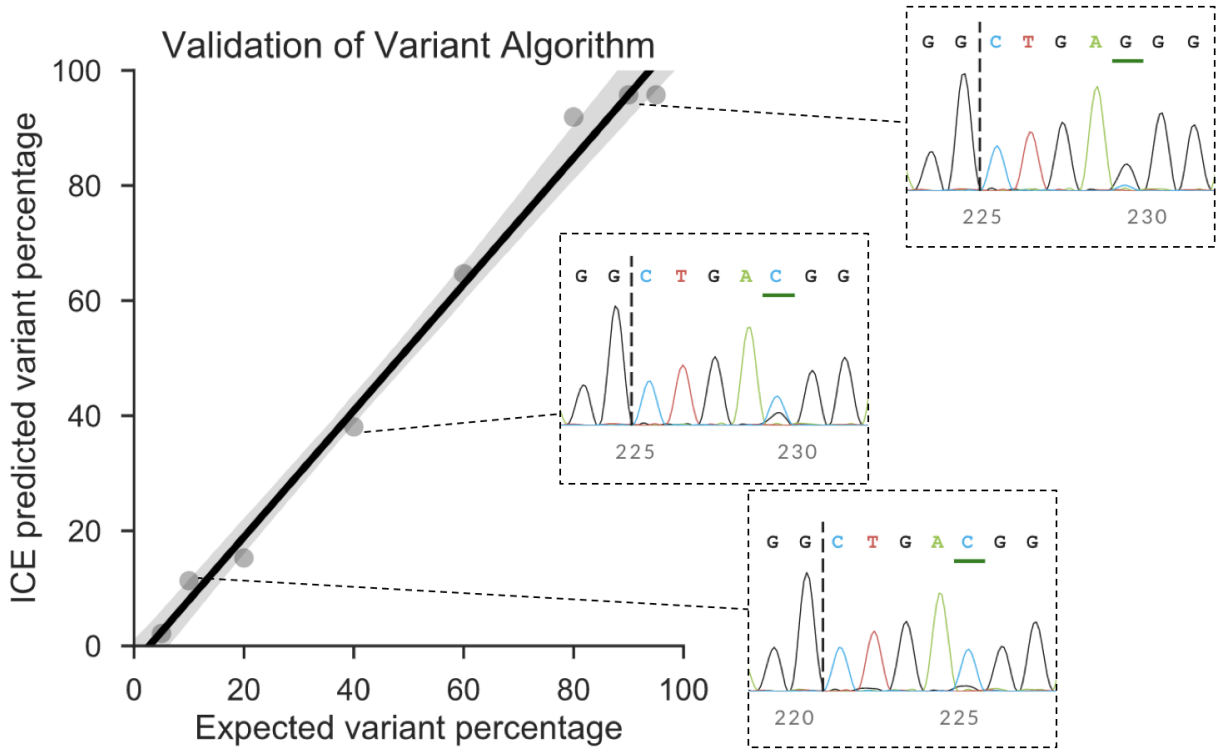


Figure 5. ICE variant prediction results correlate with expected variant percentages. Pearson r^2=0.99.

Discussion

Here we present our software tool, ICE, which uses Sanger sequencing data from CRISPR edited samples to quantify the identity and prevalence of edits. ICE is able to handle single guide, multiplex guide, base editing, and homology-directed repair experiments. A major benefit of the ICE workflow is that it is a fast and robust assay that enables experimenters to easily optimize gene editing experiments. While Amp-Seq has better sensitivity and quantitation, Sanger sequencing still remains a more widely accessible, faster, and cheaper method. Moreover, we show high correlation of ICE results with Amp-Seq results for 92 samples, suggesting that ICE can provide a reliable substitute in the vast majority of cases.

In comparison to TIDE, ICE is able to analyze more types of experiments, requires no subjective parameter tuning, and has comparable results. These advantages result in a webtool and software package that is able to easily process hundreds of CRISPR editing results in a reproducible manner. Additionally, ICE-D provides a form of insurance for the ICE proposing process by being able to capture unexpected indels. We have validated the robustness of the ICE tool by running analyses for thousands of Sanger files in one batch.

For base editing or HDR experiments, ICE offers the benefit of not requiring laborious lab-work to construct a synthetic standard and a third Sanger sequencing, unlike TIDER [3]. Our approach will allow one experimental workflow to be able to analyze single, multiplex, HDR, and base editing experiments.

There are some general assumptions made by both Sanger-based CRISPR analysis tools (ICE and TIDE) that may limit their precision. Both make the assumption that the peak signal *S* for different bases at each position are linearly proportional to the molarity of the base *m* with the relationship $S=bm$. Critically, the coefficient *b* is assumed to be the same for all bases. However, the peak height and phasing for a particular base in the Sanger trace is a function of the local sequence context. This could result in sequences where the molar ratios of bases present at a given position are not reflected by the Sanger signal ratios. Because base editing and HDR rely on the signal from single base positions, the peak height and phasing assumptions may have a larger adverse effect. It may be possible to better model the expected Sanger sequencing trace by using the approaches in [2]. However, the high correlation between ICE and Amp-Seq indicates that the assumption does not affect ICE's ability to predict insertions and deletions. We suspect this is because an indel shifts and affects the signal for all bases downstream and the effect of peak signal variance cancels out over many bases.

A caveat specific to the ICE multiplex edit analysis is that the edit proposal process assumes two cuts can happen in close proximity. For example, if there are two guides with cut sites of *n* and *n*+1 in the genome, the model will generate an edit proposal where both guides cut and dropout the intervening base. However, we know this proposal is impossible as the nuclease cannot cut in parallel due to sterics and cannot cut serially as the guide sequence in the genome would have been destroyed. The addition of this constraint and other constraints that account for biological mechanisms will make it possible to bias the edit proposal process or the regression in favor of the correct sequences.

ICE offers a new and robust method for analyzing CRISPR editing experiments. ICE can detect successful edits in just a few days after transfection, as has been validated on thousands of samples. We found that ICE is able to offer results comparable to Amp-Seq, but at a significant reduction in cost and time. The ICE workflow offers several advantages over the current state-of-the-art alternatives by offering a robust and reproducible way to analyze single guide editing experiments. It also is the only tool that can analyze multiplex editing and requires less work to analyze HDR experiments. Because ICE reduces the labor, cost, and time associated with CRISPR experiments, analysis is no longer a limiting factor for precision genome editing.

References

1. Brinkman, Eva K., et al. "Easy quantitative assessment of genome editing by sequence trace decomposition." *Nucleic acids research* 42.22 (2014): e168-e168.
2. Amir, Amnon, and Or Zuk. "Bacterial community reconstruction using compressed sensing." *Journal of computational biology* 18.11 (2011): 1723-1741.
3. Brinkman, Eva Karina, et al. "Easy quantification of template-directed CRISPR/Cas9 editing." *bioRxiv* (2017): 218156.
4. https://tide.nki.nl/

Appendix A: Examples of sequences predicted by ICE and those detected by Amp-Seq

Here we use bold and underlined text to match sequences in the ICE output and in the Amp-Seq contigs assembled from Amp-Seq. We also report the proportion of the sample that each approach predicts for the sequences.

Example 1: UCK2 multiplex editing

Edit proposal 1

51.7% -51:md-0[g1],-0[g3] **ATTATCTGCTCTGTGCTTTGCAGGA**|----------
----------|TCCTGGCCTTCTACTCCCAGGAGGTACGAGACCTGT

>G11_CG11_CONTIG_219_p1 24222 pairs of Amp-Seq reads, 40.5%

GGCTCTGTAAGACCATATTAGCCAAGTCTTTAGCCCCCGCTTGAAGTCTGTGGGGGCAAGGAACCCAGAACCcagcc
cagccagatgttctggcccttgttctctgtcccttgctagcccctgcctggcttggcc**cattatctGCTCTGTGCTT**
TGCAGGATCCTGGCCTTCTACTCCCAGGAGGTACGAGACCTGTTCCAGATGAAGCTTTTTGTGGA

Edit proposal 2

9.8% -30:md-0[g1],-0[g2] **CATTATCTGCTCTGTGCTTTGCAGGA**|----------
----|ACGTGGTGCTCTTTGAAGGG

>G11_CG11_CONTIG_240_p2 5977 pairs of Amp-Seq reads, 9.99%

GGCTCTGTAAGACCATATTAGCCAAGTCTTTAGCCCCCGCTTGAAGTCTGTGGGGGCAAGGAACCCAGAACCCAGCC
CAGCCAGATGTTCTGGCCCttgttctctgtcccttgctagcccctgcctggcttggcc**cattatctGCTCTGTGCTT**
TGCAGGAACGTGGTGCTCTTTGAAGGGATCCTGGCCTTCTACTCCCAGGAGGTACGAGACCTGTTCCAGATGAAGCT
TTTTGTGGA

Example 2: IRAK4 multiplex editing

ICE Edit proposal 1

62.8% -39:md-0[g2],-0[g1] **GTCATCAATGTCCTGTCTTTGTCACA**|----------
----------|AGAAGGTAGTG

>C12_CC12_CONTIG_231_p1 10543 pairs of Amp-Seq reads, 66.8%

TGTGCTGTGAGAATATGAGACCAACCTGTAGAAACTGGAATGATATTAAATGAACCAAGTTTCTAGTTTAACTTTTT
CACAACCActttttcttactgaaaaaccacttgtatcttacttcatttgttagatgctgttcccaaaaCTGCTAATA
CACTACCTTCT**TGTGACAAAGACAGGACATTGATGAC**ACCTGTGCAGAATCTTGAACAAAGCTATATGCCACCTGAC

Example 3: STK4

Edit proposal 1

48.4% -33:md-0[g3],-0[g1] **GCTTAATAGCAACAATCTGGCCGGTC**|----------
-------|GACCTATACATTTGGGA

>D12_CD12_CONTIG_219_p1 23309 pairs of Amp-Seq reads, 54.76%
TCAGTTGCTTGTGTTTTACCACTTCTTATATCTTGGCTTGCTTTGACTTTATAAATGTTCTTCTTCTCCCAaatgta
taggtc**gaccggccagattgttgctattaagc**aagttcctgtggaatcagacctccaggagataatcaAAGAAATCT
CTATAATGCAGCAATGTGACAGGTAAAGGCATGTGGGCTTCCTTTGGGGAGAATGTGGTTTTGAA

37.0% md+1[g3],+0[g1] **CTTAATAGCAACAATCTGGCCGGTC**n|----------
-------|GACCTATACATTTGGGA

>D12_CD12_CONTIG_220_p2 15963 pairs of Amp-Seq reads, 37.5%
TCAGTTGCTTGTGTTTTACCACTTCTTATATCTTGGCTTGCTTTGACTTTATAAATGTTCTTCTTCTCCCAAatgta
taggtca**gaccggccagattgttgctattaagc**aagttcctgtggaatcagacctccaggagataatcAAAGAAATC
TCTATAATGCAGCAATGTGACAGGTAAAGGCATGTGGGCTTCCTTTGGGGAGAATGTGGTTTTGAA