Topologically Dependent Abundance of Spontaneous DNA Damage in Single Human Cells

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ABSTRACT

In the studies of single-cell genomics, the large endeavor has been focusing on the detection of the permanent changes in the genome. On the other hand, spontaneous DNA damage frequently occurs and results in transient single-stranded changes to the genome until they are repaired. So far, successful profiling of these dynamic changes has not been demonstrated by single-cell whole-genome amplification methods. Here we reported a novel single-cell WGA method: Linearly Produced Semiamplicon based Split Amplification Reaction (LPSSAR), which allows, for the first time, the whole-genome detection of the DNA damage associated variants (dSNVs) in single human cells. The sequence-based detection of dSNVs allows the direct characterization of the major damage signature that occurred in human cells. In the analysis of the abundance of dSNVs along the genome, interestingly, we observed two modules of dSNV abundance, instead of a homogeneous abundance of dSNVs. We showed that the two modules are associated with the A/B topological compartments of the genome. This result suggests that the genome topology directly influences the genome stability. Furthermore, with the detection of the large number of dSNVs in single cells, we showed that only when the *de novo* mutations can be distinguished from the dSNVs, can a reliable estimation of *de novo* mutations be achieved.

To study single-cell genomics, various single-cell whole genome amplification (WGA) methods have been developed with the goal of detecting genomic variations in single cells, including single nucleotide variations (SNVs) (*1-6*) and copy number variations (CNVs) (*7-9*). However, we have been ignoring the fact that spontaneous DNA damage frequently occurs to the genome, resulting in single-stranded changes (*10-13*). In comparison to *de novo* mutations, single-cell measurement of the DNA lesions is greatly desired for studying genome stability and the physiology of the cells. In theory, it is possible that we can detect DNA lesions by single-cell whole genome amplification (WGA) methods since the damaged bases can lead to nucleotide misincorporation in amplification. We denote these DNA lesion associated single nucleotide variants as dSNVs.

So far, no single-cell WGA methods have been developed to detect these dynamic DNA lesions. Two major technical obstacles in achieving reliable detection of *de novo* variants are amplification errors and technically introduced damage to DNA (*5, 6*). Here we reported a new single-cell WGA method: Linearly Produced Semiamplicon based Split Amplification Reaction (LPSSAR). The reaction scheme of LPSSAR is shown in **Fig. 1** and **Fig. S1** (Supplementary **Methods**).

To evaluate the performance of LPSSAR, we amplified single cells isolated from a cultured cell line (MCF10A, a normal diploid breast epithelial cell line) and the single nuclei of human cortical neurons isolated from frozen brain samples. For MCF10A, we amplified and sequenced three single cells from the serum-free culture in the poised G0 state to warrant the diploid genome. For human cortical neurons, we amplified and sequenced 18 single cells from three brain samples. Each cell was sequenced at 30X sequencing depth with ~10X coverage for each split amplification (**Fig. S2**). On average, we achieved genome coverage of ~83% per split (**Table S1)**. Next, we combined the sequencing data of three splits and used GATK (*14*) for variant calling. Only variants that were detected in at least two splits were kept. With the two-split detection criterion, the amplification error rate is 8.3×10^{-10} per base, which corresponds to five false positives per genome, underscoring the successful filtering of the vast majority of amplification errors in our approach (Supplementary **Methods**).

In **Fig. 2A**, we plotted the number of detected heterozygous germline mutations by allele frequency (AF) values (**Table S2**) for MCF10A cells. The Gaussian distribution centered at 0.5 demonstrates the evenness of our amplification. To identify *de novo* variants, we need to filter out all the germline SNVs detected in the bulk sequencing data. Here, we implemented the zero-read criterion: only the variants not detected in the bulk sequencing data were defined as *de novo* variants in single cells. The single-cell statistics of *de novo* variants can be found in **Table S3**. Surprisingly, even after effective filtering of amplification errors as described above, we still detected 10,634±1,845 *de novo* variants per MCF10A cell. In **Fig. 2B**, we

plotted the distribution of AF values for these presumed *de novo* variants. These variants center at a low AF value (AF=0.1), in contrast to germline mutations which center at 0.5 (**Fig. 2A**). This different AF distribution strongly suggests that the detected *de novo* variants did not originate from mutations. The low AF value indicates that they are originated from single-stranded variants, which is likely to be associated with single-stranded DNA lesions. The distribution of the AF values for single-stranded variants should center at 0.25, however, considering that the AF values of half of dSNVs are less than 0.25, we expect to observe that the AF distribution of dSNVs peaks at AF=0.1 as observed in **Fig. 2B**.

For single cortical neurons, we observed similar AF distributions for germline mutations and *de novo* variants (**Fig. 2C-D**). The average level of the detected dSNVs in 18 single neurons is 16,274±725 per neuron, which is significantly higher than the dSNVs detected in single MCF10A cells. Next, based on the genome coverage in each split, we can estimate the detection rate of dSNVs (please refer to Methods for detailed procedure and formula) and the total DNA lesion level in single cells can be readily estimated (**Table S3**).

To demonstrate the ability in detecting DNA lesions, we applied LPSSAR to the cells damaged by hydrogen peroxide (H_2O_2). Only five minutes of H_2O_2 treatment was used to avoid introducing significant mutagenesis. After sequencing, we observed that the number of DNA lesions is significantly increased in H_2O_2-treated cells (**Fig. 2E**). The increase of dSNVs in all six subtypes of variants is consistent with the known DNA lesion pattern caused by H_2O_2 treatment (**Fig. S3**) (*15*).

In **Fig. 2F-G**, we plotted the complete array of the detected dSNVs for MCF10A and cortical neurons respectively. Based on the knowledge of the major forms of DNA lesions occurred to the genome, dSNVs of C->T/G->A are associated with cytosine oxidation (*16, 17*), the dSNVs of A->G/T->C are potentially associated with adenine oxidation followed by deamination (*10*), and the dSNVs of G->T/C->A are associated with 8-oxoguanine (*18, 19*) . Among all six types of dSNVs, the major contribution to dSNVs is from the transition variants: C->T/G->A and A->G/T->C. Next, we compared the trinucleotide signature of dSNVs to the COSMIC mutational signatures (*20*). In **Fig. 2H-I**, we showed the trinucleotide signature of dSNVs for MCF10A cells and neurons. The COSMIC mutational signature with the highest similarity score is SBS30 with cosine similarity of 0.86 (**Fig. 2J**). In a recent knockout study of *NTHL1* gene, SBS30 has been also shown as the mutational signature of *NTHL1* deficiency (*21*). Our result indicates that *NTHL1* gene encodes the proteins that repair the C->T/G->A damage --- the major component of dSNVs. Therefore, *NTHL1* deficiency could lead to severe mutagenesis. Indeed, it has been reported that *NTHL1* deficiency is highly mutagenic and promotes adenomatous polyposis and colorectal cancer (*22*).

Next, we calculated dSNV densities in different functional regions. In both MCF10A cells and neurons, different regions have different dSNV densities (**Fig. 3A-B, Table S4**), which strongly supports that the dSNVs detected by LPSSAR are biologically genuine. We observed that the dSNV densities in the exon and promoter regions are significantly lower than the intron and intergenic regions ($p < 0.0001$). Between exon and promoter regions, the dSNV densities in promoter regions are lower ($p < 0.0001$). Between the intron and intergenic regions, dSNV densities in the intergenic regions are lower ($p = 0.018$). The potential mechanisms for all these differences are likely due to different levels of DNA assault or different levels of repairing efficiency in different functional regions.

To further investigate the distribution of dSNVs on the genome of neurons, we plotted the histogram of the dSNV densities in 200 kb windows of genome (**Fig. 3C**). The total number of 18 neurons allows the sufficient sampling of dSNV density in each binning window. The distribution of dSNV densities can be fitted into two Gaussians with the goodness of R^2=0.99 (**Fig. 3C**), indicating the existence of two types of DNA damage modules: high-damage and low-damage modules. In the topological profiling of the genome by Hi-C experiments, A/B compartments have been observed as a generic feature of 3D genome across different cell types (*23-27*). To investigate the potential relation between our DNA damage distribution and topological compartments in human prefrontal cortex (*24*), we compared the dSNV densities in the A compartment versus B compartment. As shown in **Fig. 3D**, the significant difference between the dSNV densities in the A and B compartments suggests that the abundance of DNA lesions depends on the topological structure of the genome.

Next, we calculated the Z score of dSNV density for each window. In **Fig. 3E**, we plotted the Z scores of each window along the chromosome: the Z-score is shown as the height of the bar. The color indicates whether the window belongs to A or B compartment. We observed that the majority of the binning windows with positive Z-scores (high dSNV density) are red colored (B compartment); the majority of the binning windows with negative Z-scores (low dSNV density) are blue colored (A compartment). The consistent pattern between Z-score and the compartment assignment strongly indicates that A/B compartments influence the abundance of DNA damage along the genome. The reason for this phenomenon could be due to that DNA repair is less efficient due to limited accessibility to genome in the relatively closed B compartments. Furthermore, we showed that the human essential genes are preferentially located in A compartments (**Fig. 3F**) as well as in the low-damage regions (**Fig. 3G**).This observation indicates that the genome topology shapes the landscape of DNA damage along the genome, which results in different enrichment of essential genes in different topological compartments. The list of human essential genes was summarized based on previous studies (*28-31*).

Besides the detection of dSNVs by LPSSAR, next we showed that we can reliably estimate the level of *de novo* mutations in single cells. With the observation of the large number of dSNVs existing in single cells, it is worth pointing out that without effective filtering of dSNVs, the levels of *de novo* mutations cannot be accurately determined. In particular, when the amplification bias leads to strand dropouts as shown in **Fig. 4A**, it becomes difficult to distinguish mutations from dSNVs. To test different criterions for detecting *de novo* mutations, we performed a single-cell expansion experiment as shown in **Fig. 4B**. Firstly, by comparing the sequencing data of Bulk S1 and S2, we identified the somatic mutations existing in the original cell selected for single-cell expansion (937 somatic mutations with TsTv ratio of 0.87). Next, we performed LPSSAR for three single cells isolated from the expanded clone. With the three single-cell sequencing data, different criterions for filtering out the dSNVs were then tested. We found out that only at the loci that satisfy this stringent filtering criterion: the variants were detected in all three splits with AF larger than 0.4 and variant calling scores (Q) larger than 1000, can we separate the *de novo* mutations from dSNVs (**Fig. 4C and Fig. S4**). In contrast, without the stringent AF/Q criterion, we cannot effectively separate *de novo* mutations from dSNVs, which underscores the advantage of our split-amplification strategy and the necessity of applying the stringent AF/Q criterion (**Fig. 4D**).

We then estimated the total number of *de novo* mutations in single cells based on the detection rate of germline mutations under the same criterion. As a result, we estimated that the average level of *de novo* mutations in three single cells from the expanded clone is 1038±97 with the TsTv ratio of 0.9 (**Fig. S5**), which is consistent with the true level of *de novo* mutations detected by comparing two bulk sequencing data (Bulk S1 and S2). We also compared the three single cells (S2A-C) to the bulk sequencing data of S2 to estimate the *de novo* mutations introduced during the single-cell expansion, which are 37, 44 and 61, respectively (**Fig. S6**). This result shows that the mutation rate during the single-cell expansion is about two mutations per division.

Next, we applied the same filtering criterion and estimated that the average number of *de novo* mutations in the single cortical neurons are 90.5±24.9, 81.8±16.1 and 79.7±37.8 for the three brain samples (**Fig. S7-9)**. Our estimated *de novo* mutations are slightly lower than the recent single-cell expansion-based measurement (*32*). The reason for this difference is probably due to the introduction of new mutations during the single-cell expansion process, especially under stress condition at the early stage of expansion.

Recently, Ochs *et al*. has shown that genome topology plays important roles in safeguarding the genome integrity in response to the double strand breaks (*33*). In this report, we showed that the genome topology also influences the degree of spontaneous DNA damage. Overall, with the ability for simultaneous measurement of *de novo* mutations and dSNVs, LPSSAR offers an unprecedented approach to study

genome stability and heterogeneity in various biological and disease systems at single-cell resolution.

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Author contributions:

C.Z. designed the project. M.G. and K.S. contributed to the early development of linear preamplification with double strand conversion strategy. Q.Z. and Y.N. optimized the linear preamplification and developed pipetting-based MDA reaction. Q.Z. and Y.N. performed the LPSSAR experiments of MCF10A and cortical neurons. Y.N. and M.N. performed the single cell expansion experiment of MCF10A. C.Z. and Y.N. performed the data analysis. C.Z. wrote the manuscript. Baylor College of Medicine applied a patent that covers the chemistry of LPSSAR.

Fig. 1: The reaction scheme of LPSSAR.

Fig. 2: The measurement of dSNVs in MCF10A cells and neurons. (**A**) The histogram of allele frequency (AF) distribution of heterozygous germline mutations in single MCF10A cells. The Gaussian distribution centered at 0.5 indicates the evenness in our amplification. (**B**) The histogram of AF distribution of *de novo* variants in single MCF10A cells. The distribution centered at low AF values supports the detection of damage associated variants. (**C**) The histogram of AF distribution of heterozygous germline mutations in neurons. (**D**) The histogram of AF distribution of *de novo* variants in neurons. (**E**) The comparison of the total number of dSNVs between the MCF10A cells with and without hydrogen peroxide treatment ($P < 0.01$). (**F**) The total number of different types of dSNVs of the three single MCF10A cells at G0 phase. (**G**) The total number of different types of dSNVs of all sequenced neurons. (**H**) The trinucleotide signature of dSNVs detected in the MCF10A cells. The dSNV signature resembles COSMIC SBS30 (cosine similarity = 0.86). (**I**) The trinucleotide signature of dSNVs detected in all neurons. (**J**) The trinucleotide signature of COSMIC SBS30.

Fig. 3: The genomic distribution of dSNVs in MCF10A cells and neurons. (**A**) The dSNV densities (number per million bases) in different genomic regions (exon, promoter, intron, and intergenic regions) in MCF10A cells. (**B**) The dSNV densities in different genomic regions (exon, promoter, intron, and intergenic regions) in all neurons. (**C**) The histogram of the dSNV densities in 200 kb binning windows in the genome of neurons. The distribution is fitted into two Gaussians with $R^2 = 0.997$: $F(x) = 163.8 \times \exp\left(-\left(\frac{x-1.24}{1.601}\right)^2\right) + 378.1 \times \exp\left(-\left(\frac{x-2.315}{0.904}\right)^2\right)$. (**D**) The dSNV densities of neurons in A compartments and B compartments. A/B compartment is determined at 1 Mb resolution. (**E**) The Z-score of the dSNV density in each binning window along the genome (window size: 1 Mb). The A compartments are blue colored, and B compartments are red colored. (**F**) Essential genes are more enriched in the A compartments than the B compartments. (**G**) Essential genes are more enriched in the low-damage regions (dSNV density Z-score < 0) than the high-damage regions (dSNV density Z-score > 0).

Fig. 4: The measurement of *de novo* mutations in MCF10A cells. (**A**) Different scenarios of strand dropout causing the technical challenge in distinguishing *de novo* mutations from dSNVs. (**B**) The single-cell expansion scheme for validating the accuracy in estimating the total number of *de novo* mutations in single cells. (**C**) The histogram of the detected *de novo* variants and true *de novo* mutations on the axis of the variant calling scores with the requirement of AF > 0.4 in all three splits. (**D**) The histogram of detected *de novo* variants and true *de novo* mutations on the coordinates of variant calling scores without the AF criterion.

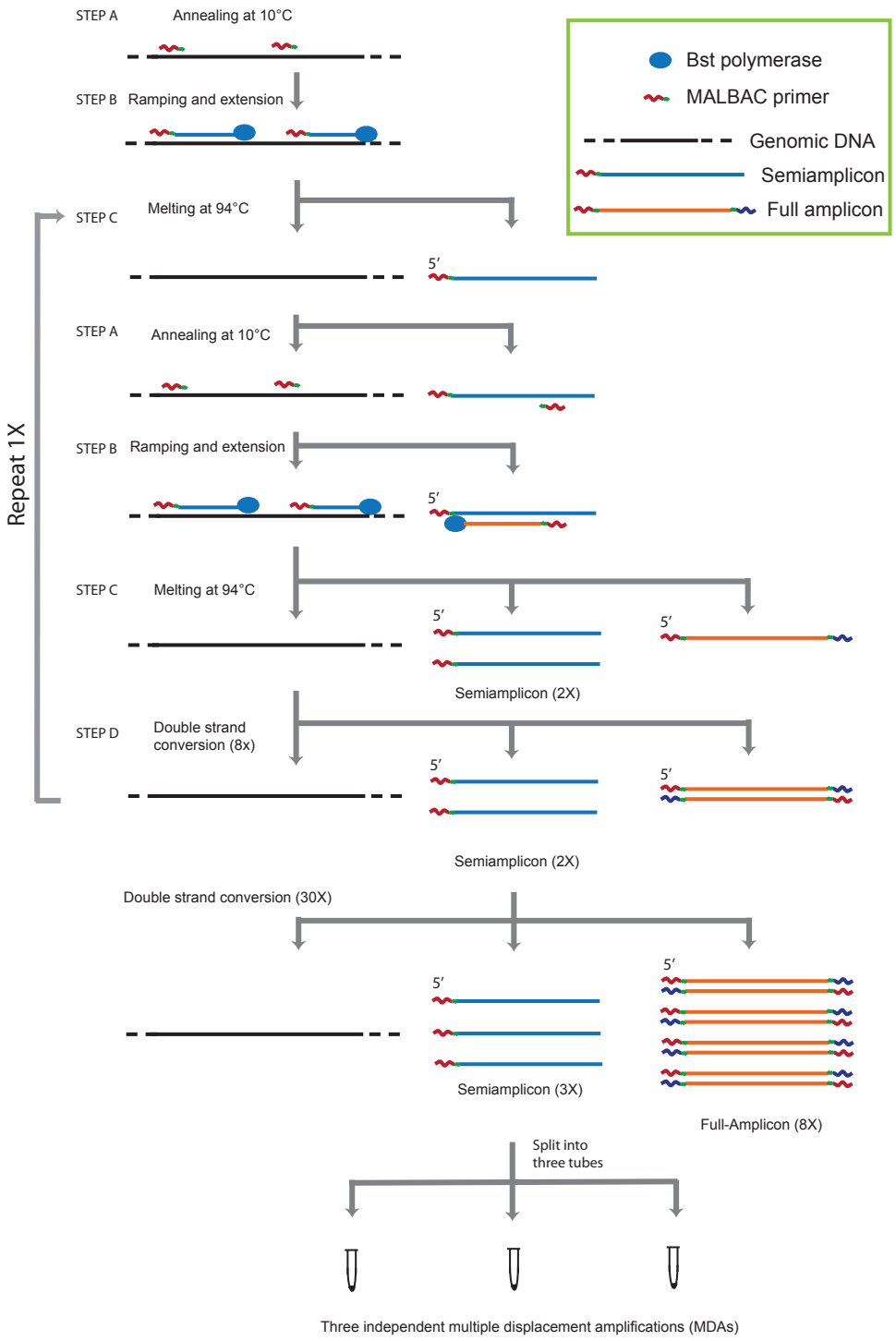
Supplementary Materials:

Materials and Methods

Fig. S1-S10

Table S1-S4

Figure 1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Full amplicon
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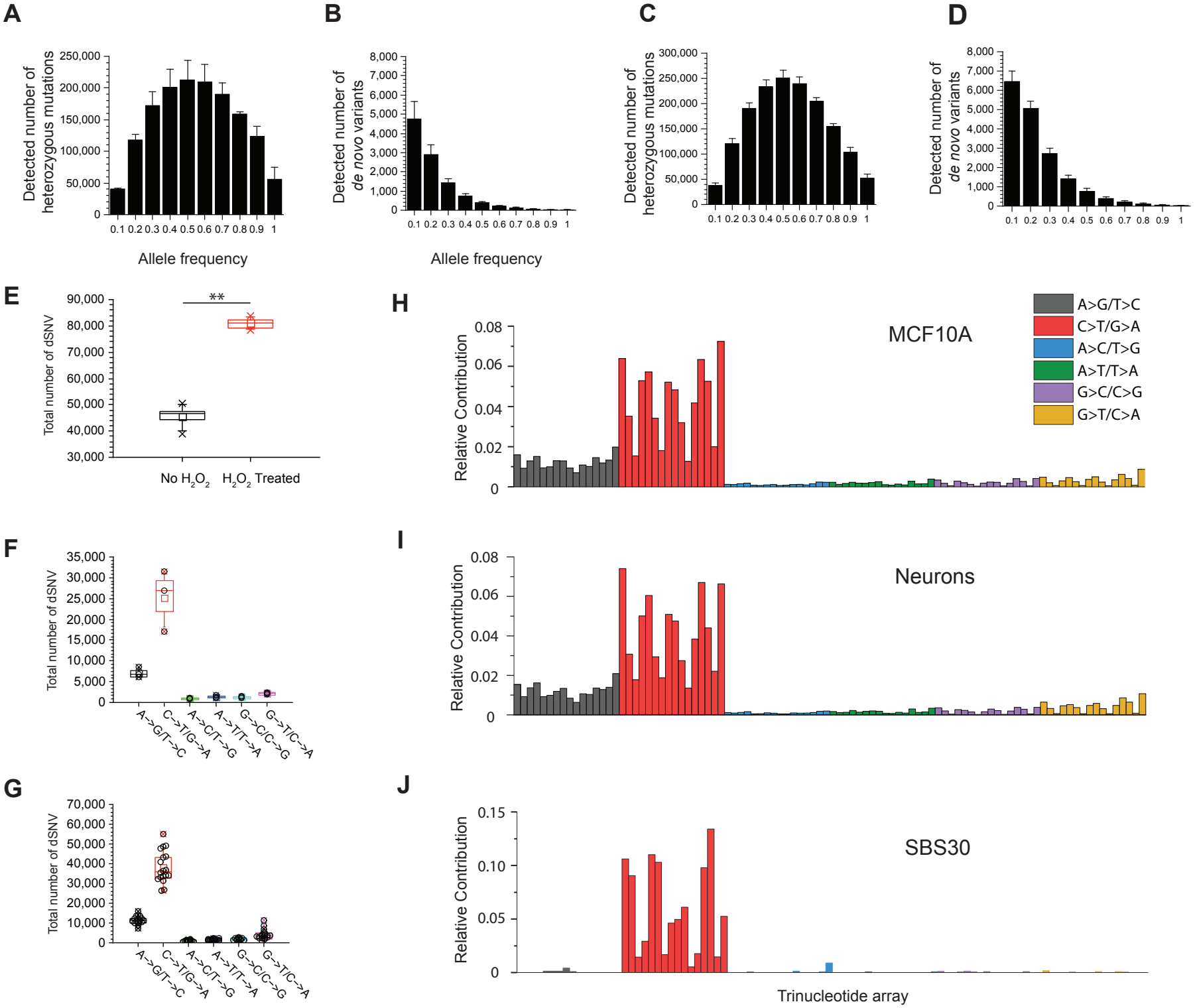
5′
STEP A
Annealing at 10°C
STEP B
Ramping and extension
5′
Repeat 1X
STEP C
Melting at 94°C
5′
5′
Semiamplicon (2X)
STEP D
Double strand conversion (8x)
5′
5′
Semiamplicon (2X)
Double strand conversion (30X)
5′
5′
Semiamplicon (3X)
Full-Amplicon (8X)
Split into three tubes
Three independent multiple displacement amplifications (MDAs)

Figure 2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Allele frequency
B
Detected number of de novo variants
Allele frequency
C
Detected number of heterozygous mutations
D
Detected number of de novo variants
E
Total number of dSNV
**
No H2O2
H2O2 Treated
F
Total number of dSNV
A->G/T->C
C->T/G->A
A->C/T->G
A->T/T->A
G->C/C->G
G->T/C->A
G
Total number of dSNV
H
MCF10A
Relative Contribution
A>G/T>C
C>T/G>A
A>C/T>G
A>T/T>A
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G>T/C>A
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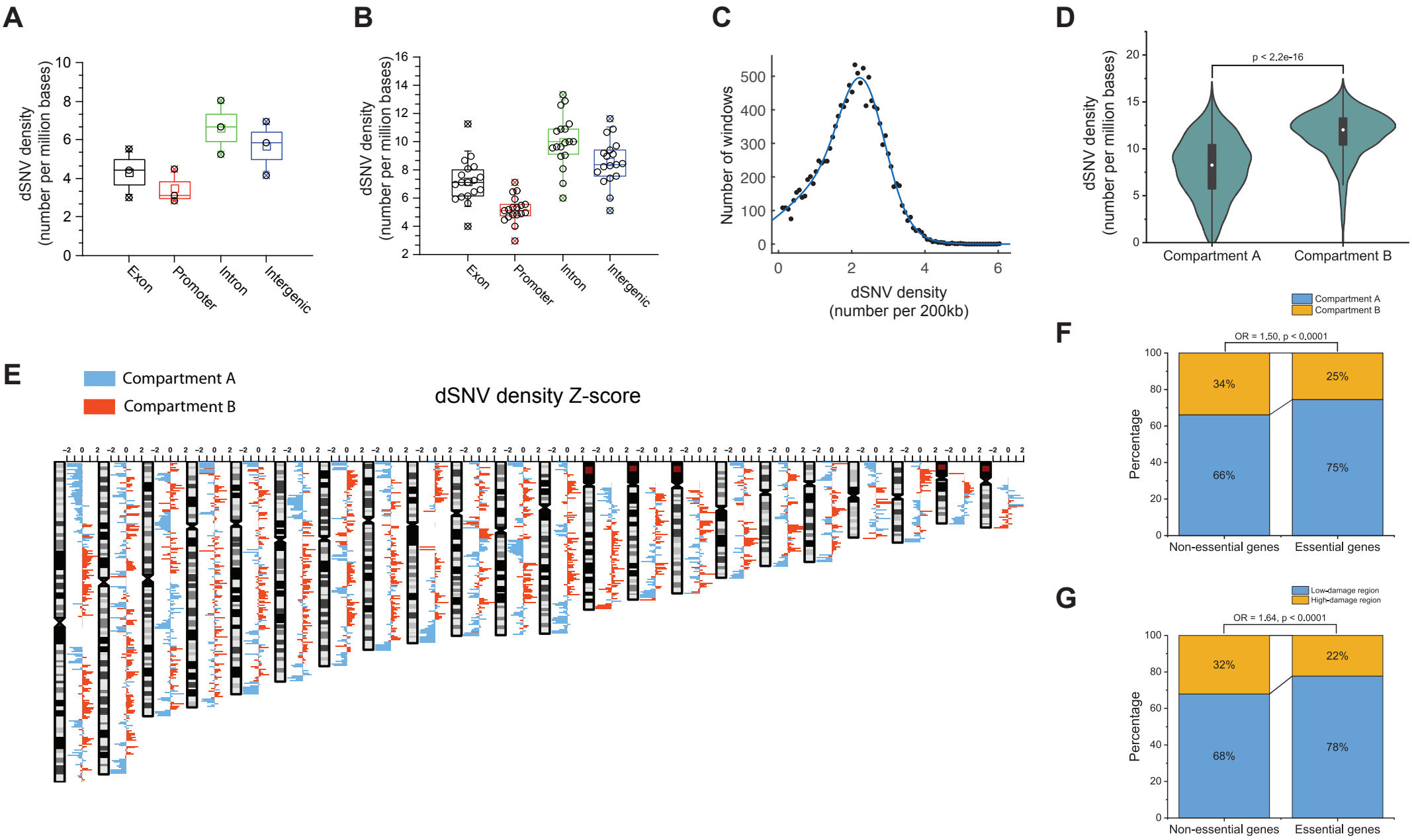
Neurons
Relative Contribution
J
SBS30
Relative Contribution
Trinucleotide array

Figure 3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(number per million bases)
Exon
Promoter
Intron
Intergenic
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dSNV density
(number per million bases)
Exon
Promoter
Intron
Intergenic
C
Number of windows
dSNV density
(number per 200kb)
D
dSNV density
(number per million bases)
p < 2.2e-16
Compartment A
Compartment B
E
Compartment A
Compartment B
dSNV density Z-score
F
Compartment A
Compartment B
OR = 1.50, p < 0.0001
Percentage
34%
25%
66%
75%
Non-essential genes
Essential genes
G
Low-damage region
High-damage region
OR = 1.64, p < 0.0001
Percentage
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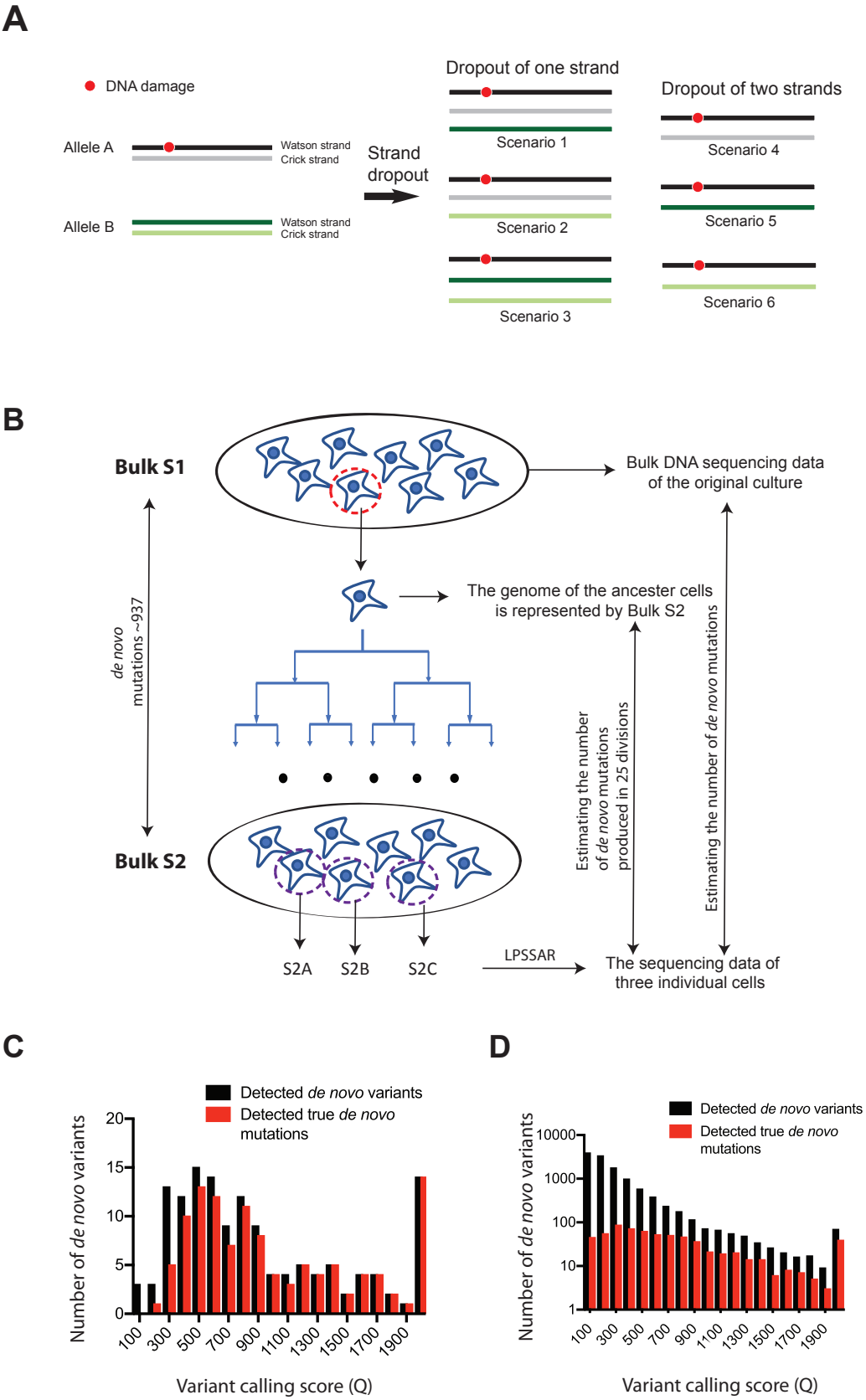
68%
78%
Non-essential genes
Essential genes

Figure 4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Watson strand
Crick strand
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Watson strand
Crick strand
Strand dropout
Dropout of one strand
Scenario 1
Scenario 2
Scenario 3
Dropout of two strands
Scenario 4
Scenario 5
Scenario 6
B
Bulk S1
Bulk DNA sequencing data of the original culture
The genome of the ancester cells is represented by Bulk S2
de novo mutations ~937
Estimating the number of de novo mutations produced in 25 divisions
Estimating the number of de novo mutations
Bulk S2
S2A
S2B
S2C
LPSSAR
The sequencing data of three individual cells
C
Detected de novo variants
Detected true de novo mutations
Number of de novo variants
Variant calling score (Q)
D
Detected de novo variants
Detected true de novo mutations
Number of de novo variants
Variant calling score (Q)