

The spectrum of NHEJ products following CRISPR/Cas9 DNA cleavage is target site dependent



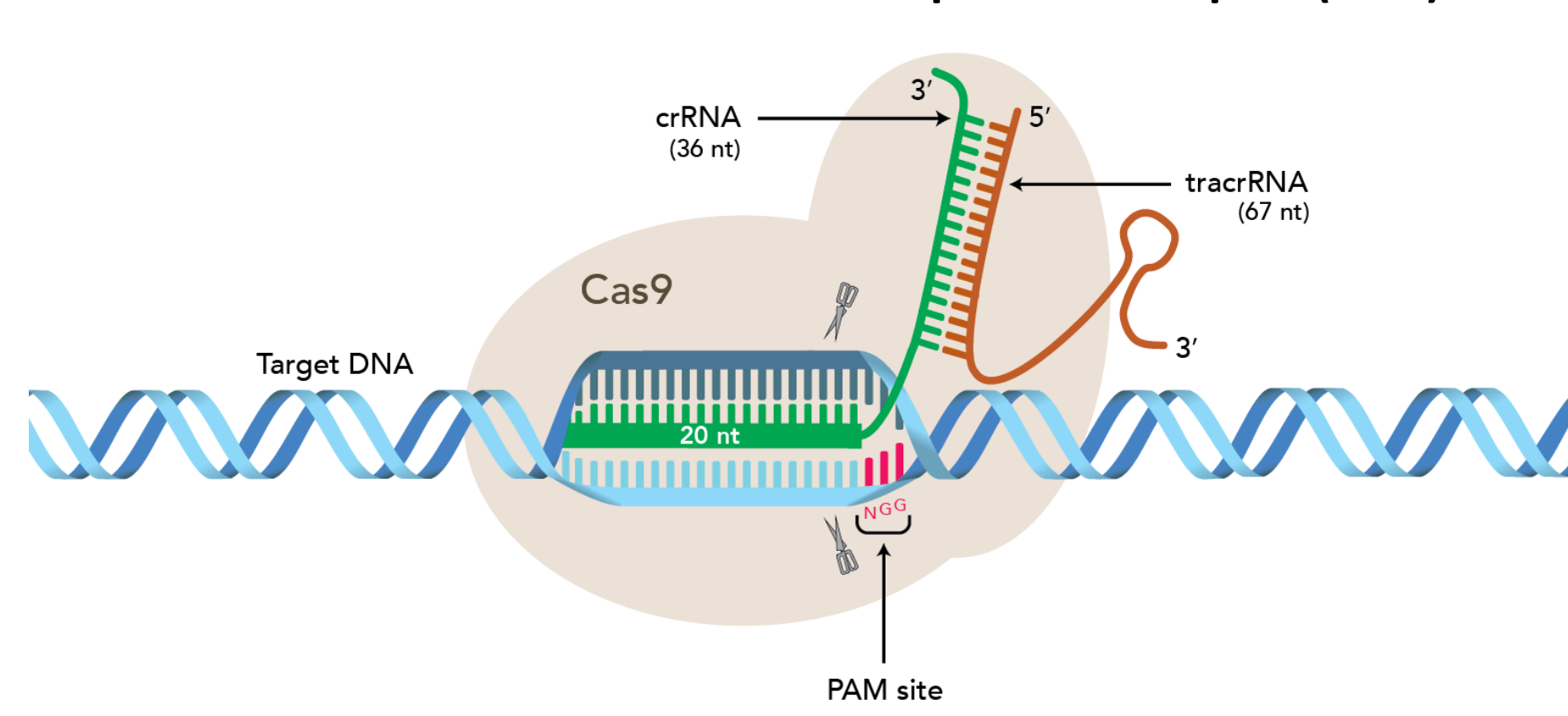
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Introduction

The *S. pyogenes* Cas9 nuclease produces a double-stranded cut in genomic DNA at a site directed by an associated guide RNA (gRNA). This double-stranded break is closed by endogenous repair mechanisms of which non-homologous end joining (NHEJ) is most prevalent. The products of this repair process vary widely and can include small or large insertion and/or deletion events (indels).

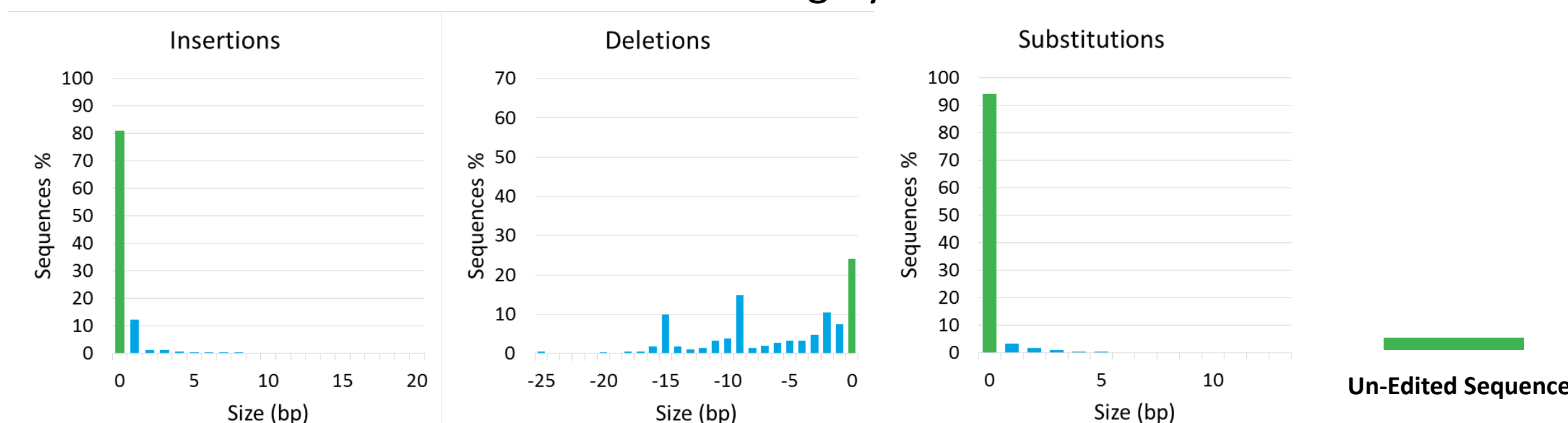
Interestingly, the products of NHEJ repair using a given gRNA results in a unique editing profile at the targeted locus. The profile of indels varies by site, and the unique profiles are reproducible which gives a "fingerprint" of mutations characteristic of that specific gRNA. We utilized next-generation sequencing (NGS) to interrogate on-target editing events for a number of gRNAs, comparing various methods of CRISPR/Cas9 delivery. NGS analysis demonstrated consistent indel profiles associated with a given gRNA that were independent of delivery mode (i.e. RNA-only vs. ribonucleoprotein complex), method of delivery (ie, lipofection vs. electroporation), and human cell line (HEK293 vs. K562). Furthermore, we see that the use of single-stranded, non-homologous carrier DNA enhances editing efficiency in nucleofection of RNP complexes into HEK293 cells as well as K562 cells without changing the editing profile.

Alt-R™ crRNA:tracrRNA Ribonucleoprotein Complex (RNP)

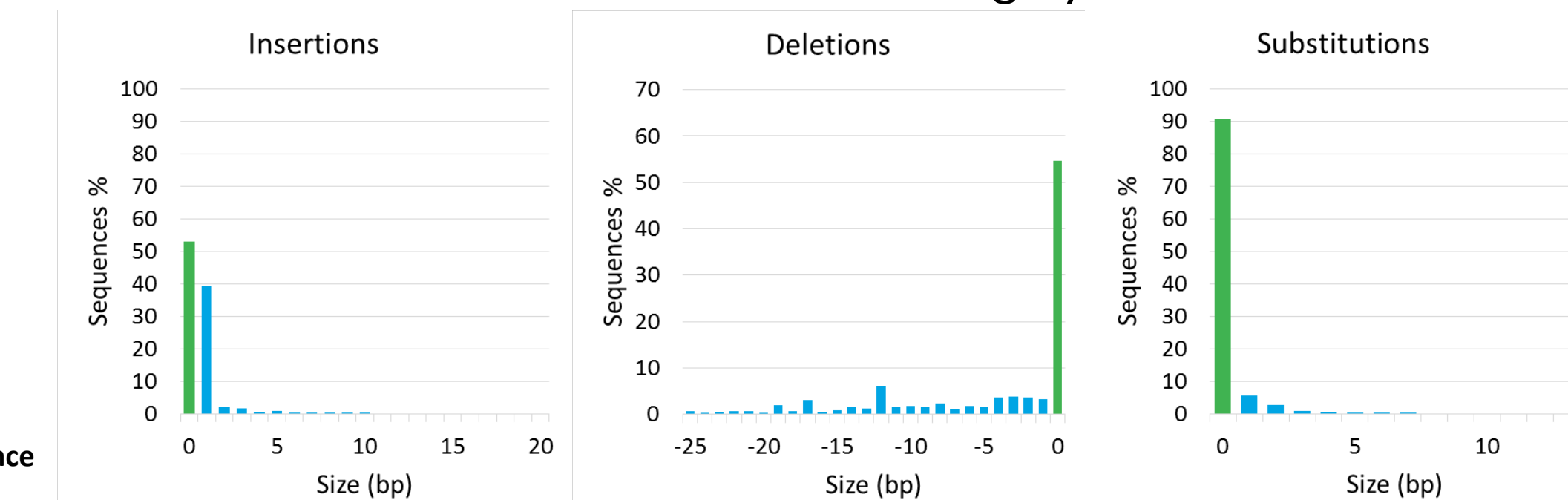


Editing profiles at multiple target sites

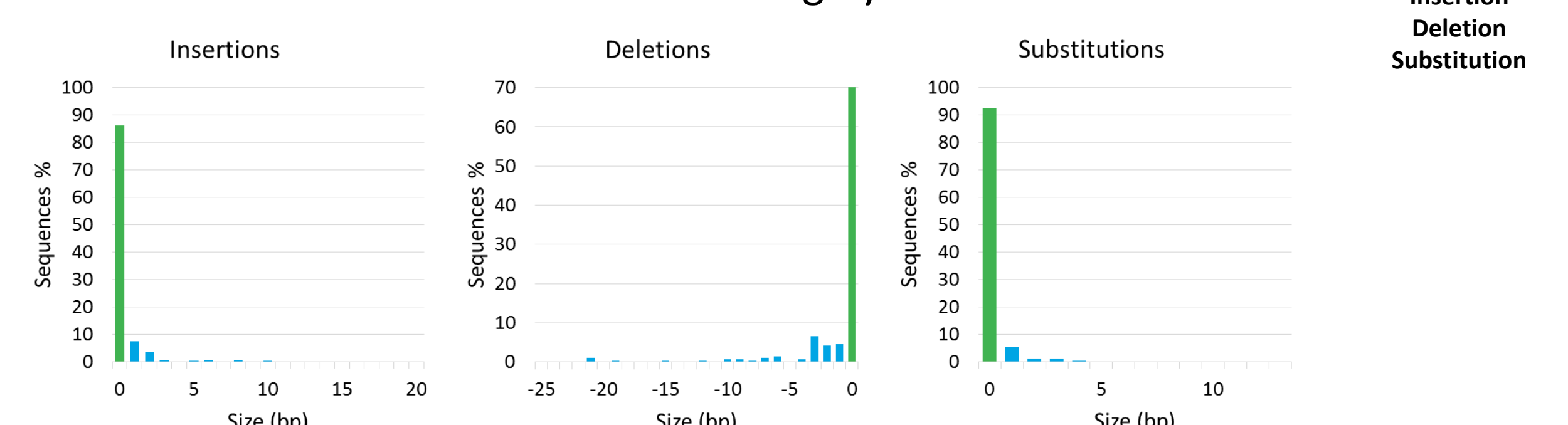
Human HPRT 38087 crRNA – 94.3% Editing by NHEJ



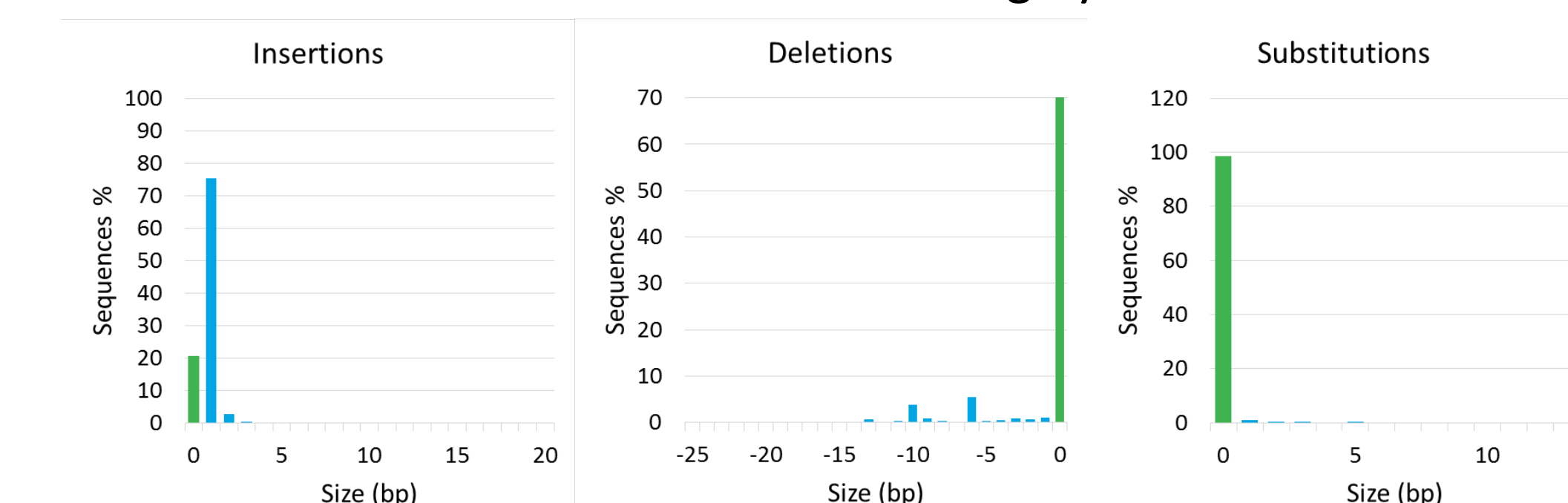
Human HPRT 38285 crRNA – 93.4% Editing by NHEJ



Human HPRT 38509 crRNA – 36.6% Editing by NHEJ



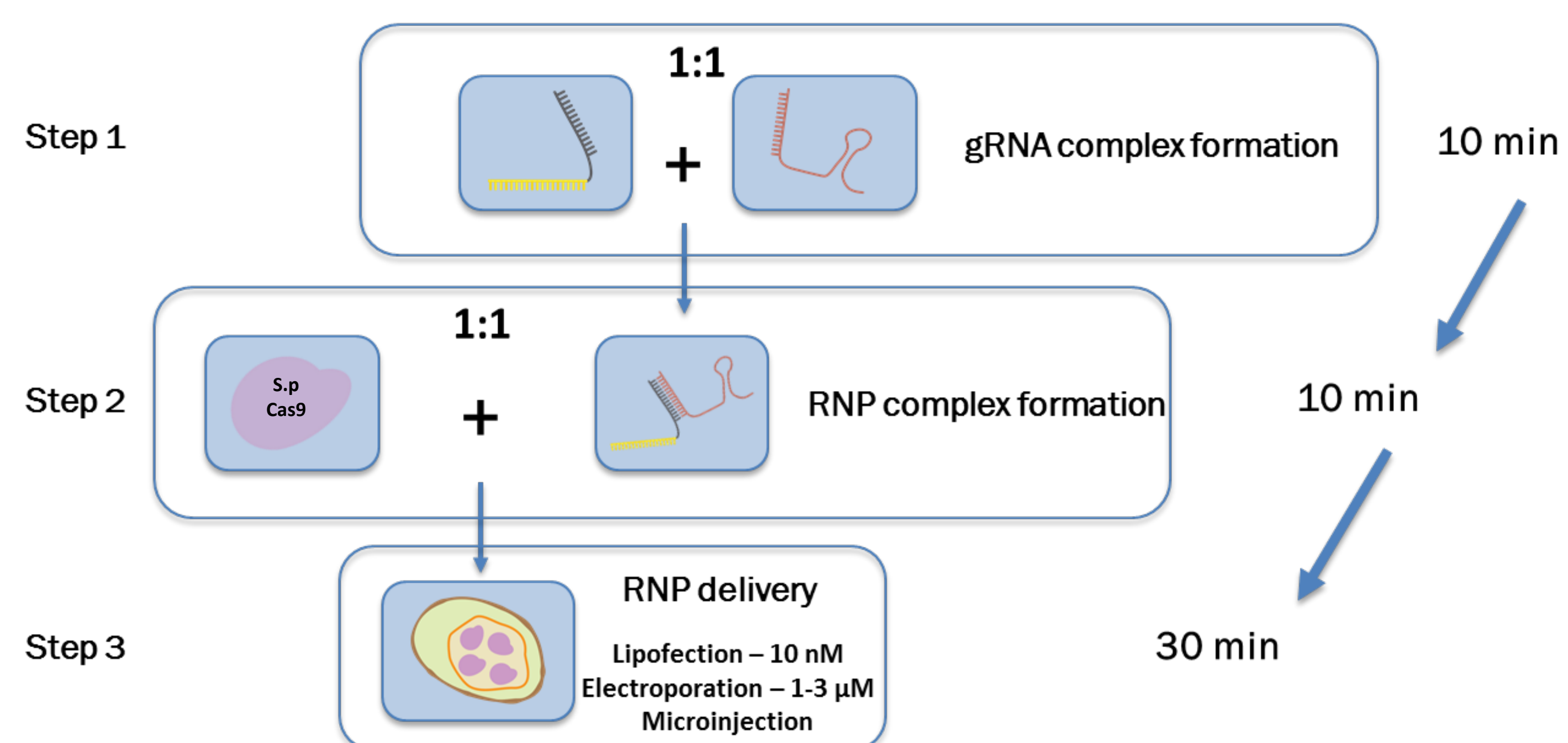
Human HPRT 38636 crRNA – 95.8% Editing by NHEJ



The overall profile of insertion/deletion/substitution events are unique for different genomic DNA target sites. Chemically modified Alt-R™ RNA oligonucleotides targeting various sites in *HPRT1* were reverse transfected using RNAiMAX™ (30 nM gRNA) into HEK293 cells that stably express *S. p.* Cas9. Total editing was assessed 48 hrs post-transfection using locus-specific amplification by KAPA HiFi Polymerase (KAPA), Nextera™ Library Prep (Illumina), and sequencing of the adapter-ligated library on a MiSeq™ (Illumina). Data was analyzed programmatically using freely available software - CRISPResso (Pinello, et al. doi: 10.1038/nbt.3583).

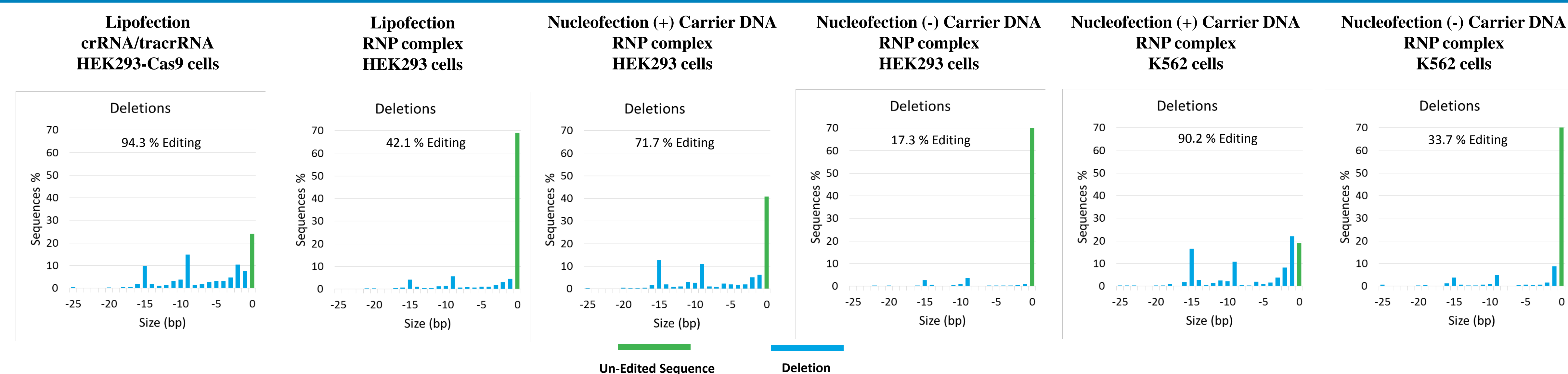
Alt-R™ RNP complex formation

Simple, 3-Step ribonucleoprotein (RNP) delivery

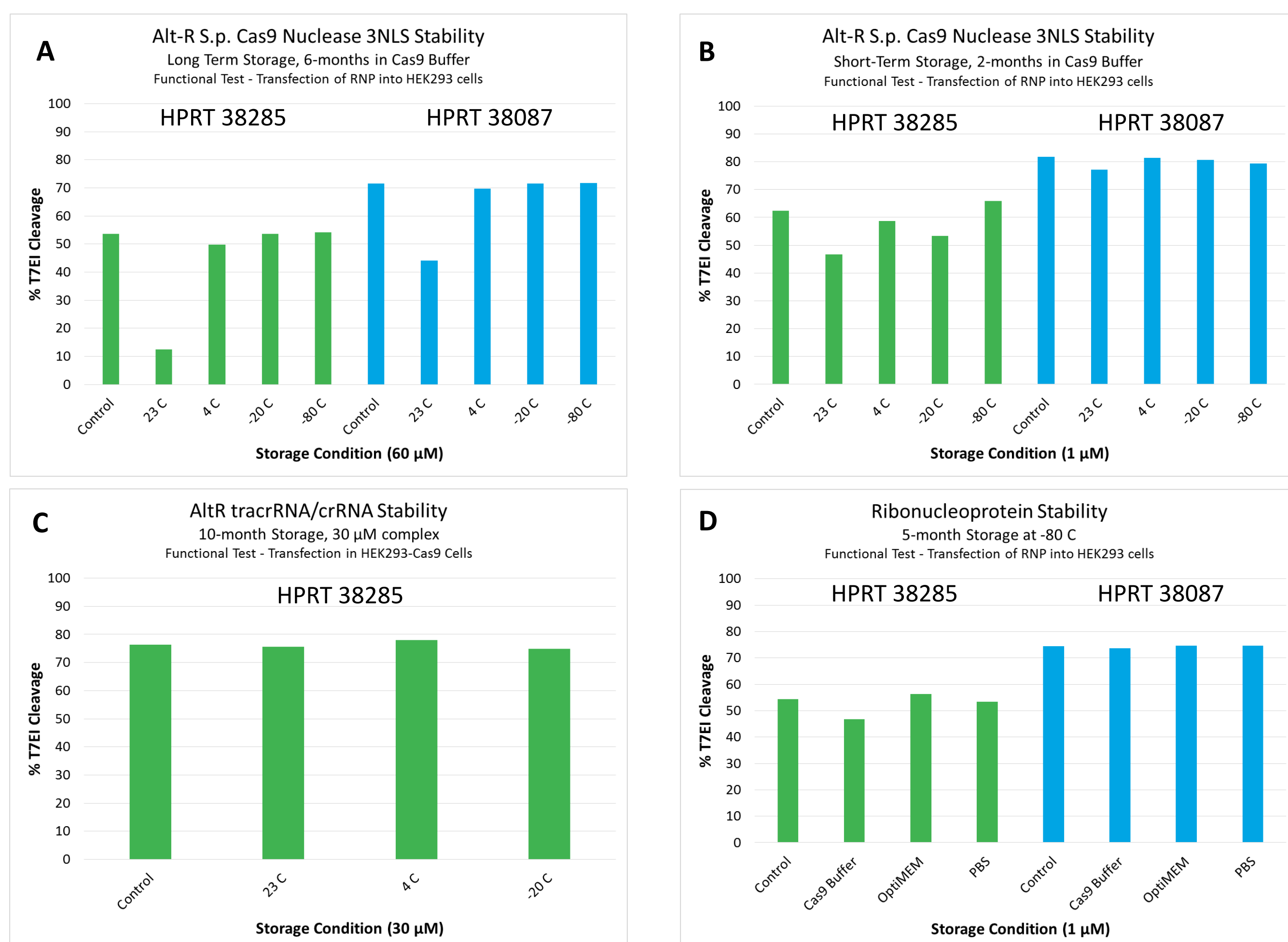


Formation of the RNP complex. The Alt-R™ crRNA and tracrRNA are combined in equimolar amounts to the desired final concentration (i.e. 1 – 50 μM), then heated to 95°C for 5 min and slowly cooled to room temperature. Next, this gRNA complex and the Alt-R S.p. Cas9 Nuclease 3NLS are combined in equimolar amounts and incubated at room temperature for 10 min to form the RNP complex.

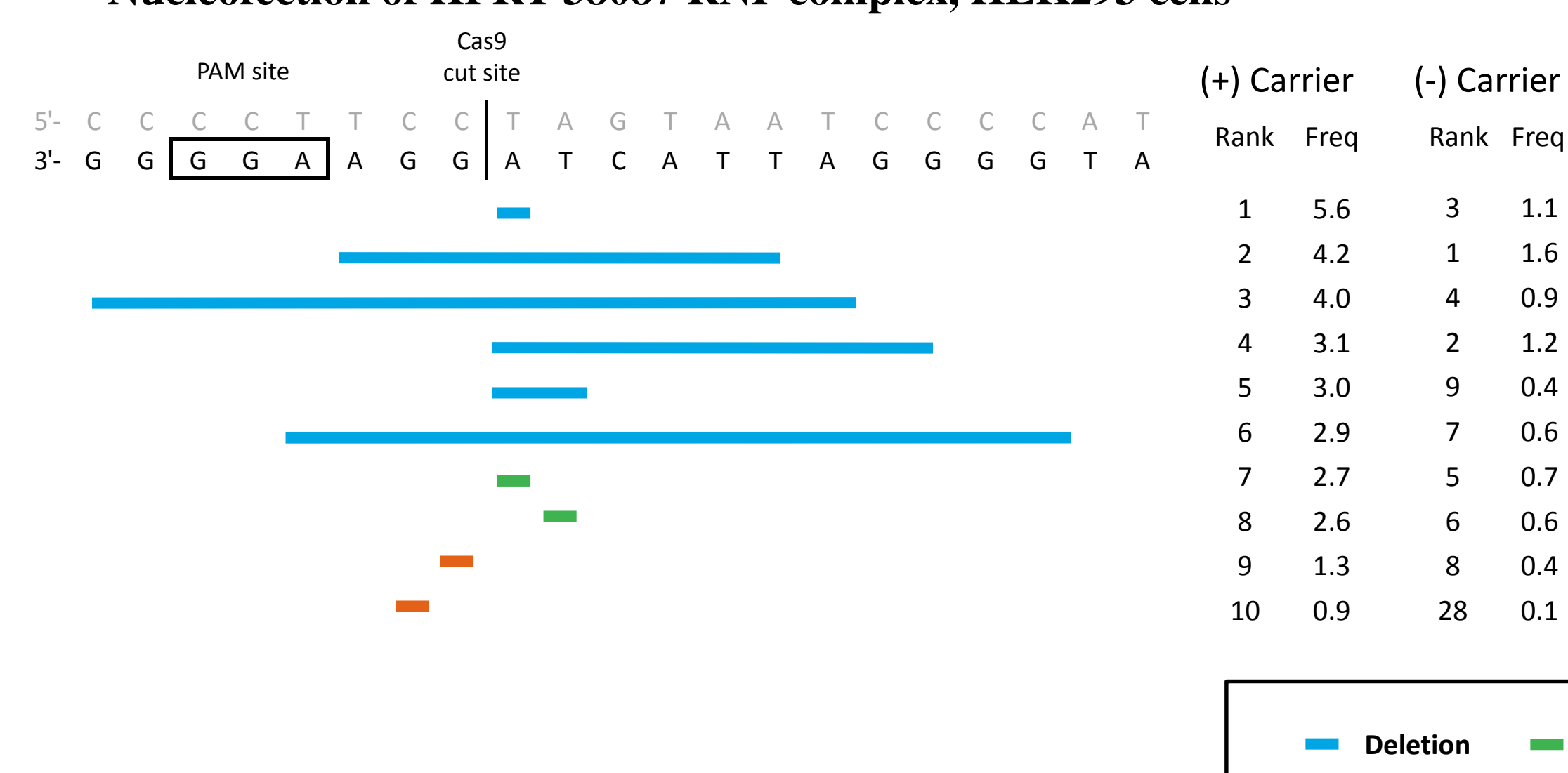
Consistent editing profile by site



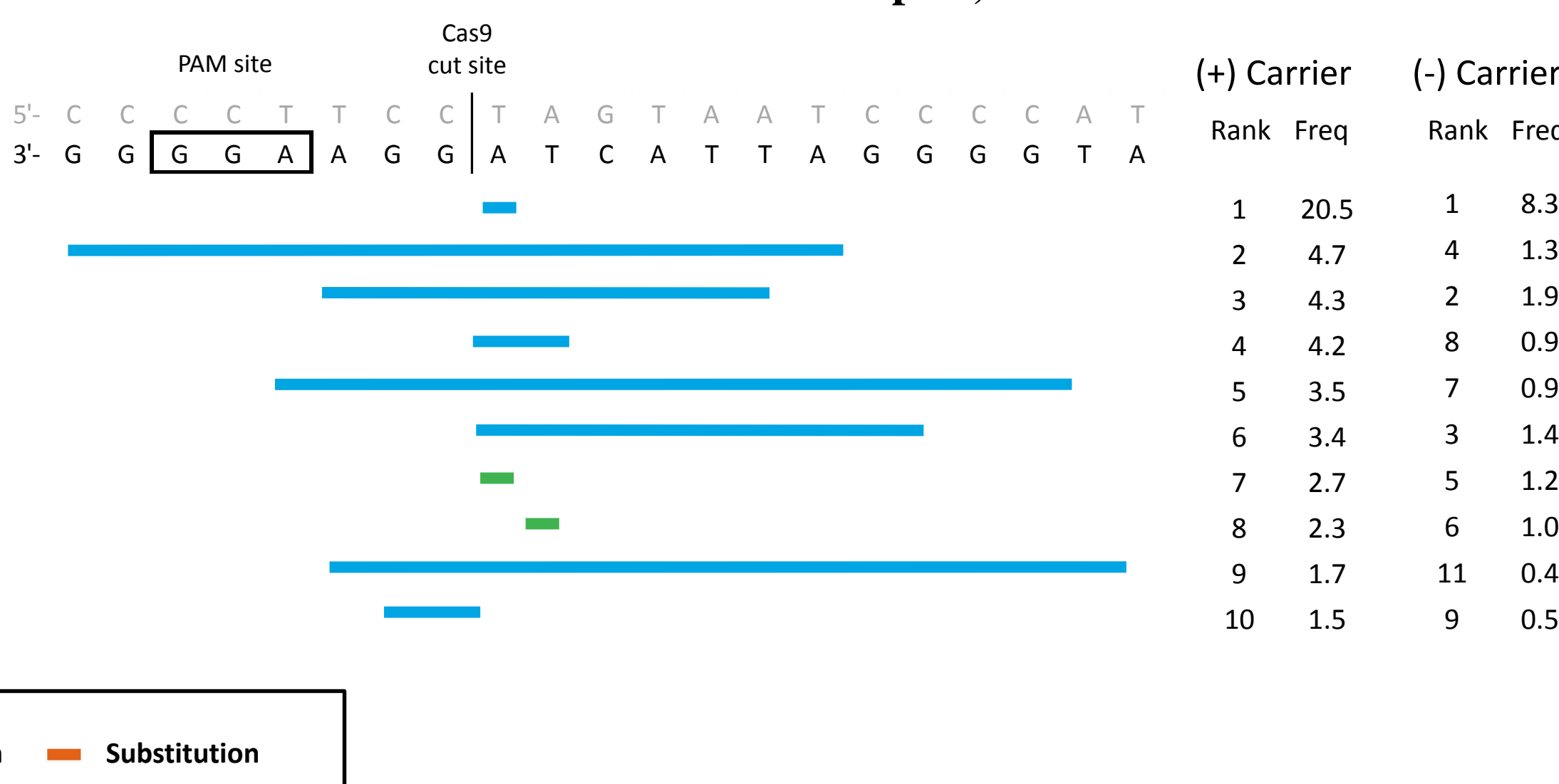
Deletion profiles from multiple treatments demonstrate highly similar editing at HPRT 38087. Prominent deletion events at -9 bp and -15 bp are consistent across multiple conditions. The non-random repair profile is in agreement with the recently published work by van Overbeek, et al (doi: 10.1016/j.molcel.2016.06.037). Alt-R™ crRNA & tracrRNA oligonucleotides were delivered to HEK293 cells via lipofection (RNA complex transfected at 30 nM with RNAiMAX™, and RNP complex delivered at 10 nM with RNAiMAX), HEK293 cells via nucleofection (4 μM RNP complex, Amaxa™ Nucleofector™ 96-well shuttle (Lonza), SF buffer +/- 4 μM non-homologous, single-stranded carrier DNA, program CM-130), and K562 cells via nucleofection (4 μM RNP complex, Amaxa 96-well shuttle, SF buffer +/- 4 μM non-homologous, single-stranded carrier DNA, program FF-120). Total editing was assessed 48 hr post-transfection using locus-specific amplification by KAPA HiFi Polymerase (KAPA), Nextera™ Library Prep (Illumina), and sequencing on a MiSeq™ (Illumina). Data was again analyzed programmatically using CRISPResso.



Nucleofection of HPRT 38087 RNP complex, HEK293 cells



Nucleofection of HPRT 38087 RNP complex, K562 cells



Editing events and relative frequencies of editing at HPRT 38087 are highly consistent in the presence or absence of nucleofection-enhancing, single-stranded DNA. The tables above indicate specific editing events following nucleofection of the RNP complex. Edits are ordered by most frequent events for the (+) carrier condition; the corresponding rank order and frequencies for the (-) carrier condition are listed for comparison showing that 9 of the 10 most abundant edits occur with and without carrier. Nucleofection of HEK293 cells and K562 cells is described above. Data was analyzed via an in-house NGS analysis pipeline. Following a soft trim, all reads were compared to a reference sequence using BLAST. From the detailed alignment data, variations were identified and their frequencies computed.

Conclusions

- Functional studies indicate that the cr/tracrRNA complex, the Cas9 nuclease, as well as the RNP complex are quite stable under multiple storage conditions and over an extended time course which makes for improved ease-of-use.
- NGS results for the four *HPRT1* target sites above demonstrate unique indel profiles.
- Different delivery methods show variations in the overall editing frequency at HPRT 38087; however, the indel profile is consistent for all treatments.
- We observe very little variation in the most frequent editing events when RNP nucleofection is carried out in the presence of carrier DNA compared to nucleofection in the absence of the carrier DNA. This finding is consistent in multiple cell lines.

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Functional testing of the Alt-R™ Cas9 nuclease, Alt-R RNA oligonucleotides and RNP complex. *S.p.* Cas9 nuclease retains full activity when stored at temperatures < 4° C for at least 6 months at 60 μM (A), and for at least 2 months when stored at -80° C as a 1 μM working dilution in Cas9 buffer (20 mM HEPES, 150 mM KCl, pH 7.5) (B). When stored at 30 μM, Alt-R RNA oligonucleotides are stable for 10 months (C). Panel D shows that the full RNP complex can be reliably stored at -80 C diluted to 1 μM in multiple buffers. Functional testing in panels A, B and D involve the formation of RNP complexes at 1 μM in Cas9 buffer and reverse transfection into HEK293 cells (RNAiMAX™, Thermo Fisher at 1.2 μl /well; 96-well plate) at a final RNP concentration of 10 nM. In C, Alt-R™ RNA complexes were formed (as noted above) and reverse-transfected (30 nM; RNAiMAX at 0.75 μl /well; 96-well plate) into HEK293 cells constitutively expressing *S.p.* Cas9. Genomic DNA was isolated for all samples using QuickExtract™ (Epicentre) after cells were incubated at 37°C, 5% CO₂ for 48 hr. Total editing efficiency was measured using a T7E1 assay (New England Biolabs).