Genome Editing-Mutant Screening

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Where to start……

Method

Crop?

Tissue culture?

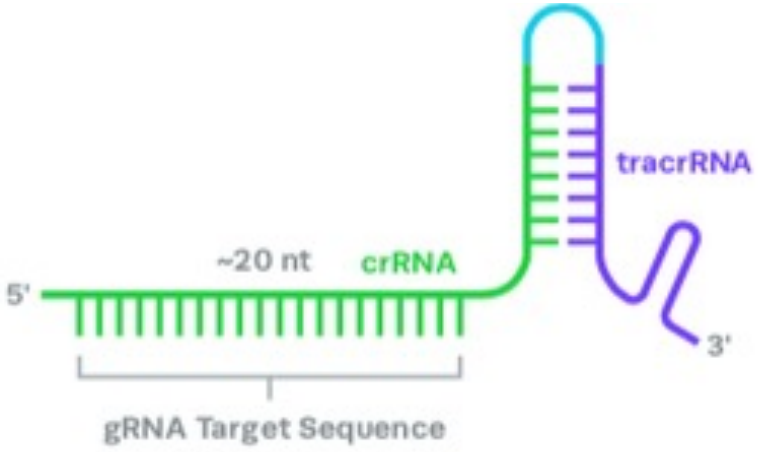
Gene Information and gRNA Design?

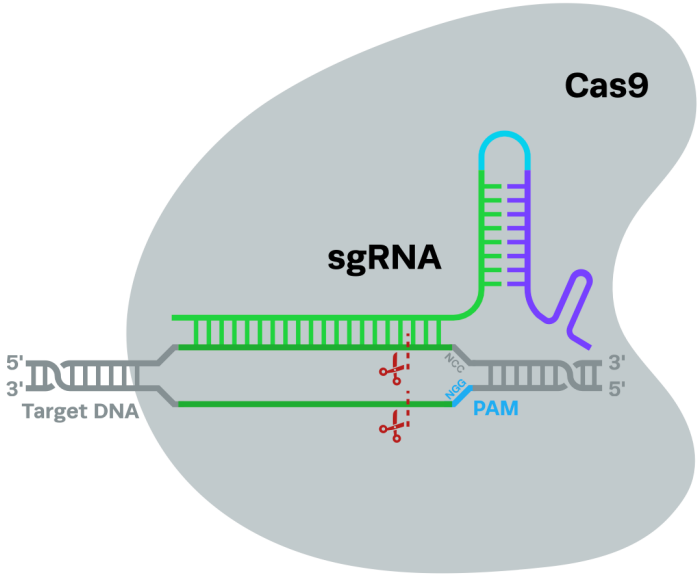
Mutant screening?

Regulation?

New Breeding Techniques (NBTs)-Targeted Gene Editing

- Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein-9 (CRISPR-Cas9)
- Directing a guide RNA (gRNA) complementary to a defined target, together with a CRISPR-associated endonuclease, Cas9, to create double-stranded DNA cleavage
- gRNA: crispr RNA (crRNA), a 17-20 nucleotide sequence complementary to the target DNA, and a tracrRNA, which serves as a binding scaffold for the Cas nuclease





https://www.synthego.com/

Molecular characterization of editing events

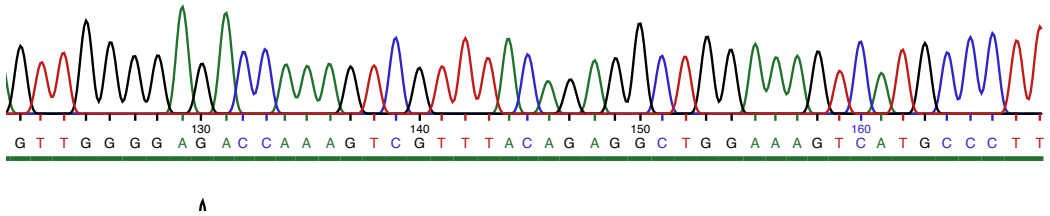
Medium-throughput assays

- T7 Endonuclease 1 (T7E1) Assay
- High-resolution fragment analysis (HRFA)

High-throughput assays

Sanger Sequencing

Amplicon Sequencing (NGS)



DNA Extraction and PCR for Initial Screening

Plant Genotyping Without DNA Purification

Requirements

- ❖ Dilution Buffer from Thermo Scientific Phire Plant Direct PCR Kit
- ❖ Sterile pipette tips (100 or 200 µL)
- ❖ Young leaves (0.5 – 1 cm)

Protocol

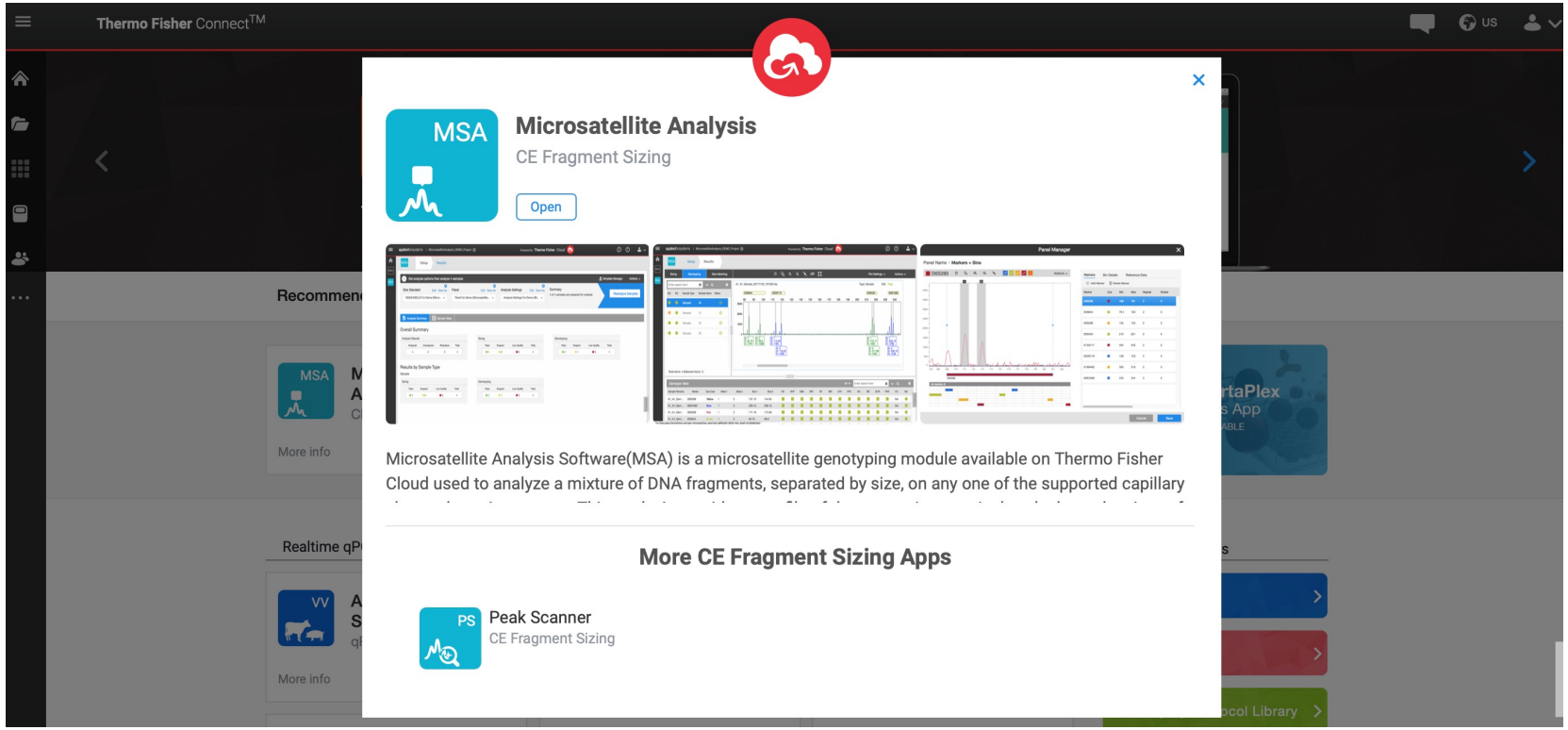
1. Take a leaf and place it in a 0.5 mL microcentrifuge tube, then add 15 µL of Dilution Buffer.
2. Crush the leaf sample with a pipette tip by pressing it briefly on the bottom or wall of the tube. The solution should have a greenish color.
3. Then, add 25 µL of sterile water. Centrifuge the tube until the leaf material settles to the bottom, and then transfer the supernatant to a new tube.
4. Use a 1.5- to 2-µL template for a 20-µL PCR reaction.
5. DNA Samples in dilution buffer can be stored for up to 4 weeks at 4 °C. For long-term storage, store at −20 °C (up to 6 months).

Tips for PCR

- ➢ Use 98°C for denaturation for 5 min.

High Resolution Fragment Analysis

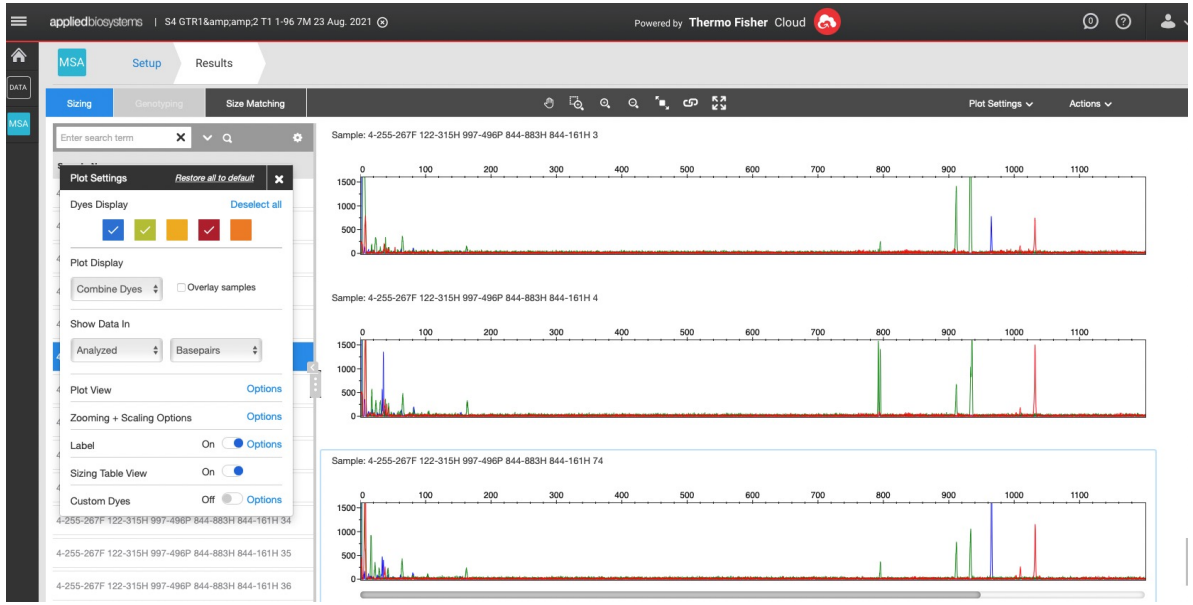
- 3500 Genetic analyzer
- PCR with one of the primer attached Florescent dyes
- 6 different dyes at the same time
- GeneMapper™ analysis
- Thermo Fisher Connect™

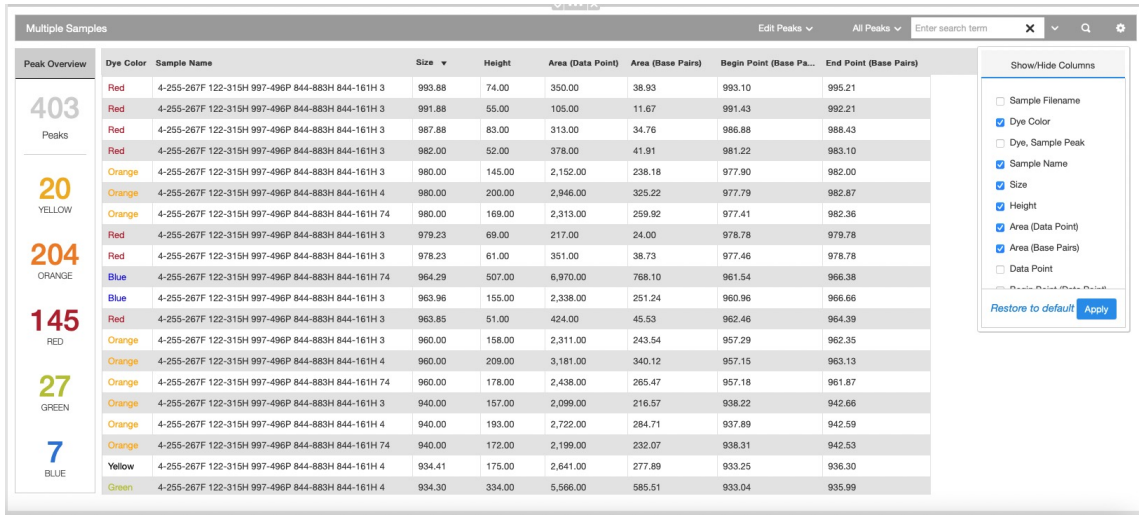


https://apps.thermofisher.com/apps/spa/#/dashboard

High Resolution Fragment Analysis

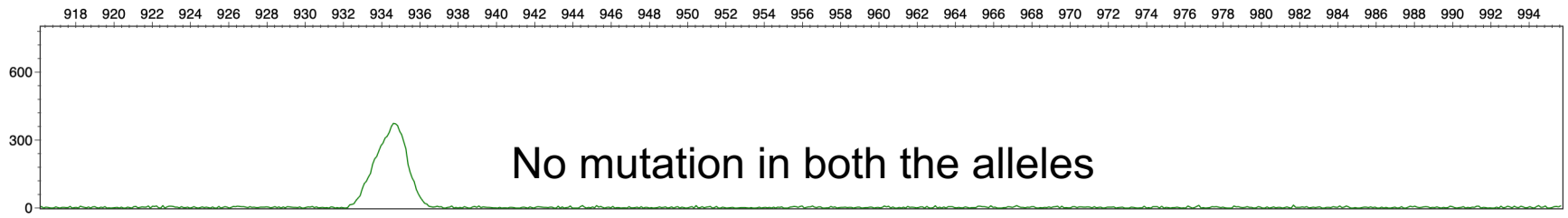
Fragment analysis using Thermo Fisher Connect™

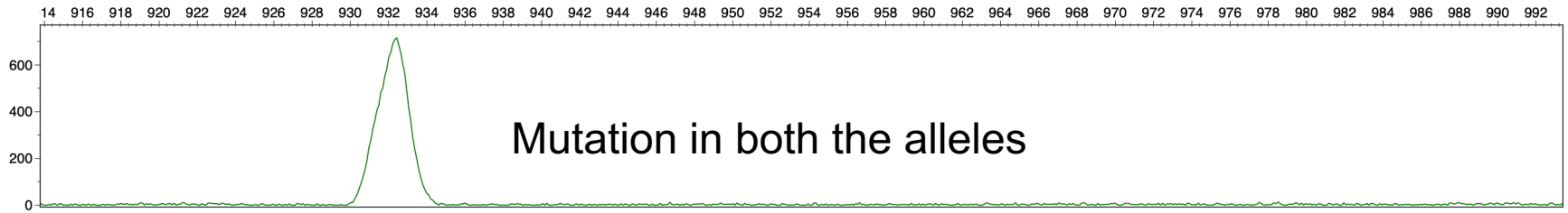


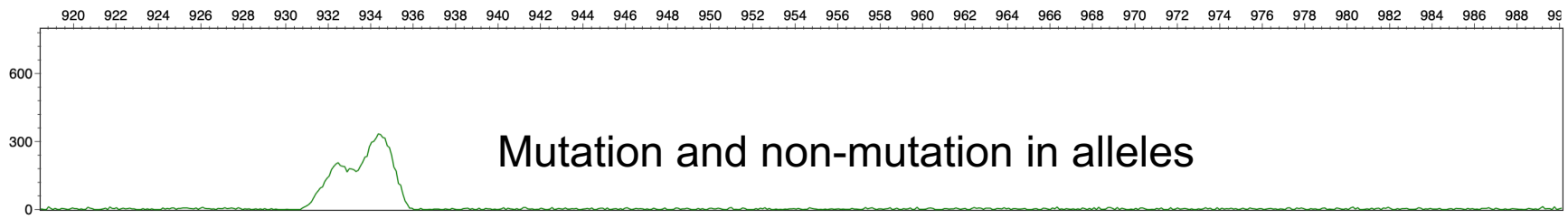


Dye Color	Sample Name	Size	Height	Area (Data Point)	Area (Base Pairs)	Begin Point (Base Pa...)	End Point (Base Pairs)
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	993.88	74.00	350.00	38.93	993.10	995.21
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	991.88	55.00	105.00	11.67	991.43	992.21
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	987.88	83.00	313.00	34.76	986.88	988.43
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	982.00	52.00	378.00	41.91	981.22	983.10
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	980.00	145.00	2,152.00	238.18	977.90	982.00
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	980.00	200.00	2,946.00	325.22	977.79	982.87
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	980.00	169.00	2,313.00	259.92	977.41	982.36
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	979.23	69.00	217.00	24.00	978.78	979.78
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	978.23	61.00	351.00	38.73	977.46	978.78
Blue	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	964.29	507.00	6,970.00	768.10	961.54	966.38
Blue	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	963.96	155.00	2,338.00	251.24	960.96	966.66
Red	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	963.85	51.00	424.00	45.53	962.46	964.39
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	960.00	158.00	2,311.00	243.54	957.29	962.35
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	960.00	209.00	3,181.00	340.12	957.15	963.13
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	960.00	178.00	2,438.00	265.47	957.18	961.87
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 3	940.00	157.00	2,099.00	216.57	938.22	942.66
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	940.00	193.00	2,722.00	284.71	937.89	942.59
Orange	4-255-267F 122-315H 997-496P 844-883H 844-161H 74	940.00	172.00	2,199.00	232.07	938.31	942.53
Yellow	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	934.41	175.00	2,641.00	277.89	933.25	936.30
Green	4-255-267F 122-315H 997-496P 844-883H 844-161H 4	934.30	334.00	5,566.00	585.51	933.04	935.99

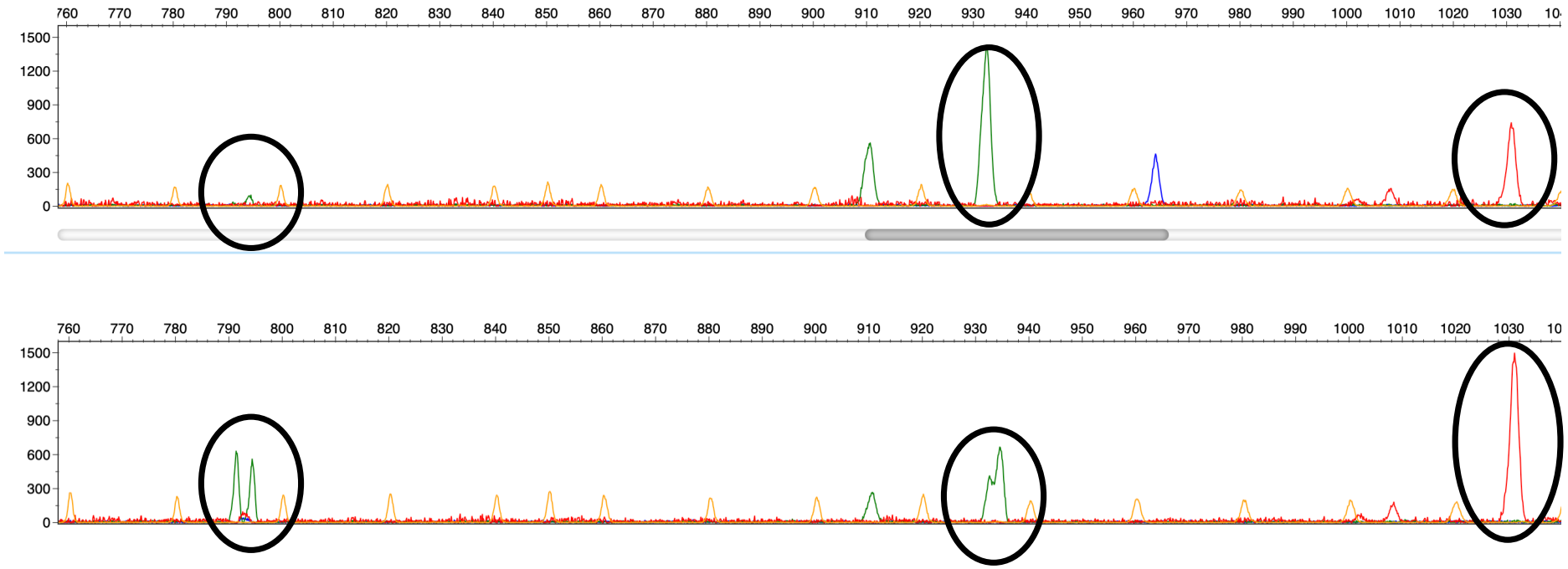
High Resolution Fragment Analysis





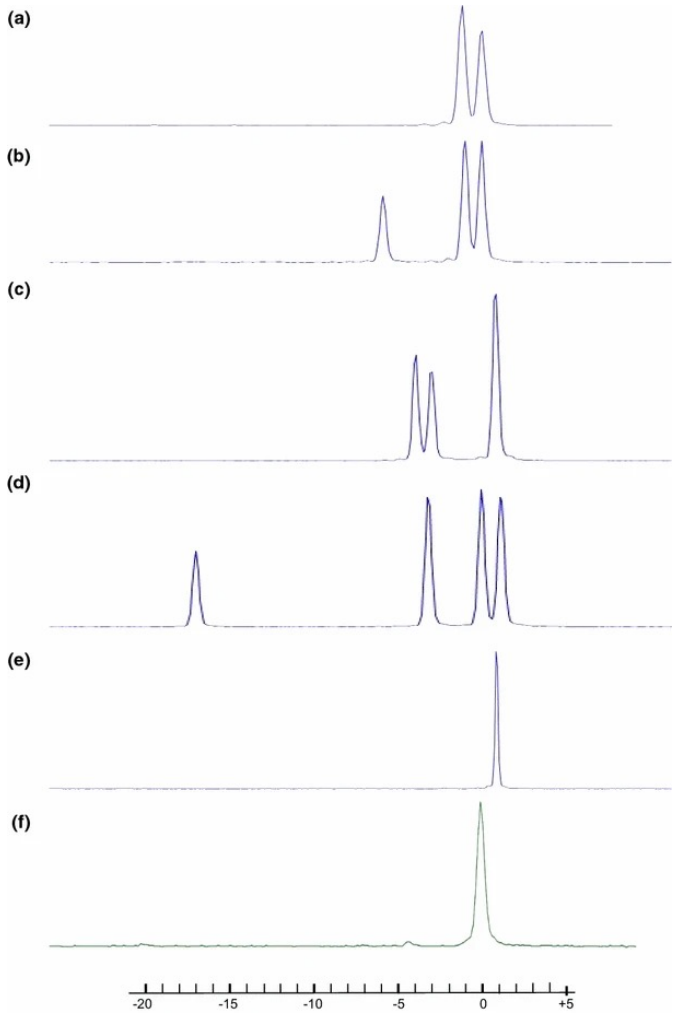


High Resolution Fragment Analysis



Multiple PCR products in one sample

High Resolution Fragment Analysis (HRFA) to Detect Mutations in Potato



a 1–2 allele mutated in the target region

b 2–3 alleles mutated in the target region

c 4 alleles mutated in the target region

d 3 alleles mutated in the target region

e 4 alleles mutated in the target region

f Wild type

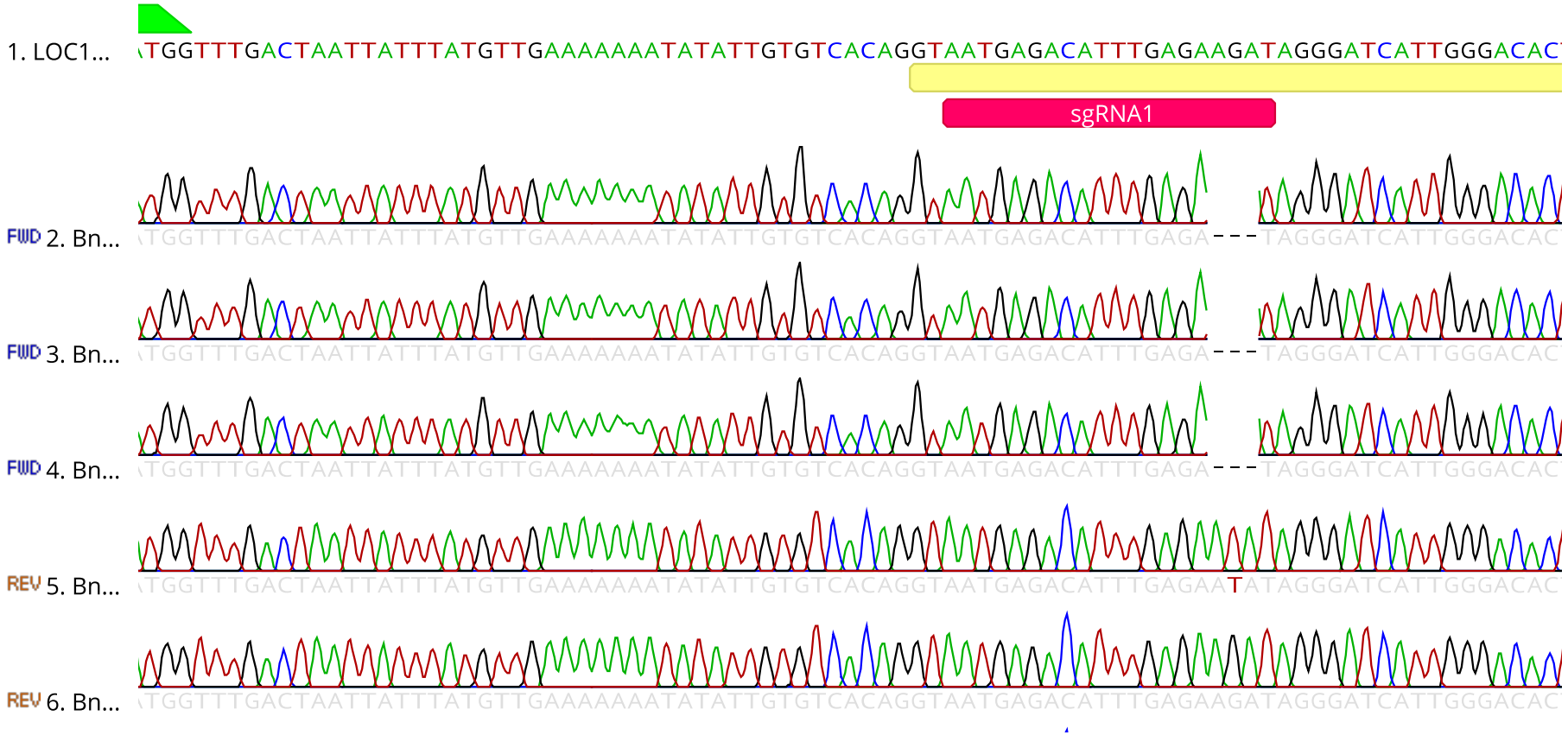
Wild-type fragment is set to 0 on the bp scale.

Andersson, M., Turesson, H., Nicolia, A. *et al.* 2017. *Plant Cell Rep* **36**, 117–128 (2017). https://doi.org/10.1007/s00299-016-2062-3

Sanger Sequencing Result Analysis

PCR with gene-specific primers amplifying gRNA targeting DNA

Cloning and transformation



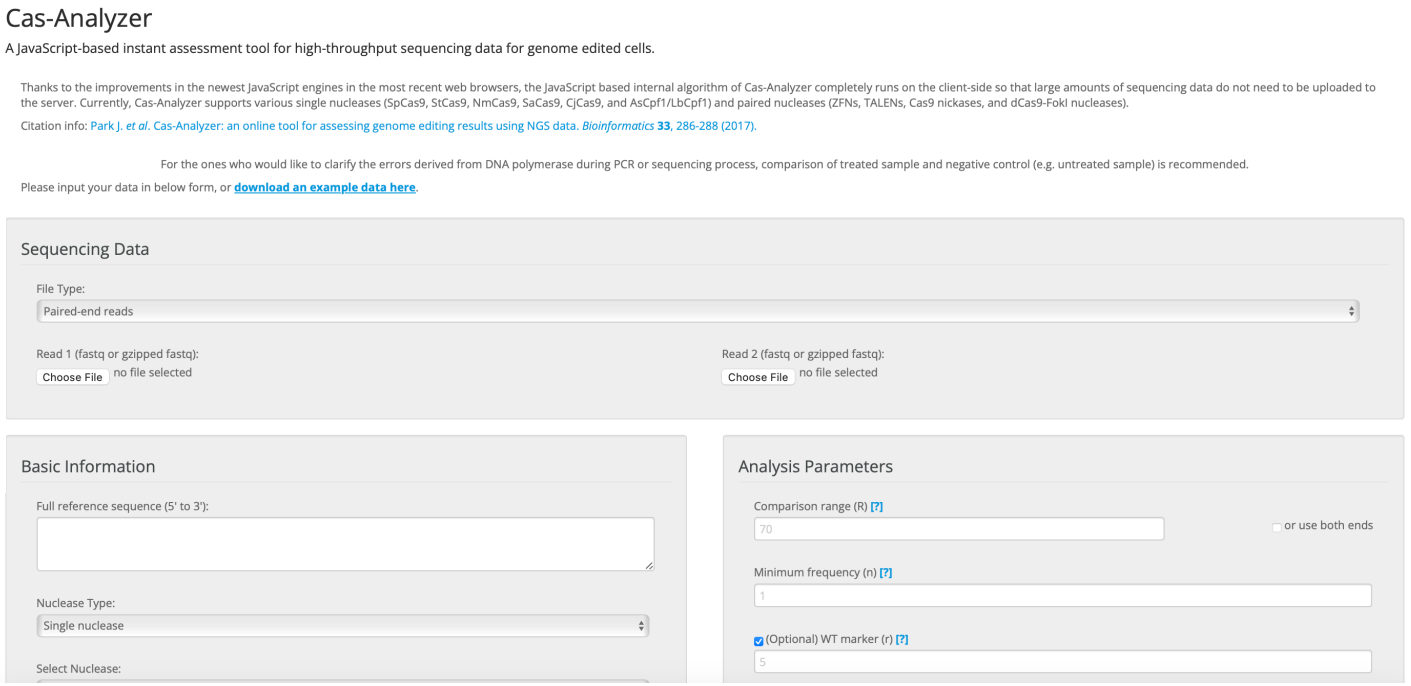
Molecular characterization of editing events

NGS-Illumina-Next Generation Sequencing Technologies

CRISPResso2

Analysis of genome editing outcomes from deep sequencing data

CRISPResso2



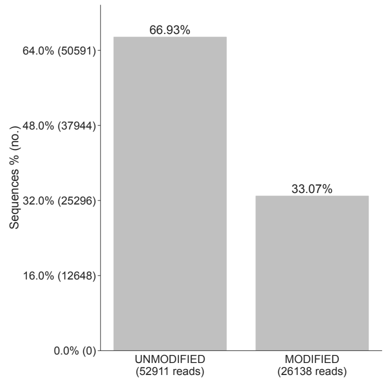
Amplicon Sequencing Analysis

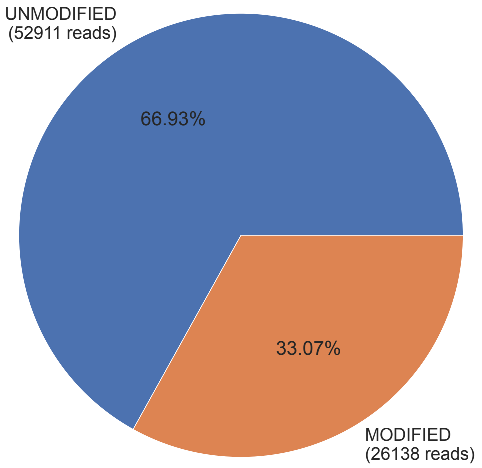
Molecular characterization of editing events

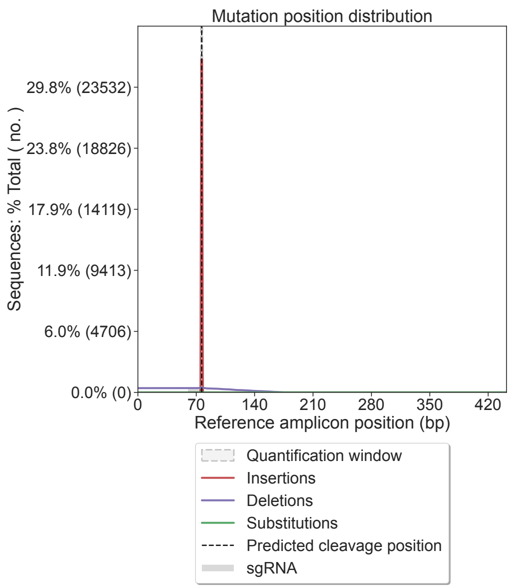
CRISPResso2

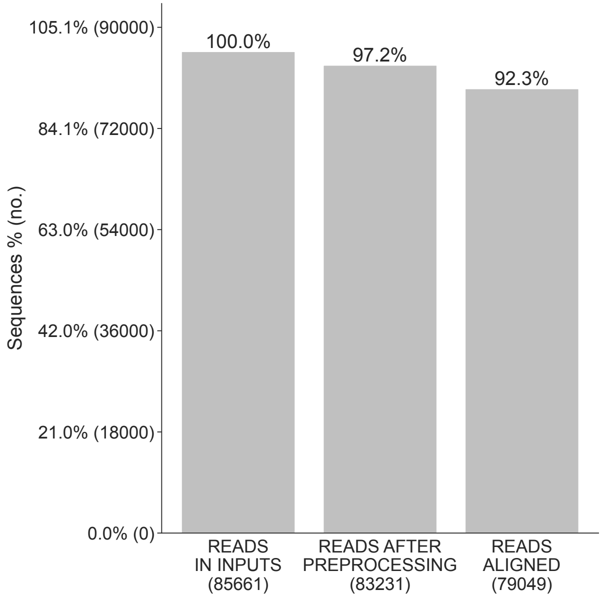
Analysis of genome editing outcomes from deep sequencing data

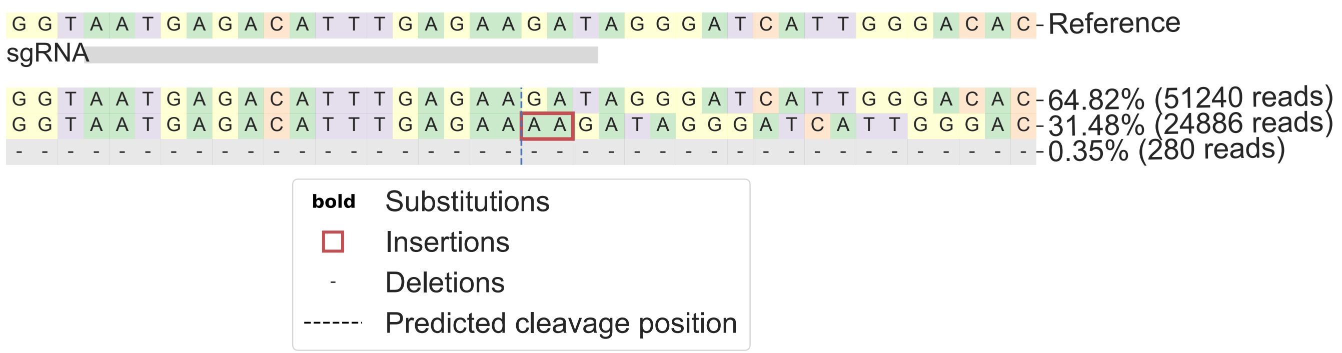
CRISPResso2











Molecular characterization of editing events

Cas-Analyzer

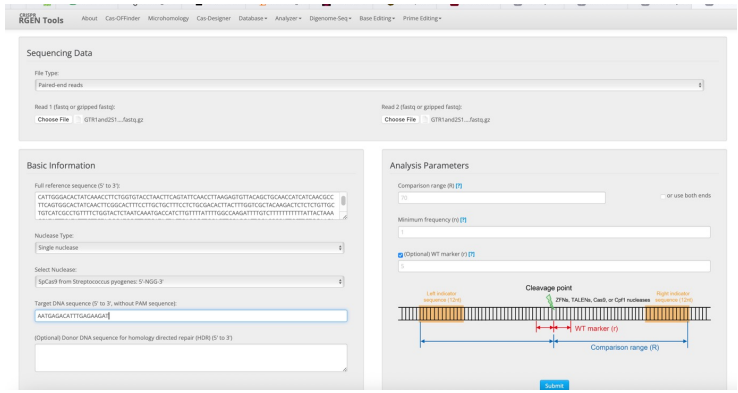
A JavaScript-based instant assessment tool for high-throughput sequencing data for genome edited cells.

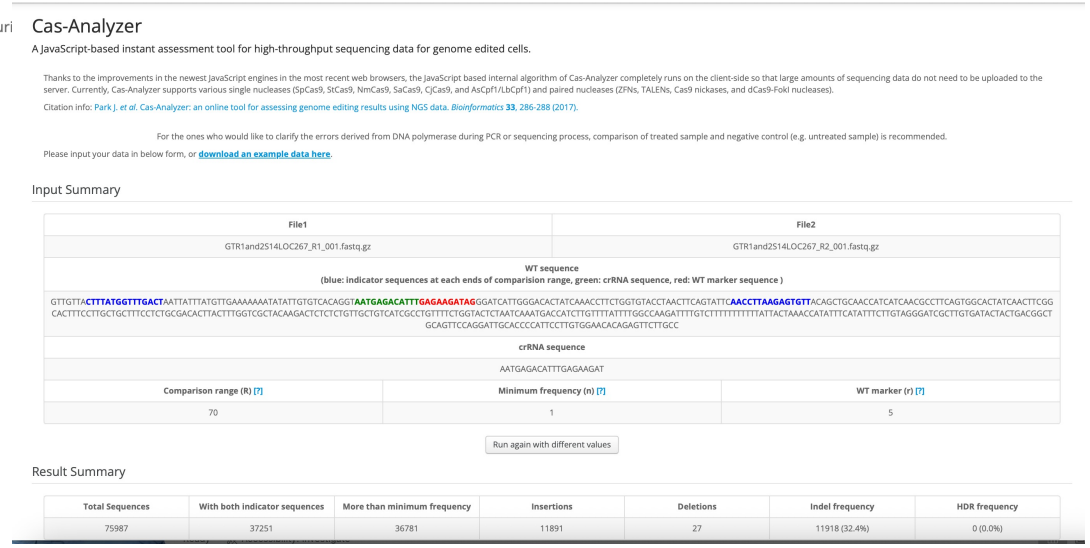
Thanks to the improvements in the newest JavaScript engines in the most recent web browsers, the JavaScript based internal algorithm of Cas-Analyzer completely runs on the client-side so that large amounts of sequencing data do not need to be uploaded to the server. Currently, Cas-Analyzer supports various single nucleases (SpCas9, StCas9, NmCas9, SaCas9, CjCas9, and AsCpf1/LbCpf1) and paired nucleases (ZFNs, TALENs, Cas9 nickases, and dCas9-FokI nucleases).

Citation info: Park J. *et al*. Cas-Analyzer: an online tool for assessing genome editing results using NGS data. *Bioinformatics* **33**, 286-288 (2017).

For the ones who would like to clarify the errors derived from DNA polymerase duri

Please input your data in below form, or **download an example data here**.





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For the ones who would like to clarify the errors derived from DNA polymerase during PCR or sequencing process, comparison of treated sample and negative control (e.g. untreated sample) is recommended.

Please input your data in below form, or **download an example data here**.

Input Summary

File1	File2
GTR1and2S14LOC267_R1_001.fastq.gz	GTR1and2S14LOC267_R2_001.fastq.gz

WT sequence
(blue: indicator sequences at each ends of comparision range, green: crRNA sequence, red: WT marker sequence)

GTTGTTA**CTTTATGGTTTGACT**AATTATTTATGTTGAAAAAAATATATTGTGTCACAGGT**AATGAGACATTT**GAG**AAGATAG**GGGATCATTGGGACACTATCAAACCTTCTGGTGTACCTAACTTCAGTATTC**AACCTTAAGAGTGTT**ACAGCTGCAACCATCATCAACGCCTTCAGTGGCACTATCAACTTCGG
CACTTTCCTTGCTGCTTTCCTCTGCGACACTTACTTTGGTCGCTACAAGACTCTCTCTGTTGCTGTCATCGCCTGTTTTCTGGTACTCTAATCAAATGACCATCTTGTTTTATTTTGGCCAAGATTTTGTCTTTTTTTTTTTATTACTAAACCATATTTCATATTTCTTGTAGGGATCGCTTGTGATACTACTGACGGCT
GCAGTTCCAGGATTGCACCCCATTCCTTGTGGAACACAGAGTTCTTGCC

crRNA sequence

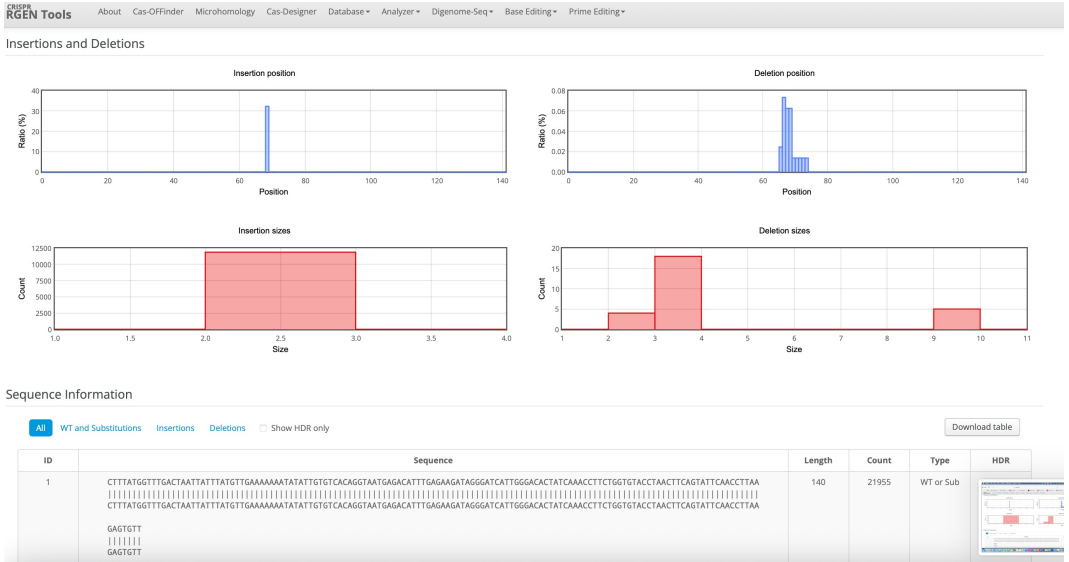
AATGAGACATTTGAGAAGAT

Comparison range (R) [?]	Minimum frequency (n) [?]	WT marker (r) [?]
70	1	5

Run again with different values

Result Summary

Total Sequences	With both indicator sequences	More than minimum frequency	Insertions	Deletions	Indel frequency	HDR frequency
75987	37251	36781	11891	27	11918 (32.4%)	0 (0.0%)



Questions?

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