

CRISPR/Cas9 Optimization by Electronic Genome Mapping

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Abstract

Cas9's ability to bind both targeted and non-targeted sites, and its functional interactions with other proteins and domains, have been studied using many technologies. We show how electronic genome mapping (EGM), which can detect DNA nicks in single molecules covering the whole genome, can be used to determine how gRNA mismatches affect target versus off-target binding and how Cas9 interacts with other proteins when bound to DNA. Cas9 binding to perfectly matched gRNA sites was examined and compared with binding to sites with mismatches at each position in the gRNA. The PAM site and nearby bases in the seed region were found to be critical for binding activity, while single mismatches in positions toward the 5' end of the recognition region allowed significant off-target binding. The impact of mismatches varied by gRNA. In addition, we used EGM to determine, at base-pair resolution, how physically close the bound CRISPR/dCas9 complex can be relative to nearby nickase recognition sites and still allow activity. Using gRNAs that each bind many different sites in the human genome, the distance between bound CRISPR/dCas9 and its ability to inhibit nicks with recognition sites > 4 bp away from the gRNA/PAM was determined. Sites 4-7 bp from the gRNA impact activity, while those 8 bp or more from the gRNA had minimal impact. Coupled with EGM's ability to simultaneously evaluate hundreds of singly and doubly mismatched off-target sites, this insight into Cas9 activity provides insight that may be extended to other CRISPR systems.

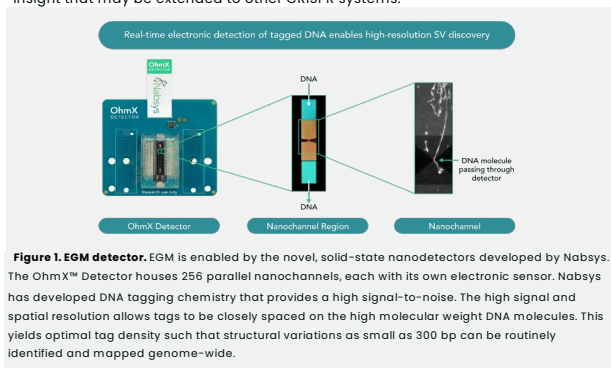
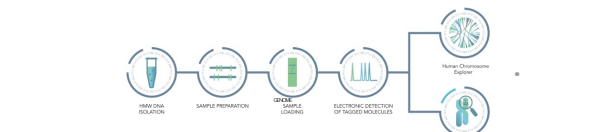


Figure 2. EGM workflow overview. The EGM workflow consists of the following steps: (1) high molecular weight (HMW) DNA isolation, (2) genome preparation using the OhmX Velocity Optimized Labeling and Tagging (VOLT™) Kit, (3) sample loading, (4) electronic detection of tagged molecules using the OhmX Analyzer, and (5) data analysis.

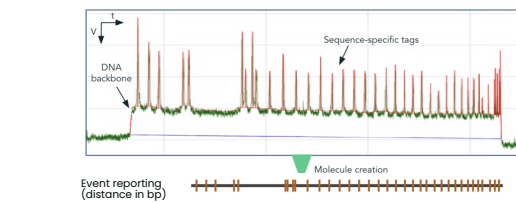


Figure 3. Electronic profile for tagged DNA molecules over 150 kb. The baseline voltage is shown on the left. When the DNA molecules enter the nanochannel, a change in voltage is observed. Whenever a site-specific tag on the DNA is also present, the voltage changes again. The time between tags is converted to distance in base pairs.

Cas9 Variants Can Be Adapted for EGM

Different Cas9 variants can be used to customize EGM assays and to analyze protein function. When new sequence-specific nicks are needed in genomic regions of interest, D10A Cas9 can be used to introduce them. When nicks need to be blocked, or protein behavior studied in the presence of other proteins, dCas9 can be used for stable, sequence-specific binding.

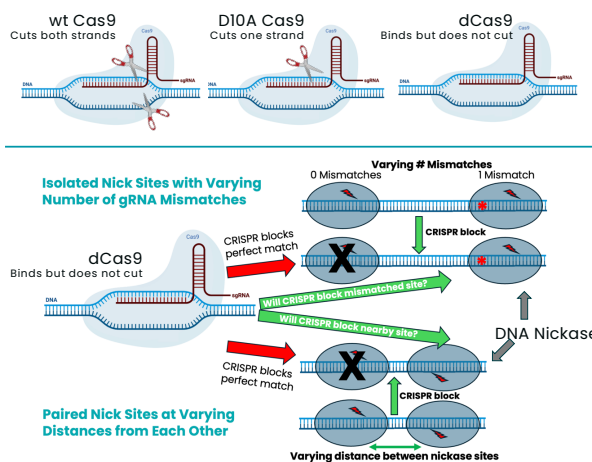


Figure 4. Schematic diagram of dCas9-induced blocking of DNA nicks. dCas9 was used in two modes to examine CRISPR activity. As shown in the top part of the figure, nicking at sites with zero or one mismatch was compared to assess dCas9 binding. In the lower part of the figure, the behavior of pairs of closely spaced nick sites was examined to determine the physical extent to which bound dCas9 can impact nearby activity.

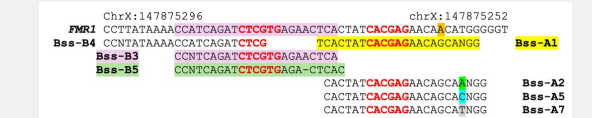


Figure 5. Sequences of repetitive gRNAs used in analysis. Each of the gRNAs tested has hundreds to thousands of perfect matches in the human genome and many more sites with 1, 2, or 3 mismatches. These sites can be examined simultaneously for binding or nicking activity.

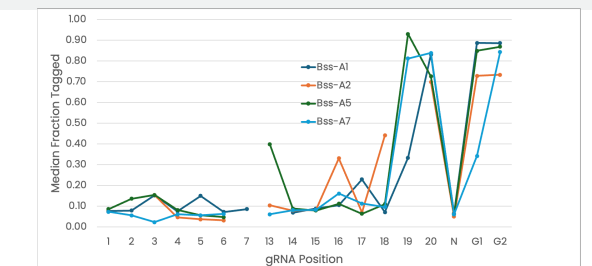


Figure 6. Median tagging frequency for mismatched genomic sequences. Median tagging frequency for all positions with four gRNAs, Bss-A1, Bss-A2, Bss-A5, and Bss-A7, is listed starting with position 1 most distal from the PAM region. Mismatches within the Nb.BssSI recognition site could not be measured (positions 8-13 for Bss-A1 and 7-12 for the other gRNAs).

Cas9 Binding Can Affect Other DNA Modifiers

Specific binding of dCas9 can prevent the activity of other DNA modifiers in a distance-dependent manner. The ability of DNA nicksases Nt.BspQ1 and Nb.BssSI to nick near a bound CRISPR/Cas9 complex was examined as a function of difference from their recognition site.

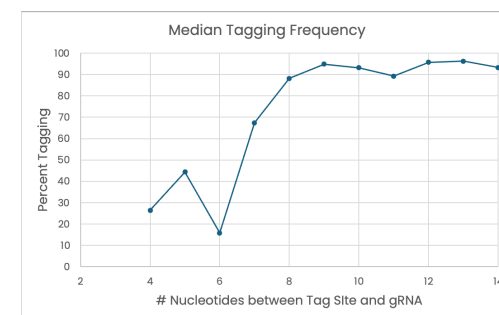


Figure 7. Length dependence of median tagging frequency for paired sites. The combined data for six gRNAs are shown graphically for lengths between 4 and 14 nt.

Distance from gRNA (bp)	Bss-B3	Bss-B3	Bss-B5	Bss-A1	Bss-A2	Bss-A5	Bss-A7	All
6 °C Pre-incubation	25 °C Pre-incubation	6 °C Pre-incubation	25 °C Pre-incubation	6 °C Pre-incubation	25 °C Pre-incubation	6 °C Pre-incubation	25 °C Pre-incubation	6 °C Pre-incubation
#	Median % Tagged	#	Median % Tagged	#	Median % Tagged	#	Median % Tagged	#
4	19	55.4	19	41.7				4
5					14	12.4	2	75.2
6							1	21.6
7							1	40.5
8							1	67.2
9	46	96.8	47	96.8				9
10	4	94.8	4	93.2				4
11	1	86.6	1	100				1
12	28	94.3	28	96.8				28
13								
14	2	91.3	2	97.5				2

Table 1. Length dependence used in Figure 7.

Conclusions

- EGM can be used to generate detailed information about the activity of DNA-modifying proteins
- Judicious study design enables functional differences in protein activity to be resolved at base-pair resolution.
- Whole genome assessment provides broader information than with many other technologies.
- Flexibility of being able to add or block nick sites nearly at will provides many opportunities to study DNA-modifying proteins and their interactions with DNA and other proteins.
- Differences in mismatch discrimination can be observed among Cas variants

For additional info, see the preprint at:

<https://www.biorxiv.org/content/10.64898/2026.09.14.751601v1>



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