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How specific is CRISPR/Cas9 really?

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The specificity of RNA-guided nucleases has gathered considerable interest as they become broadly applied to basic research and therapeutic development. Reports of the simple generation of animal models and genome engineering of cells raised questions about targeting precision. Conflicting early reports led the field to believe that CRISPR/Cas9 system was promiscuous, leading to a variety of strategies for improving specificity and increasingly sensitive methods to detect off-target events. However, other studies have suggested that CRISPR/Cas9 is a highly specific genome-editing tool. This review will focus on deciphering and interpreting these seemingly opposing claims.

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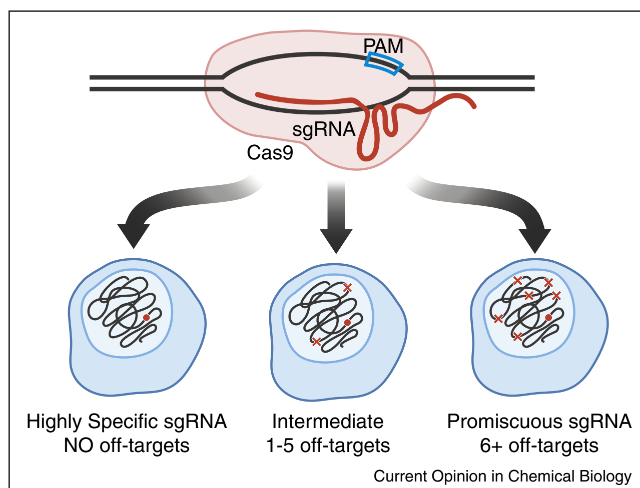
First generation methods to detect potential off-target sites: computational prediction and *in vitro* screens

The RNA-guided clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) endonuclease system has taken the genome editing field by storm. The complex consists of the Cas9 nuclease protein and a single guide RNA (sgRNA) that targets a specific DNA sequence through RNA-DNA base pairing (Figure 1) [1]. The most widely used Cas9, derived from *Streptococcus pyogenes*, targets a 20 nucleotide DNA sequence immediately followed by a 5′-NGG-3′ protospacer-adjacent motif (PAM) [1]. The first studies of Cas9 specificity focused on off-target cleavage activity at genomic regions that were identified by computational prediction based on similarity to the target sequence [2••,3••,4,5], *in vitro* cleavage assays [6], or high-throughput reporter screens [7] (Figure 2, top). Predicted sites were analyzed for cleavage using a PCR-based assay. Some studies suggested high frequency of CRISPR/Cas9 activity [2••,5], which alarmed the entire field and established expectations for follow-up studies to identify high off-target activity. However, other studies found modest or low off-target activity at predicted genomic sites [3••,6] (Table 1). Results varied widely, even within a single research study. For example, among the six target sites tested by Fu and colleagues [2••], no off-target sites were identified for two targets (RNF2 and FANCF), only one off-target site was detected for EMX1, while 4, 12 and 7 off-target sites were observed for VEGFA sites 1, 2 and 3, respectively. Thus, there was a clear distinction between very high target specificity of some sgRNAs (RNF2, FANCF), while others were very promiscuous (VEGFA sites 2 and 3) (Table 1). Despite this variance, the promiscuous VEGFA sgRNAs became the archetypal poster child for off-target activity and would be used in many subsequent studies. Although it certainly makes sense to use promiscuous sgRNAs to test new methods for off-target site detection and avoidance, one should not assume that CRISPR/Cas9 *per se* has high off-target activity. Perhaps the most accurate conclusion from these early studies would be that CRISPR/Cas9 has the potential to be highly specific or lead to high-frequency off-target activity depending on the choice of sgRNA.

This conclusion notwithstanding, concerns about specificity led to several strategies to reduce off-target effects while retaining efficient on-target cleavage (reviewed in [8]). Heterodimeric Cas9 variants, such as paired Cas9 nickases and dimeric Cas9-FokI nucleases rely on targeting via two sgRNAs significantly enhanced specificity [9,10]. Modified sgRNAs can effectively reduce off-target activity by, paradoxically, the addition of two extra guanine nucleotides to the 5′ end (GGN$_{20}$-NGG) of the traditional sgRNA design (GN$_{19}$-NGG) [4], or the use of truncated sgRNAs (GN$_{17}$-NGGor GN$_{18}$-NGG) [11,12]. In addition to mismatches, some sgRNAs can also tolerate DNA sequences with an extra base (DNA bulge) or a missing base (sgRNA bulge) [13••] (Figure 3).

There is an expanding list of algorithms available that search the genome for similar sites adjacent to the Cas9 PAM, allowing a certain number of mismatches to the target site [3••,14•,15–17]. However, since predictive first generation methods could only survey a subset of potential off-target sites, a much larger number of off-target sites in the entire genome was expected. This assumption highlighted the need for unbiased and genome-wide detection of Cas9 off-target activity.

Figure 1



CRISPR/Cas9 specificity is dependent on its sgRNA. The Cas9 protein (pink) complexes with a single guide RNA (sgRNA, red) at a DNA target site that contains a protospacer adjacent motif (PAM, blue box). The 5′ end of the sgRNA forms a 20-bp heteroduplex with one strand of the DNA. The binding or cleavage events facilitated by the Cas9/sgRNA complex can be categorized as highly specific (no off-targets), intermediate (1 to 5 off-targets) and promiscuous (6 or more off-targets).

Second generation methods: genome-wide binding specificity of nuclease-inactive dCas9

The catalytically inactive dCas9 has been used as a simple programmable DNA-binding platform for many applications including transcriptional activation and repression (CRISPRa and CRISPRi, respectively) [18–23]. Since dCas9 regulators do not possess nuclease activity, several groups performed ChIP-seq (Chromatin Immunoprecipitation followed by high throughput sequencing) to determine CRISPR/dCas9 binding specificity on a genome-wide scale [24–27] (Figure 2, middle). Virtually all studies observed the highest intensity binding at the target site, suggesting a strong binding preference for the target. However, less-intense off-target sites were detected varying from a few to hundreds or thousands of binding sites. The wide variation in off-target sites observed between different groups was likely due to differences in their experimental and analytical methods, in addition to any differences between individual sgRNAs. Off-target sites often contained motifs that were identical to the PAM proximal target sequence. Overall, these studies suggested that ChIP-seq identifies stable dCas9 binding to genomic target sites as well as transient binding of dCas9 to regions with partial complementarity as it scans the genome. Transient binding of Cas9 and dCas9 had been demonstrated *in vitro* and, interestingly, DNA cleavage only occurred at target sites and not at sites of transient interaction [28]. The lack of cleavage at transient sites *in vitro* was borne out *in vivo*. In fact, some studies were only able to observe cleavage at none or one off-target site [24,25,27] (Table 1).

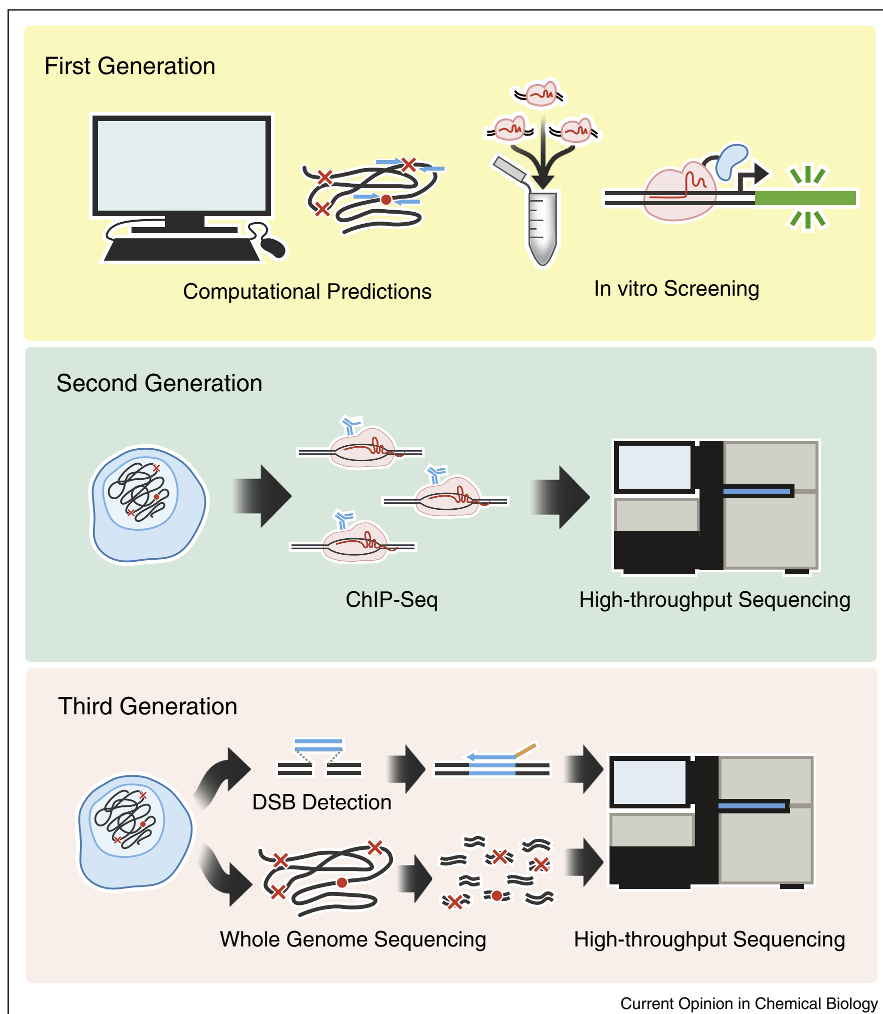
However, subsequent third-generation methods would show that the near-perfect specificity of dCas9 in ChIP-seq assays might be an underestimation of the true off-target behavior of catalytically active Cas9. This discrepancy might be attributed to different determinants for Cas9 binding and nuclease activity, or structural differences between Cas9 and dCas9. Indeed a structure of dCas9 bound to DNA showed the HNH endonuclease domain located away from the scissile phosphate group of the target DNA strand, suggesting activity-dependent conformational rearrangements [29]. However, the results of these studies are still valid for all dCas9 applications, and generally showed that binding of dCas9 was very highly specific.

Third generation methods: genome-wide detection of Cas9-induced double strand breaks

Double strand breaks (DSB) in the DNA of most species can be repaired by a highly efficient but error-prone nonhomologous end-joining pathway, leading to the accumulation of mutations at the breakpoint. Therefore, the most intuitive and comprehensive approach to identify DSB induced by catalytically active Cas9 across the whole genome is to search for mutations using whole genome sequencing (WGS, Figure 2, bottom). WGS of induced pluripotent stem cell (iPSC) clones generated by CRISPR/Cas9 treatment suggested high specificity of CRISPR/Cas9 [30,31]. In addition to the on-target site, one WGS study identified one high-frequency off-target that was not present in the reference genome, but rather created by a single nucleotide variation in that particular iPSC line [32]. This brings up the issue of sequence variation between individual genomes that will need to be addressed moving forward. Each genome is unique, possibly leading to off-targets that are not present in one individual but may be present in another. However, while WGS can readily detect high-frequency events, it is limited by the need of extensive sequencing depth. The typical 30x-60x coverage of the genome is not sufficient to identify low-level mutations. Digenome-seq also relies on WGS sequencing of nuclease digested genomic DNA, but Cas9-induced insertion and deletions are identified by their sequence signature rather by divergence from the reference genome [33]. However, sequencing depth and cost remains a limiting factor, especially when using non-human cells.

The need for accurate and unbiased detection of Cas9-induced off-targets on a genome wide scale has led researchers to adopt and develop new methods (reviewed in [8,34]). Integrase-deficient lentivirus vectors (IDLV) were able to identify off-target sites by integrating a marker gene at Cas9-induced DSBs [35], based on

Figure 2



A diversity of methods has been used to predict or detect off-target sites. Each successive methodology attempted to be less biased and interrogate more of the genome than the previous generation.

methods developed earlier for zinc finger nucleases [36]. Depending on sgRNA used, detected off-targets varied from zero to seven (Table 1), but no off-target sites were observed with paired Cas9 nickases. A similar approach, genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq), identifies DSB by inserting small barcoded pieces of DNA followed by high throughput sequencing [37]. Among seven sgRNAs, off-target activity varied widely from zero (RNF2) to as many as 151 off-targets (VEGFA site 2) (Table 1). Thus, these studies recapitulated the same basic message that we learned from the first generation methods, that CRISPR/Cas9 cleavage has the potential for high or low specificity depending on the sgRNA.

In addition to insertion and deletion mutations, Cas9 also induces chromosomal translocations between breakpoints at on-target and off-target sites and double strand break hotspots that are independent of Cas9. Translocation events can be determined by several methods, such as GUIDE-seq [37] and high-throughput, genome-wide, translocation sequencing (HTGTS) [38]. No translocation events were detected by HTGTS for two of the four sgRNAs targeting the RAG1 locus. By contrast, a large number of translocations was observed with the promiscuous sgRNA (VEGFA site 2), whose high off target activity has previously been reported [2••,33,37] (Table 1). DSB hot spots can vary between cell types, which may contribute to cell-type specific off-target effects. Recently, DSB hotspots of individual genomes have been mapped revealing common and unique hotspots [39]. It will be interesting to define determinants of DSB hotspots to reduce the risk of deleterious consequences by irreversible changes to the genetic information.

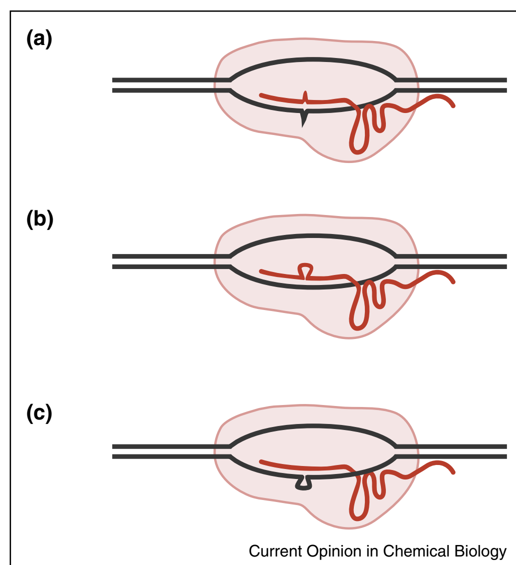
Table 1

Categorized sgRNA specificity

	Study	Off-target prediction/detection method	Validation rate[a]	sgRNA specificity based on validated off-target activity		
				Highly specific (0 off-targets)	Intermediate (1–5 off-targets)	Promiscuous (>5 off-targets)
Computational prediction & *in vitro* screen	Mali *et al*. 2013	Reporter gene screen	<1%	n.d.[b] (artificial target sites)		
	Cho *et al*. 2013	Computational & exome capture	<1%	C4BPB CCR5		
	Fu *et al*. 2013	Computational	<1%	RNF2 FANCF	EMX1 VEGFA site 1	VEGFA site 2 (12) VEGFA site 3 (7)
	Hsu *et al*. 2013	Computational	<1%		EMX site 1 EMX site 3	
	Pattanayak *et al*. 2013	Computational & *in vitro* screen	<1%		CLTA4 (3)	
	Cradick *et al*. 2013	Computational	<1%	9 sgRNAs[c]	10 sgRNAs[c]	
	Lin *et al*. 2014	Computational (gRNA and DNA bulges)	<1%	n.d.[b] (focus on DNA/RNA bulges, not mismatches)		
Genome-wide detection of dCas9 binding	Wu *et al*. 2014	ChIP-seq	<1%[d]	Nonog-sg3 Phc1-sg1 Phc1-sg2	Nanog-sg2	
	Cecnic *et al*. 2014	ChIP-seq	<1%[d]	sgp53-3	sgp53-1	
	Kuscu *et al*. 2014	ChIP-seq	<1%[d]			
	O'Geen *et al*. 2015	ChIP-seq & sequence capture	<1%[d]	S1	S2	
	Gersbach *et al*. 2015	ChIP-seq & RNA-seq	3–17%	IL1RN HBG1/2		
Genome-wide detection of DSBs and NHEJ	Tsai *et al*. 2015	GUIDE-seq	80%	RNF2	HEK 293 site 2 HEK 293 site 3	VEGFA site 1 (21) VEGFA site 2 (151) VEGFA site 3 (59) EMX1 (15) FANCF (8) HEK293 site 1 (9) HEK 293 site 4 (133)
	Kim *et al*. 2015	Digenome-seq	7–10%		HBB	VEGFA (81)
	Ran *et al*. 2015	BLESS	14–41%	Pcsk9	EMX1-sg1	EMX-sg2 (12)
	Frock *et al*. 2015	HTGTS (focus on translocation)	n.d.[b]		RAG1B	RAG1A
	Wang *et al*. 2015	IDLV	100%[e]	WAS CR-3 TAT CR-4 TAT CR-6	WAS CR-5 TAT CR-1	WAS CR-4 (12)

[a] Validation rate serves only as a reference and is highly dependent on the sensitivity of the method, the number of sgRNAs tested, and the number of potential off-target sites used for validation.
[b] n.d., not determined.
[c] Only one potential off-target site was examined.
[d] ChIP-seq detection is based on DNA binding while validation is based on DNA cleavage activity. Both methods have different specificity determinants and cannot be directly compared.
[e] Additional sites that were not identified by IDLV were validated after computational prediction.

Figure 3



Some sgRNAs allow binding or cleavage at variants of the target site. In addition to single-base mismatches **(a)**, some sgRNAs can tolerate DNA sequences with an extra base (**(b)**, DNA bulge) or a missing base (**(c)**, sgRNA bulge).

Conclusions and prospectus

It is evident that major differences in CRISPR/Cas9 specificity arise from sgRNAs themselves. Although some sgRNAs have the potential to be highly specific, others are promiscuous leading to hundreds of off-targets (Table 1). Therefore, it would seem inappropriate to suggest that the CRISPR/Cas9 platform *per se* is specific or non-specific. The current challenge is to anticipate which sgRNA will provide high on-target activity while having minimal off-target effects. In this regard, CRISPR/Cas9 maintains a technological advantage over zinc finger and transcription activator-like effector (TALE) proteins, which can also be highly specific but require more effort to assembly each new protein to test.

A deeper analysis of Cas9 orthologs from other species may reveal greater or less specificity for a given target site. Cas9 orthologs often vary in target site and PAM requirements [22,40,41•]. The genome-wide nuclease activity of the *S. aureus* Cas9 was assessed using BLESS (direct *in situ* breaks labeling, enrichment on streptavidin and next-generation sequencing) [41•]. Interestingly, *Sa*Cas9 displayed higher specificity than *Sp*Cas9. Furthermore, no off-target activity was observed in the mouse neuroblastoma cell line or mouse liver after AAV delivery of *Sa*Cas9 and Pcsk9 sgRNAs [41•]. Further analysis is also required to understand how chromatin structure and sequence context contribute to target site accessibility, as well as on-target and off-target site recognition. Off-target binding correlated with DNase I hypersensitive sites (DHS) characteristic for accessible chromatin regions, and preferentially localized to regions void of DNA methylation [24–26]. Sequence features that contribute to sgRNA efficiencies have been systematically assessed in order to construct a predictive sequence model for the design of CRISPR/Cas9 knockout experiments [14•,42]. There was a distinct preference for a guanine nucleotide immediately preceding the PAM site and nucleotide composition downstream of the PAM site also contributed to sgRNA efficiency. More recently, sequence context on sgRNA efficiency was also assessed for CRISPRi/CRISPRa [14•]. These studies again found the sequence preference of CRISPRi/CRISPRa to be distinctly different from CRISPR knockout experiments. Although these models are not perfect, they are a step towards improvement of sgRNA design for gene editing and regulation. Solving the challenge of optimal sgRNA selection will likely require large data sets of many sgRNAs in different cell types. Cell types for which large amounts of genomic data are already available, such as the ENCODE Tier 1 cell lines [43], would be more informative than the HEK293 and U2OS cells used frequently in the past.

The number of off-target events that could be tolerated by any sgRNA may also depend on the application. Changes to the genome by a Cas9 endonuclease are irreversible at off-target sites, which could lead to deleterious effects. One could argue that a single off-target site is too much when genomic DNA is permanently altered. Introducing CRISPR/Cas9 into a patient for gene therapy, with the potential to modify millions of cells and cell descendants, would require the highest specificity. However, there may be few off-target events in any single Cas9-treated iPSC, which could be clonally expanded and off-target events verified by WGS of that one genome. A modest number of off-target bindings might also be acceptable when using dCas9 to regulate transcription without altering the genetic content [18–20,23], even in clinical applications.

In conclusion, the current data suggest that careful selection of the sgRNA used with *Sp*Cas9 can produce a very highly specific DNA nuclease that would be appropriate for most if not all applications. Today's computation design programs can help find target sites with minimal similarity to off-targets in a reference genome, but empirical testing in the appropriate cell type will likely be required to ensure optimal specificity performance. With more data sets of sgRNAs with *Sp*Cas9 and others in well annotated genomes and epigenomes, improved computational approaches will likely reduce the need for empirical testing to produce specific CRISPR/Cas9 nucleases and gene regulators.

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