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# ONLINE AND OFFLINE TOOLS: CRISPR/CAS OFF-TARGET DETECTION

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**ABSTRACT:** Genome engineering field is a new revolution in biological research and to this new field several restriction endonucleases are available. Out of all these, based on easier construction, sensitivity and specificity Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) preferred by scientists for research and industrial purposes. However, off-target DNA cleavage causes limitation to researchers who are interested in using engineered nucleases in gene or cell therapy. To overcome this issue, there is a need to design gRNAs which will produce CRISPR/Cas products with high specificity. Till writing this manuscript, there is no compile details available on such bioinformatics tools. Therefore, in this review we are trying to provide platform of information for research scientist who are working on CRSPR-Cas technology so that they can use these online and offline tools to prevent CRISPR/Cas off-targeting in research and industrial field depending on the type of experiment.

**INTRODUCTION:** Genome engineering is defined as "direct or indirect tailored changes in manipulation of genetic makeup of organism to cause desired effects which is highly desirable in basic". This new field originated a new revolution in biological research [1].

The traditional method for gene modification is homologous recombination which is widely used in mouse embryonic stem cells to generate germline knockout or knocking mice. However, this method is not applicable in genetically modified mouse and humans.

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To overcome this problem, currently there are several restriction endonucleases are available like Zinc-Finger nucleases (ZFNs), Transcription activator-like effectors nucleases (TALEN), Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) [2].

Out of these genome editing tools, we are focussing on widely used Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) because Cas9 is a nuclease guided by small RNAs through Watson-Crick base pairing with target DNA which represents a system that is markedly easier to design, highly specific, efficient and well-suited for high-throughput and multiplexed gene editing for a variety of cell types and organisms [3].

Clustered, regularly interspaced, short, palindromic repeats (CRISPR)—Cas (CRISPR-associated)

system is an adaptive, sequence specific, nucleic acid restriction machinery found in many bacteria and archaea which provide prokaryotes with an effective defence against mobile genetic elements like bacteriophages, plasmids, and transposons [4]. Three types of (I–III) of CRISPR systems have been identified across a wide range of bacterial and archaeal hosts. Each system comprises a cluster of CRISPR-associated (*Cas*) genes, noncoding RNAs and a distinctive array of repetitive elements (direct repeats). These repeats are interspaced by short variable sequences20 derived from exogenous DNA targets known as protospacers, and together they constitute the CRISPR RNA (crRNA) array. Each protospacer is always associated with a protospacer adjacent motif (PAM), which can vary depending on the specific CRISPR system [5].

Following Cas9 binding and subsequence target site cleavage, the double strand breaks (DSBs) generated are repaired by either non-homologous end joining (NHEJ) or homologous recombination directed repair (HDR), resulting in indels or precise repair respectively [6]. Recent study by Ran et al., (2013) published that 20 nucleotide sequence present in the gRNA contributes to overall specificity but there are multiple mismatches that could be well tolerated if present only at the 5' end of the gRNA on other hand any mismatches towards the 3' end of the gRNA are not tolerated [3].

Applications of engineered nucleases in research, biotechnology, and medicine are hampered by their off-target effects because too much off-target DNA cleavage causes not only cellular toxicity but also the repair of off-target DSBs gives rise to unwanted chromosomal rearrangements such as deletions, inversions, and translocations which results in the activation of oncogenes or inactivation of tumour suppressor genes. This sets limitation to researchers who are interested in using engineered nucleases in gene or cell therapy. To overcome this issue, we need to design gRNAs which will produce CRISPR/Cas products with high specificity. Till writing this manuscript, there is no compile detail available on such tools.

Therefore, in this review we are trying to provide platform of information for research scientist who are working on CRSPR-Cas technology so that they can use these online and offline tools to prevent CRISPR/Cas off-targeting.

**1. CHOPCHOP:**

Initially, we need to retrieve a particular nucleotide sequence or accession number of a gene of interest and then need to enter it into CHOPCHOP software. The user can choose this tool to target coding region, exonic sequence (including 5' and 3' UTR), splice sites, 5' UTR, 3' UTR, specific exon/subset of exons and introns. The software will automatically analyse the sequence and identifies all possible 20bp sequences which are immediately followed by the PAM sequence (5'-NGG) and allows gRNA scores from best scoring to lowest scoring according to a pre-set code, which looks at GC content and off target sites. This tool not only provide more information on a particular gRNA potential off-target sites but also provides primer sequences that could be used to screen these off-target sites for potential mutations through the SURVEYOR assay [7].

**2. CRISPR design:**

This web based tool provides simple process of CRISPR guide selection. In this tool, initially we need to choose target DNA sequence then need to provide target genome. It BLASTS every gRNA sequence and job page will provide a detailed report about its off-target positions and the number of mismatches with the designed gRNA and presented in the order of their score from zero to 100% roughly indicating the faithful on-target activity of each guide. The gRNA sequences of score greater than 50% are coloured green and should be considered as a candidate targeting sequences. The gRNA sequences which are coloured yellow should be considered backups for specific targeting in the case where no suitable green guides are available for high-scoring, genic off-targets while red coloured gRNA sequences should be avoided as it will provide maximum off-targets. This software is superior when designing two gRNAs for paired nickase activity as it will automatically find two gRNAs that are within close proximity to one another [8, 9].

**3. CRISPR gRNA design tool:**

In this tool, we can construct gRNAs by providing target gene name, genomic region or target

sequence of interest and the design tool will identify all Cas9 target sites within the input sequence. The results contain a rank ordered list of target sites based on predicted specificity.

This software will also tell us gRNA targeting on either top strand or bottom strand. The algorithm used in the program is based on the occurrence of the 12 base pair seed sequence preceding the NGG and NAG protospacer-adjacent motif (PAM). The advantage of this tool is that it provides vectors list in which you want to clone our gRNA [10].

**4. CRISPR MultiTargeter:**

CRISPR MultiTargeter web based tool developed by Sergey Prykhozhij at the IWK Health Centre and Dalhousie University which automatically searches CRISPR guide RNA targets. The advantage of this tool is that it can find highly similar or identical target sites in multiple genes or transcripts or design targets unique to particular genes or transcripts. The search for common targets is based on generating a multiple sequence alignment, and unique targets are found using a string comparison algorithm among all possible targets for each sequence. The basic algorithm implemented in CRISPR MultiTargeter is versatile and can accommodate almost any possible target specificity of CRISPR/Cas system, which is important because new target specificities are discovered and will be used in applied studies in different model systems [11].

**5. CRISPR-ERA:**

CRISPR-ERA is an automated and comprehensive sgRNA design tool for CRISPR-mediated editing, repression, and activation (ERA) which utilizes a fast algorithm to search for genome-wide sgRNA binding sites and evaluates their efficiency and specificity using a set of rules summarized from published data for CRISPR editing, repression and activation.

This tool searches all targetable sites in that particular organism for patterns of N20NGG (N=any nucleotide) and each target sequence is then calculated for two scores namely, an efficacy score (E-score) and a specificity score (S-score) based on the genome-wide off-target binding sites [12].

**6. CRISPR direct:**

CRISPR direct is a simple and functional web server for selecting rational CRISPR/Cas targets from an input sequence. The CRISPR/Cas system is a promising technique for genome engineering which allows target-specific cleavage of genomic DNA guided by Cas9 nuclease in complex with a guide RNA (gRNA), that complementarily binds to a ∼20 nt targeted sequence. First, the 5′-NGG protospacer adjacent motif (PAM) sequence must be located adjacent to the target sequence. Second, the target sequence should be specific within the entire genome in order to avoid off-target editing. CRISPR direct enables users to easily select rational target sequences with minimized off-target sites by performing exhaustive searches against genomic sequences. The server currently incorporates the genomic sequences of human, mouse, rat, marmoset, pig, chicken, frog, zebrafish, *Ciona*, fruit fly, silkworm, *Caenorhabditis elegans*, *Arabidopsis*, rice, *Sorghum* and budding yeast [13].

**7. fly CRISPR Optimal Target Finder:**

It is mainly for research work focused on Drosophila. This is online tool for identifying CRISPR target sites and evaluating their specificity using transparent rules based on empirical data in the literature so one can easily select the best target(s) for project. This fly CRISPR Target Finder uses TagScan and algorithms based on the large-scale analyses of CRISPR/Cas9 specificity in cell lines and animals published to date to identify potential off-target cleavage sites for a given CRISPR target. The algorithms consider both the number and location of mismatches to evaluate all potential off-target cleavage sites. It also has links for CRISPR/Cas9 in Drosophila, Drosophila CRISPR target finders, General CRISPR/Cas9 resources. It was designed by three labs namely O'Connor-Giles Lab, Wildonger Lab, Harrison Lab [14].

**8. E-CRISP:**

E-CRISP is online tool to design and evaluate target sites for use with the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) system. It has option of DE novo and Evaluation, one can design CRISPR to knock out gene or in tagging experiment which is included in advance

options of De novo. The web application uses fast algorithms to identify sgRNA target sequences in any nucleotide sequence for use in CRISPR/Cas mediated genome editing. E-CRISP analyzes target specificity of the putative designs and assesses their genomic context (e.g. exons, transcripts, CpG islands). Numerous of options are available depending on the need one can design CRISPR constructs for experiments. This was recently updated in Jan 2015. It contains 25 reference genomes including *Homo sapiens sapiens* [15].

**9. Desk GEN:**

This tool works on Autoclone software tools desiged by own Desktop genetics team. Using DESKGEN, scientists can design any genome-editing experiment in any cell line, and generate tailored CRISPR library designs for their research. It is London based lab having partners with three other reputed companies. It will very supportive for those who are looking out for outsourcing CRISPR/CAS designing [16].

**10. CROP IT:**

CROPIT is GUI based It helps to identify off targets sites and gives numbering for the same according to chances of occurrence and it also incorporates whole genome level biological information from existing Cas9 binding and cleavage data sets. Utilizing whole-genome chromatin state information from 125 human cell types [17].

**11. CRISPR direct**:

CRISPRdirect is GUI based online tool enables users to easily select rational target sequences with minimized off-target sites by performing exhaustive searches against genomic sequences. It is a web server for selecting rational CRISPR/Cas targets from an input sequence. The server currently incorporates the genomic sequences of human, mouse, rat, marmoset, pig, chicken, frog, zebrafish, Ciona, fruit fly, silkworm, C. elegans, Arabidopsis, rice, Sorghum, and budding yeast [18].

**12. CRISPRer:**

CRISPRer (Version 1.0) tool for genome-wide selection and assessment of CRISPR/Cas protospacers. It is available on the Galaxy server of the Martin Luther University Halle-Wittenberg. It can be installed on the local system and used by command line [19].

**13. CRISPR scan:**

This tool is based on a large scale analysis of sgRNA mutagenesis activity in zebrafish, it can predict sgRNA activity *in vivo* model which is very unique characteristic of this tool. It is created by Department of Genetics, Yale University School of Medicine, New Haven, Connecticut, USA [20].

**14.CRISPR seek**:

It is an open source software package to identify gRNAs that target a given input sequence while minimizing off-target cleavage at other sites within any selected genome. Created at (Program in Gene Function and Expression and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, United States of America [21].

**15. GT-Scan:**

GT-Scan (Genomic Target Scan) aid in the selection of optimal genomic targets for genome editing or transcriptional control via systems based on CRISPR/Cas and other systems. GT-Scan is a flexible, web-based tool that scans a user defined genomic region for candidate targets and ranks them in terms of the number of exact or approximate off-targets in the genome. GT-Scan allows the user to define a "target rule" in a simple format that specifies target length, constrained positions and positions with high, low or no target (and off-target) specificity. GTScan output is interactive, allowing the user to examine candidate targets and the characteristics of their potential off-targets (number of mismatches, positions of mismatches, genomic locations). The GT-Scan website currently supports target selection in over 25 Ensembl genomes [22].

**16. Off spotter:**

Off-Spotter quickly and exhaustively identifies all genomic sites that satisfy the PAM constraint and are identical or nearly-identical to the provided gRNA. Off-Spotter achieves its extreme performance through purely algorithmic means and not through hardware accelerators such as graphical processing units (GPUs). It also allows the user to identify on-the-fly how many and which

nucleotides of the gRNA comprise the "seed". Then output includes a histogram showing the number of potential off-targets as a function of the number of mismatches. The output also includes for each potential off-target the site's genomic location, a human genome browser hyperlink to the corresponding location, genomic annotation in the vicinity of the off-target, GC content [23].

**17. sgRNAcas9:**

This tool is a software package developed for fast design of CRISPR sgRNA with minimized off-target effects. This package consists of programs to perform a search for CRISPR target sites (protospacers) with user-defined parameters, predict genome-wide Cas9 potential off-target cleavage sites (POT), classify the POT into three categories, batch-design oligonucleotides for constructing 20-nt (nucleotides) or truncated sgRNA expression vectors, extract desired length nucleotide sequences flanking the on- or off-target cleavage sites for designing PCR primer pairs to validate the mutations by T7E1 cleavage assay. Importantly, by identifying potential off-target sites in silico, the sgRNAcas9 allows the selection of more specific target sites and aids the identification of bona fide off-target sites, significantly facilitating the design of sgRNA for genome editing applications [24].

**18. SS Finder:**

SS Finder is a comprehensive tool for the identification of specific CRISPR-Cas target sites with high reliability. It is a freeware, easy to edit, and low memory demand tool compatible with many commonly used operating systems. Our tool is very useful in high throughput inhouse screening applications of large genomes in limited time. This can accelerate the functional genomics research based on the application of CRISPR-Cas system [25].

**19. TIDE:**

TIDE is a simple, rapid and cost-effective strategy that accurately quantifies the editing efficacy and simultaneously identifies the predominant types of insertions and deletions (indels) in the targeted pool of cells. The method, named TIDE (Tracking of Indels by DEcomposition), requires only two parallel PCR reactions followed by a pair of standard capillary sequencing analyses. The two resulting sequencing traces are then analysed using specially designed software. TIDE greatly facilitates the testing and rational design of genome editing strategies [26].

**20. WGE:**

Wellcome Trust Sanger Institute Genome Editing database (WGE) uses novel methods to compute, visualize and select optimal CRISPR sites in a genome browser environment. The WGE database currently stores single and paired CRISPR sites and pre-calculate off-target information for CRISPRs located in the mouse and human exomes. Scoring and display of off-target sites is simple, and intuitive, and filters can be applied to identify high quality CRISPR sites rapidly. WGE also provides a tool for the design and display of gene targeting vectors in the same genome browser, along with gene models, protein translation and variation tracks. WGE is open, extensible and can be set up to compute and present CRISPR sites for any genome [27].

**21. ZiFiT (Zinc Finger Targeter):**

ZiFiT is a simple and intuitive web-based tool that provides an interface to identify potential binding sites for engineered zinc finger proteins (ZFPs) in user-supplied DNA sequences. It identifies potential sites for ZFPs made by both the modular assembly and OPEN engineering methods. In addition, ZiFiT now integrates additional tools and resources including scoring schemes for modular assembly, an interface with the Zinc Finger Database (ZiFDB) of engineered ZFPs, and direct querying of NCBI BLAST servers for identifying potential off-target sites within a host genome. It facilitates the design of ZFPs using reagents made available to the academic research community by the Zinc Finger Consortium [28].

**22. AZIMUTH:**

The steps carried out in this software are simple. Initially, collection and classification of guides available can be carried out and tested to determine their knockout potential and accordingly rated. Then, training data set is thus prepared and by way of machine learning models the performance of each guide is noted and compared. Using this learned model, the guide efficiency can be generalized and a clear view can be obtained of

using genes which were not used while preparing the data set, thus saving enormous amount of time. Features most essential for such predictions are nucleotide identity and so on. Also factors concerning thermodynamics are helpful and things like microhomology is usually considered redundant. Off target effects are not known using this software [29].

**23. COSMID:**

COSMID software searches the genome sequences. The input contains the guide RNA and the PAM sequence. Then based on our requirements it provides base mismatches with an insertion or deletion or base mismatches without any insertion or deletion. The users can ask for the output to contain the primer which are designed by COSMID if required. The output contains target sites, off target sites which are then ranked according to whats ideal along with reference sequences and primer designs. These can be then used for sequencing or mutation detection assay. The output of this software is preferred as it include links to test the results and if not satisfactory the output can be reformatted. It is in the form of text, file or a spreadsheet. It also provides guidelines while working on this software so evaluating and choosing guide strands becomes easier. On performing various experiments, it is observed that the proximity to PAM of the off target sites affected the results. Comparative studies with other softwares like Cas Online Designer, ZiFit, CRISPR, Cas OFFinder and COSMID was carried out and the results obtained favoured COSMID over others [30, 31].

**24. Cas OFFinder:**

It is available as a command line program. This software is written in OpenCl enabling operation in diverse platforms such as control processing units, graphics processing units and digital signal processors. Initially the data containing the guide RNA sequence and PAM sequence is collected. It allows variations in PAM sequences. This data is then compared with the query sequence and the mismatches can be counted. It is not limited by the number if mismatches. The genome sequence file read in FASTA format. Since the memory of the device is not large enough for big data analysis, it is divided to genome data with size permissible for analysis by wrapper. These divided sequences are then collected and searched with all the sites containing that PAM sequence by the searching kernel. Then wrapper 2 delivers the information and comparing kernel does the work of counting mismatches. The processes on the calculation units in all these steps is carried out simultaneously. Wrapper 3 is involved in detecting the potential off target sites having fewer mismatches. The output obtained consists of chromosome number, position, direction, number of mismatches bases and potential off target DNA sequences with mismatches [32].

**25. CasOT:**

This is a Perl script which can be run on windows, nix and Mac OS. There are three searching modes. One that contains the single gRNA by an input FASTA file including the target sites. Second, the off target effects are detected by the genome sequence files. Third, it can be found out that the potential off target sites are located on exons. In this software there is a seed region in which mismatches can be limited and the non-seed region which has no limit. So the input may contain the PAM type and number of mismatches. Data is then compiled that contain all possible 12nt sequences with their mismatches. If all criteria are met of the PAM type and mismatch number after scanning nucleotide by nucleotide, the position and strand of the fragment in the genome or sequence is recorded. The output contains the entire information of locations, sequences, PAM type, numbers, mismatches as a separate file. Gene id and gene symbol is also obtained on providing annotations in the input. A statistic file is also a part of the output which makes it easier to count the potential site. The paired gRNA searching mode is similar. It has an additional benefit of keeping a preferred distance between the two potential off target sites [33].

**26. CCTop:**

The input is in the form of a query sequence. PAM sequence can be targeted using this software however there may be target site 5’ and target site 3’ limitations. Off target sites are also predicted on selecting the required PAM sequence. Mismatches closer to the PAM do not allow DSBs to take place. Maximum core mismatches and maximum total

mismatches can be set according to the requirements. The output consists of a forwarded result that can be downloaded as it is or in a FASTA file or also the query sequence can be visualized with colour coded sgRNA, however this option has its limitations. The input characters are also given and the desired target sites are ranked according to their preference. It takes into account the number of off target sites, mismatches and proximity to exons. The potential off target sites are found out along with its complete information on the genomic coordinates to the gene ID [34].

## CONFLICT OF INTEREST: None.

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