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REVIEW

A PLATFORM TO AID SELECT THE OPTIMAL TOOL TO DESIGN GUIDE RNAS

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KEYWORDS

CRISPR/Cas, *Aid-TG*, gene editing, sgRNA design, web server

HIGHLIGHTS

- Summaries on sgRNAs design.
- Overview of the features of 43 web sgRNA designers.
- A platform to select optimal sgRNA design tool.

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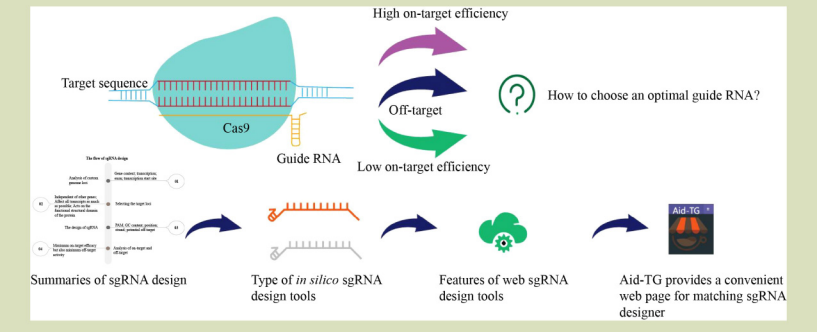
GRAPHICAL ABSTRACT



ABSTRACT

CRISPR-mediated gene-editing technology has arguably driven an unprecedented revolution in biological sciences for its role in elucidating gene functions. A multitude of software has been developed for the design and analysis of CRISPR/Cas experiments, including predictive tools to design optimally guide RNA for various experimental operations. Different *in silico* sgRNA design tools have various application scenarios and identifying the optimal design tools can often be a challenge. This paper describes the sgRNA design workflow in experiments, the classification of sgRNA designers, previously published benchmarking work of *in silico* designers, and the criteria involved how to select an sgRNA web server. Through basic testing, this paper comprehensively overviews and compares the features of 43 web server designers to provide a reference for the readers. Ultimately, the project developed an integrated platform, called *Aid-TG*, which helps users find appropriate tools quickly.

1 BACKGROUND

With the boom in sequencing technology, the relationship between genes and phenotypes can be revealed through a variety of experimental techniques. CRISPR-mediated gene editing is currently the most convenient and rapid technique for observing phenotypic effects by knocking out (knocking down) or activating genes to regulate gene expression[1,2]. Steered by a single guide RNA (sgRNA), CRISPR-associated (Cas) nucleic acid proteins can target and complement near the site where a protospacer adjacent motif (PAM) appears[1]. At targeted genomic loci, Cas proteins generate insertion or

deletion by cellular DNA repair pathways after a DNA double break (DSB)[3,4]. Since the first discovery of the CRISPR/Cas editing system, the CRISPR toolbox continues to expand for better application in various cell types and organisms[5]. Cas9 is the major nuclease in CRISPR-based gene editing, mutants of this Cas enzyme offer additional application scenarios as well as improved editing efficiency[3]. Cas9 nickase is a mutant form of Cas9 that can be created by mutating one of the two nuclease active regions, RuvC1 and HNH. This form of mutation produces a single-strand nick rather than a DSB at the target DNA loci. Using Cas9 nickase, the prime editor efficiently generates accurate base conversion, insertion and deletion effects without the DSB and exogenous DNA templates[6]. Dead Cas9 (dCas9) is a simultaneous mutation of the RuvC1 and HNH nuclease active regions of Cas9. As a result, dCas9 retains only the ability to be guided into the genome by sgRNA, but the cleavage activity is lost. By fusing the dCas9 with a base modification enzyme that operates on single-stranded DNA, the base editor can enable the precise substitution of a single base[7]. In addition, CRISPR interference and activation editors can be generated for transcriptional downregulation and upregulation by integrating dCas9 and transcriptional regulators[2]. Also, CRISPR off/on editing systems was developed to regulate targeted gene expression by adjusting DNA methylation conditions and modifying histone proteins with long-term memory[8]. Likewise, other Cas nuclease families offer additional application scenarios to facilitate their development in medicine and other fields[9,10].

The on-target specificity of all these CRISPR-based editing systems is mainly determined by the guiding component, guide RNA[11]. Since a segment of 20 nucleotides can occur multiple times in a given genome, and some mismatches may be accepted by CRISPR/Cas system, off-target could be produced[12]. Meanwhile, the differential editing efficiency of sgRNAs at distinct locations of the same gene, and hence maximizing on-target and minimizing off-target is essential for the application of the CRISPR/Cas system[13]. One of the most accurate methods is to conduct experiments to screen candidate sgRNAs one by one. However, each step is costly in terms of time, funding and labor. Various experiment data for CRISPR/Cas editing have been available with the application and development of the technology, which can be used for *in silico* analysis for sgRNA design[11,13]. Dozens of predictive tools have been devised in recent years, either in a web server or in a stand-alone program[14–17]. Web-based methods are user-friendly, especially for those without deep understanding of computers. Even so, there are a number of predictive tools with distinctive design propose and frameworks that would confuse users[17–20]. In addition, some tools do not work due to a lack of continuous maintenance and updates by the developers. Here, we characterized the currently available on-target design algorithms in web form, and developed a web-based selection tool, named Aid for Target Guide RNA design (*Aid-TG*), to help users quickly select a system suitable for their purpose[21].

2 sgRNA DESIGN FLOW

When conducting CRISPR-related experiments, there are several key points to note during the sgRNA design (Fig. 1(a)). The first step is to query the database for information about the target gene. It is important to consider the selection of species and to determine the registration number of the target gene in the database in order to avoid searching for the incorrect target gene. Once the target gene information is acquired, further attention should be given to the upstream and downstream sequence context of the targeted loci, the number of transcripts, the number and length of exons, the transcriptional start and stop sites. This information will then be taken into considerable account for further sgRNA design. The next step is to pick the appropriate target areas. For efficient editing of the targeted genes, the following tips should be considered. (1) Avoid selecting regions that overlapped with other genes. (2) Cover as many transcripts as possible and avoid the promoter region, with the target site preferably in the first 50% of the coding region[4]. (3) Act on the functional domain of the protein. The third step is to perform sgRNA design, in which PAM sequence, GC content, positional information, strand, potential off-target sites, is considered[13]. Following aforementioned steps, experiments are performed using the sgRNAs designed in the previous step. Evaluating their efficiency and selecting one or more sgRNAs with maximum on-target efficiency but also minimum off-target efficiency. It was worth noting that each step in the experimental screening process of sgRNA is time-consuming, costly and labor-intensive. These drawbacks prompted the emergence of software tools based on experimental data sets.

3 OVERVIEW OF *IN SILICO* sgRNA DESIGN TOOLS

With the investigation of CRISPR-mediated editing tools, *in silico* design methods based on various frameworks and algorithms have been developed. Depending on different design principles, these sgRNA designers can be divided into three categories (Fig. 1(b))[12,22]. (1) Sequence pairing-based (Table 1)—the Cas protein binding is confined to a DNA target

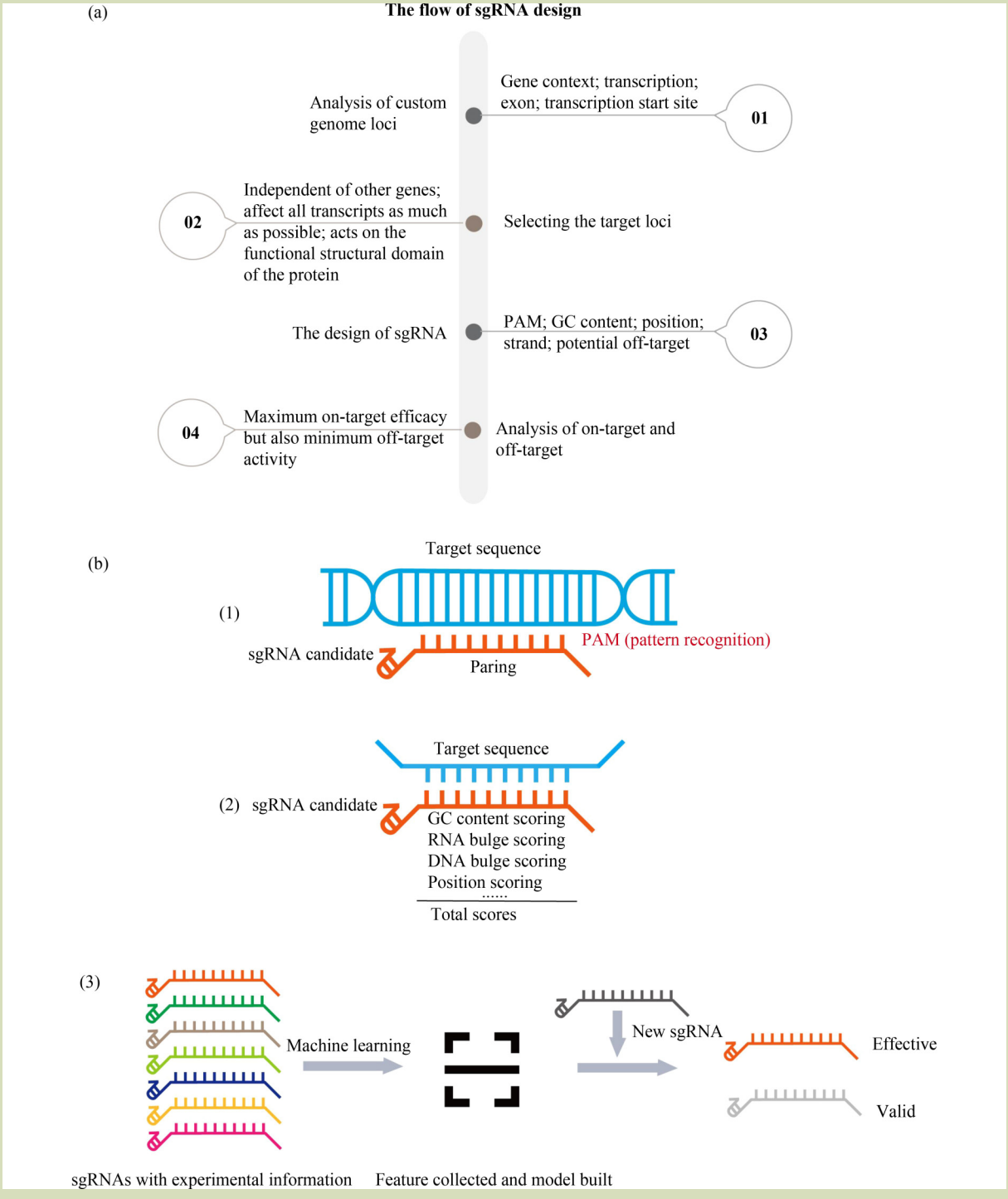


Fig. 1 (a) Schematic diagram showing the workflow of sgRNA design for CRISPR/Cas adaptive immune system. (b) The classification of *in silico* sgRNA design tools. They are sequence pairing-based (1), feature scoring-based (2), and machine learning-based (3), respectively.

site adjacent to the PAM, which is diverse in different species and nucleases. Any the better performing candidate sgRNAs often have fewer mismatches. Also, the type of promoters is an influencing factor, as the U6 and T7 promoters require GG and G at the 5′ end of the sgRNA, respectively[38,39]. As indicated in previous studies, the Cas-OFFinder is mainly designed for potential off-target sites prediction using Bowtie2, while flyCRISPR is designed for *Drosophila* research with an

Table 1 Comparison of the features of sequence pairing-based sgRNA design websites

Tools	Species	Cas effector	Function	Input	Off-target	Additional	Reference
GT-Scan	105 kinds of vertebrate, invertebrate, and plant	Cas9	KO/KI	Sequence; coordinates	Yes	Provide off-target filter	[23]
Cas-Designer	Any species	Cas9	KO/KI	Sequence	Yes	Batch mode	[24]
BE-Designer	Any species	CBE; ABE; CGBE	Base editing	Sequence	Yes	Batch mode	[25]
CHOPCHOPv3	Any species	Cas9; Cas12a; Cas13: CRISPRi; CRISPRa	KO/KI activation; repression	Sequence; gene	Yes	No	[18]
Cas-OFFinder	Any species	Cas9	KO/KI	Sequence	Yes	No	[26]
PE-Designer	Any species	Cas9	KO/KI; base editing	Sequence	Yes	No	[27]
CRISPR-Cereal	Wheat; maize; rice	Cas9; Cas12a	KO/KI	Sequence; coordinate	Yes	No	[28]
Breaking-Cas	Any species	Cas9; Cas12a	KO/KI	Sequence	Yes	Batch mode	[29]
pegFinder	Human	PE3/PE3b	KO/KI; Base editing	Sequence	Yes	No	[30]
CRISPR-PLANT v2	7 kinds of plants	Cas9	KO/KI	Sequence; coordinate	Yes	No	[31]
flyCRISPR	37 kinds of fly	Cas9	KO/KI	Sequence	Yes	Mainly for *Drosophila*	[32]
CRISPy-web2	Any bacterial or fungal	CRISPR-BEST; Cas9	KO/KI; Base editing	Gene	Yes	No	[33]
E-CRISP	55 kinds of vertebrate, invertebrate, and plant	Cas9	KO/KI	Sequence; gene	Yes	Visualization of results	[34]
Off-Spotter	Human; mouse; yeast	Cas9	KO/KI	Sequence	Yes	No	[35]
CRISPRscan	24 kinds of vertebrate and invertebrate	Cas9; Cas12a	KO/KI	Sequence; gene; transcription	Yes	Visualization of results	[36]
CRISPR multitargeter	12 kinds of vertebrate, invertebrate, and plant	Cas9	KO/KI	Sequence; gene; transcription	Yes	Visualization of results	[37]

Note: KO, knockout; KI, knock-in.

alignment design purpose[2,26,32]. (2) Feature scoring-based (Table 2)—editing activity has been found to vary across target loci, suggesting inherent differences in the sensitivity of certain targets to cleavage, leading to a series of explorations to find key features that influence targeting effectiveness[11,48]. Examples include the percentage of GC in candidate sgRNA, position-dependent nucleotide features, position-independent nucleotide motifs and exon position[13,49,50]. (3) Machine learning-based (Table 3)—the system can learn the weights of multiple features from an existing data set. However, the performance of sgRNA design tools based on different frameworks and algorithms vary considerably, especially on training sets from diverse sources[12]. For example, sgRNA Scores v2.0 using a support vector machine as its backend in sequencing data from human HEK293T cells, while the developer of DeepCRISPR chose convolution neural network for both on-target and off-target editing prediction[55]. In addition to the various algorithms on which they are based, the range of editing systems and the features considered contribute to the diversity of sgRNA design tools[65]. The pgRNAFinder is a web tool designed specifically for the guide RNAs of prime editing, while BE-Hive is a tool based on deep learning for sgRNA design of base editing[54,61]. In addition, these tools are either web server and stand-alone program according, with the advantage of online tools is ease of use for those who lack coding skills.

3.1 Previous benchmarking

Nearly 60 predictive tools have been developed in recent years, and a number of them offer both website and stand-alone programs, which makes it challenging to select appropriate

Table 2 Comparison of the features of Feature scoring-based design websites

Tools	Target species	Cas effector	Function	Input	Off-target	Additional	Reference
CRISPR search	Human; mouse	Cas9	KO/KI	Sequence; gene	Yes	Visualization of results	[40]
CRISPR-ERA	9 kinds of vertebrate and invertebrate	Cas9	KO/KI	Sequence	Yes	Visualization of results	[41]
CRISPR-RT	Any species	Cas13a	RNA editing	Sequence	Yes	No	[42]
CRISPR-GE	Any plant species	Cas9; Cas12a	KO/KI	Sequence; gene	Yes	No	[43]
CCTop	Any species	Cas9; Cas12a	KO/KI	Sequence	Yes	Batch mode; visualization of results; T7/U6/Custom promoter	[19]
CRISPick	Human; mouse; rat	Cas9; Cas12a;	KO/KI; activation; repression	Sequence; gene; coordinates	Yes	Batch mode	[13]
GuideScan2	6 kinds of vertebrate and invertebrate	Cas9; Cas12a	KO/KI; base editing	Sequence; gene; coordinate	Yes	Batch mode	[44]
CRISPR-P 2.0	49 kinds of plants	Cas9; Cas12a	KO/KI	Sequence; coordinate	Yes	Visualization of results	[45]
CRISPOR	Any species	Cas9	KO/KI	Sequence; coordinates	Yes	Visualization of results	[46]
FORECasT	Human	Cas9	KO/KI	Sequence	No	Predicting the generated mutations	[47]

Note: KO, knockout; KI, knock-in

tools for guide RNA design[4]. Thus, benchmarking the performance of existing tools and highlighting their applicability scenarios is important for their application[22]. In an attempt to evaluate the performance of various tools, there have been several benchmarking studies done with diversity methods. Hanna and Doench used the human gene *HPRT1* (hypoxanthine phosphoribosyltransferase 1) to compare the on-target and off-target prediction of sgRNAs by four methods, CHOPCHOP, CRISPick, E-CRISP and GUIDES, and found that these methods gave virtually no matching output[4,13,18,34,66]. They also conducted a comparison of guides predicted by CHOPCHOP, E-CRISP and CRISPick for six protein-coding genes, and found the rankings of sgRNAs predicted by the four methods varied considerably. Another benchmarking study was conducted on 17 available *in silico* tools for genome-wide off-target prediction[22]. Through a fair comparison, they found CRISPRoff to provide the best performance and then developed a one-stop integrated genome-wide off-target cleavage search platform (iGWOS), which has demonstrated improved predictive performance[67]. Another study evaluated nine typical targeting design tools using six data sets across five separate cell types[68]. In the end, they recommended different CRISPR sgRNA design tools for diverse application scenarios. They also recommend that users choose E-CRISP and CRISPick first for sgRNA targeting design, as they are well balanced in terms of prediction accuracy, prediction coverage, tool usability and adaptability to different cell types[13,34]. These case studies highlight the common phenomenon of the variation in the predictive performance of forecasting tools due to divergent design principles.

3.2 Criteria for selecting web sever

A list of criteria is needed to help select a tool that matches the particular experiments neatly when facing these predictive tools with different purposes. The first considerations are the diversity of the genome, the type of Cas effector and the function of the editing system being considered. The majority of tools offer sgRNA design mainly for the human and mouse genome, however, there will be significant limitations for those intending to target other genomes[18,24,69]. CRISPR-PLANT v2 will be a better choice in terms of targeting plant genomes, while flyCRISPR is equally suitable for those targeting *Drosophila*[31,32].

Additionally, several tools support hundreds or any species genome, and some even allow the user to provide any

Table 3 Comparison of the features of machine learning-based design websites

Tools	Target species	Cas effector	Function	Input	Off-target	Additional	Reference
ACEofBASEs	Any species	CBE; ABE	Base editing	Sequence	Yes	Batch mode	[51]
DeepHF	Human	Cas9	KO/KI	Sequence	No	T7/U6 promoter	[52]
BEdeep	Human	ABE; CBE	Base editing	Sequence	Yes	No	[52]
inDelphi	Human	Cas9	KO/KI	Sequence	No	Visualization of results; batch mode	[53]
BE-Hive	Human	ABE; CBE	Base editing	Sequence	Yes	Predicting the generated mutations	[54]
SSC	Human; Mouse	Cas9	KO/KI; activation; repression	Sequence	Yes	No	[20]
sgRNA scorer 2.0	Any species	Cas9	KO/KI	Sequence	Yes	No	[55]
WU-CRISPR	Any species	Cas9	KO/KI	Sequence; gene	Yes	No	[17]
DeepSpCas9	Human	Cas9	KO/KI	Sequence	No	Batch mode	[56]
DeepCpf1	Human	Cas12a	KO/KI	Sequence	No	Batch mode; chromatin accessibility	[15]
BE-smart	Human	Cas9	Base editing	Sequence	No	No	[57]
CRISPRETa	Any species	Cas9	KO/KI	Sequence; gene	Yes	No	[58]
CRISPRdirect	Any species	Cas9	KO/KI	Sequence; gene	Yes	Visualization of results	[59]
EuPaGDT	Any species	Cas9	KO/KI	Sequence	Yes	No	[60]
pgRNAFinder	10 kinds of vertebrate and invertebrate	Cas9	KO/KI	Sequence; gene; coordinate	Yes	No	[61]
TUSCAN	105 kinds of vertebrates and plants	Cas9	KO/KI	Sequence; coordinate	No	No	[62]
DeepBaseEditor	Human	Cas9	Base editing	Sequence	No	Yes	[63]
BE-DICT	Human	Cas9	Base editing	Sequence	Yes	Yes	[64]

Note: KO, knockout; KI, knock-in.

genomes[70]. PAM recognition sites differ according to the type of Cas enzymes, although the options provided by most tools for Cas9 or Cas12a and their mutants are likely to be sufficient for most users, more comprehensive PAM options will be more helpful accompanied by the development of the CRISPR toolkit. The function of an editing system under consideration is fundamental in the choice of a guide RNA designer. For example, if a transformation of a specific base is needed BE-Hive or BE-smart are recommended[13,54]. Also, the input and output provided by the website are important criteria. Some websites only support sequence input whereas others provide gene symbols and/or coordinates. The major output of these websites is often a table with the corresponding analysis values but some offer additional visualization of the results that may be more intuitively interpreted[19,41]. Some users are more interested in machined learning-based tools for prediction, consequently the design principle is also worth considering.

4 PLATFORM FOR SELECTING THE OPTIMAL sgRNA DESIGN TOOL

Notably, the constantly updated and maintained website can be onerous for developers, and so certain tools are no longer maintained probably as they have few users, such as a notice of CrispRGold has been posted that they went offline in March 2021 due to server-side issues[71]. Together with the advantages of the website design tools described above and the wide range of tools currently available, we propose a solution for choosing the optimal tool for users. First, we tested almost all available web servers for sgRNA design that could be found at the time, mainly by using the test data given on the website, and excluded those that were not working properly. According to our previously summarized criteria for choosing an sgRNA designer, we carefully characterized 43 post-selection website tools. A detailed comparison is given in three tables

(Tables 1–3), which provides a relatively comprehensive reference. As no one tool is a panacea, it is critical to fully consider the prerequisites and intended purpose of an sgRNA designer before selecting. To obtain accurate results, the user

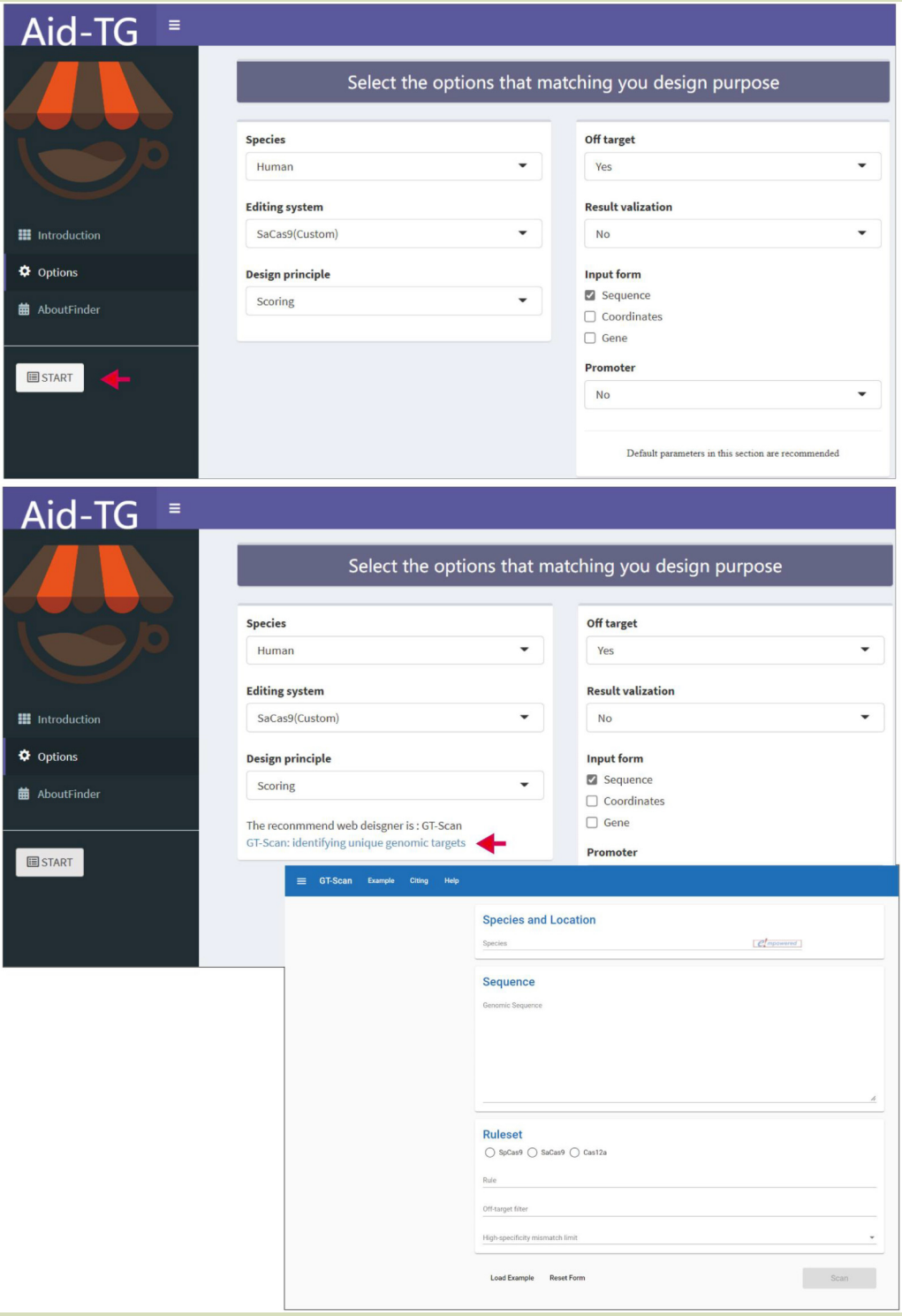


Fig. 2 The display of *Aid-TG*'s mainly panel. Here, select your matching options and click "START", then the recommend designer will be returned. And you can click the "Click here" to go directly to the usage page of the recommend tool[21].

will need to mix and match the result of multiple tools sometimes, where our summarized work could be useful.

In the end, a platform, namely *Aid-TG*, which integrates the features including species genomes, Cas effectors, and functions of 43 web servers is provided to help users find the optimal guide RNA design tool easily and quickly (Fig. 2). The user-friendly interface of *Aid-TG* offers a simple selection of options with buttons and outputs the most recommend web server tools with their introduction and address. In brief, users can choose their target genome, PAM sequence, Cas enzyme and the function of the gene-editing system according to their experimental purpose from a series of options that integrates the main information of 43 websites designer. Another advantage of *Aid-TG* is that it covers a wide range of messages, which is likely sufficient for most application scenarios, hence greatly avoiding the hassle of searching for the matching information of purpose one by one. For example, if a person wants to design an sgRNA that targets the human *Tyr* gene for knockdown based on the Cas9 enzyme, he just needs to open *Aid-TG* and click the selection, and then a recommended designer with its address will be output. Overall, *Aid-TG* provides a convenient web page for matching sgRNA designer neatly.

5 CONCLUSIONS

The CRISPR-mediated editing system is a powerful toolkit for gene engineering and has been applied to research in a number of areas including medicine, agriculture and basic life science. However, the on-target efficiency, which needs to be improved, and the potential off-target effect hinder the application in the clinic. Choosing an appropriate sgRNA is one of the effective strategies to increase the on-target efficiency with minimized off-target effect. A large number of *in silico* designers have been developed based on various algorithms and frameworks, but their results and application scenarios varied dramatically, which makes it confusing for users to choose the optimal designer for their project. In this study, we provide an overview of the conventional design of sgRNAs and the major genres of *in silico* tools. We also summarized benchmarking studies of sgRNA designers and provided principles to follow for selection of a guide RNA design tool. After testing 43 sgRNA design algorithms, we present here a table with key information on 43 web designers. We also developed a web server platform for the user to choose the optimal designer that matched their particular experiments in a simple and convenient way, which displays helpful guidance for sgRNA design.

Compliance with ethics guidelines

Qianqian Yang and Lei Ma declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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