

Instruction Guide (05.05.2026)

Breaking-Cas Tool

Spanish National Centre for Biotechnology (CNB-CSIC)

Getting started:

- By opening the link to Breaking-Cas¹ on our homepage, you will be redirected to the Breaking-Cas¹ website. The system will allow you to use your reference genome, based on the organism you are working with (vertebrates vs bacteria, protists, fungi, plants and invertebrate metazoan).
- To test the system, you can also click on "**Fill with example**" button at the bottom left of the page.
- When you paste your FASTA sequence, please make sure you do NOT also paste the header of your FASTA.
- Based on the Cas enzyme you are going to use for your experiment, you can change the nuclease settings (point 3) accordingly. Also, you can specify your PAM, again, based on the CRISPR system you are going to use. Breaking Cas¹ also offers the option "Argonaute", if you are going to use this system. To do so, change the nuclease settings to "Custom".
- Please note that the default settings for Guide length is **20 nucleotides** and the allowed mismatches are set to "**Up to 4**". Only expert users should change these settings. The mismatches are set to 4 because CRISPR- Cas enzymes can recognize and cut sequences having up to 4-5 mismatches, compared to the intended target sequence.
- Once you click on "submit", you will be redirected to a "Waiting Room" page. Once the system has finished calculating your CRISPR guide, you can either download the report or click on "**View online**".

The result page:

- **On the left side** of the page you can see a list of the potential guide sequences, their position within the sequence you have imported, number of on-target and off-target sequences, the number of genes involved in the off-target and a final score.
- **The final score** is calculated considering the probability for the off-target sequence to act as a real Cas-9 secondary site. The higher the score on the result page, the better the suggested guide sequence should work. For more information on the actual algorithm used, please refer to the original publication¹.
- Certain sequences are represented with stretches of red Ts. This depends on the fact that **a stretch of 4Ts** represents the termination signal for RNA polymerase III. Therefore, you should consider avoiding the 4 stretches-gRNA if you are going to use gRNA expressing plasmids having U6 promoters.
- **The PAM** is shown next to the suggested gRNA in orange. By clicking on a specific guide, the guide will be shown on a mini gene-browser on the right side. The PAM will be shown here in brackets. Remember that you should **NOT include** the PAM in your gRNA, when ordering gRNAs.

- You can zoom in and out in the gene browser by position your mouse on the browser and scroll down and up. Alternatively, you can click on the +/- buttons on the right side.
- By clicking on the e! symbol, you will be redirected to e! Ensembl website.
- To help you designing the remaining portion of your sgRNA, please visit our CRISPR-Cas introductory page.

Breaking-Cas¹ also offers **Tutorial Guidelines**. Just click on the button "Tutorial" on the top right corner of the Breaking Cas¹ Welcome page.

References:

1. Oliveros JC., Franch M., Tabas-Madrid D., San-León D., Montoliu L., Cubas P., Pazos F. **Breaking-Cas-interactive design of guide RNAs for CRISPR-Cas experiments for ENSEMBL genomes**. *Nucleic Acids Res.* 2016 Jul 8;44(W1):W267-71.