SUPPLEMENTARY INFORMATION

An enhanced assay to characterize anti-CRISPR proteins using a cell-free transcription-translation system

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SUPPLEMENTARY TABLES

Table S1. Plasmids and oligos used in this paper.

Lab Number	Description	Type	Source	Link
pCB663	sgRNA-non-targeting SpCas9	plasmid	[1]	https://benchling.com/s/seq-OqAfM7vHlaOfDyuU0hkl
pCB672	sgRNA-pos9 SpCas9	plasmid	[1]	https://benchling.com/s/seq-ApSabJv5XAKmGY9GHAjK
pCB843	SpCas9 no tracr Cm p15a	plasmid	[1]	https://benchling.com/s/seq-clUsq1SVDNr3EbHlmpi0
CBS-011	P70a-T7RNAP	plasmid	Noireaux Lab	https://benchling.com/s/seq-euiov04Zj34mdP6RctWu
CBS-107	CjCas9 targeted plasmid, p70a-deGFP-NT	plasmid	This study	https://benchling.com/s/seq-YRvmrbZpy3VrHWmAKGBi
CBS-117	COCjCas9 plasmid	plasmid	This study	https://benchling.com/s/seq-ThKBJYznZVaiWUayNG0m
CBS-311	CjCas9 targeting sgRNA plasmid	plasmid	This study	https://benchling.com/s/seq-PVDukn2gHMVMi1WhJr34
CBS-312	CjCas9 non-targeting sgRNA plasmid	plasmid	This study	https://benchling.com/s/seq-I8FoaBTskhGmLqKyu1hu
CBS-338	P70a-deGFP	plasmid	Noireaux Lab	https://benchling.com/s/seq-QOLPWKrpy5ZAaBhQBpp6
-	J23119-AcrIIA4	oligo	-	https://benchling.com/s/seq-PsAeT851gLCZ2UJbdZ3l
-	J23119-AcrIIA3	oligo	-	https://benchling.com/s/seq-T1sSujUdHPd1frWYeQnD
-	J23119-AcrIIA3-2	oligo	-	https://benchling.com/s/seq-Coau54DANm2kOk3GIRK7
-	J23119-AcrIIA3-4	oligo	-	https://benchling.com/s/seq-UqAACHh4C8BB865dulWi
-	J23119-AcrIIA4-1	oligo	-	https://benchling.com/s/seq-qwq8PhYMq8Y12N3AwOf8
-	Forward primer to amplify Acr gBlocks	oligo	-	https://benchling.com/s/seq-f8O85aAm5zyzZySVtPxi
-	Reverse primer to amplify Acr gBlocks	oligo	-	https://benchling.com/s/seq-DlsyUfOCSBgzjBc0Ls5C
-	T500 terminator	-	-	https://benchling.com/s/seq-KxF5C2No3aAMnSj6cNWv

SUPPLEMENTARY METHODS

Echo 525 Cleavage Assay

All shown data was produced using the Echo525 Liquid Handling system. The cleavage assays were therefore scaled down to 3-µl reactions per replicate, with 4 replicates each. The pre-expression reactions were performed manually, as depicted in the manuscript.

- Protocol
    1. Perform pre-expressions as described in the manuscript.
    2. To program the Echo, use either PlateReformat or CherryPick.
    3. It should be programmed to produce four replicates of each reaction.
    4. Use a 96-well V bottom plate as destination plate.
    5. Prepare cleavage assay, scaled down to 3-µl total volume. To estimate the volume of MyTXTL needed for this experiment, consider the dead volume of the respective source plate and the number of reactions.
    6. Load the reagents into the source plate.
    7. Let the Echo run the prepared protocol.
    8. Seal the destination plate with a cover mat to prevent evaporation of the reactions.
    9. Place the plate in a plate reader to measure GFP fluorescence (Ex 485 nm, Em 528 nm). The plate reader should be pre-warmed to 29°C (see **Note 1** in main text).
    10. Incubate the reactions for 16 h at 29°C (see **Note 1** in main text) and measure GFP fluorescence every three min.
- Data Processing
    1. Export the data in an excel spreadsheet after the plate reader run is finished. It should include time points, temperature and fluorescence intensity values for each well and each time point.

2. Subtract the background fluorescence. Background fluorescence was measured for each plate reader separately using a TXTL reaction containing a plasmid that doesn't encode deGFP. Fluorescence values were measured for 16 h at 29°C, identical to the measurement settings of the cleavage assay.
3. Perform Grubb's test with the values after 16 h to identify outliers between replicates (alpha = 0.1). If no outliers were identified, standardize which three of the four replicates you choose, e.g. the first three replicates.
4. If needed, calculate the deGFP concentration for each timepoint by using a deGFP standard curve (see **Note 3** in main text).
5. Calculate the average of the replicates and visualize the data on a graph by plotting the fluorescence over time.
6. Calculate standard deviations for the replicates and use them to show error bars for each time point in the graph.
7. Calculate the fold-reduction for the reporter construct using the ratio of deGFP concentrations after 16 h of the reaction containing non-targeting sgRNA over the reaction containing targeting sgRNA.

SUPPLEMENTARY REFERENCES

[1] R. Marshall, C.S. Maxwell, S.P. Collins, T. Jacobsen, M.L. Luo, M.B. Begemann, B.N. Gray, E. January, A. Singer, Y. He, C.L. Beisel, V. Noireaux, Rapid and scalable characterization of CRISPR technologies using an *E. coli* cell-free transcription-translation system, Mol. Cell. 69 (2018) 146–157.e3.