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Engineering of crRNA sequences for MAD7 cleavage efficiency optimization

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Background. Class 2 type V-A CRISPR nucleases are promising effectors for CRISPR-based genome modulation, featuring unique properties. They are directed for cleavage by a single short (42 bp) crRNA processed from transcript by intrinsic RNAse activity, thus enabling multiplex gene editing. The structural direct repeat (DR) configuration and chemical modification of crRNAs have been proven to influence the nuclease ability to process crRNA and stability of crRNA itself. MAD7 (ErCas12a) is a recently discovered Cas12a ortholog, which has high structural similarity to other Class 2 type V-A CRISPR nucleases. **Aim.** Comparison of native and chemically modified synthetic crRNAs in complex with MAD7 nuclease for reporter knockout in eukaryotic cell lines. Evaluation of alternative crRNA array formats on MAD7-mediated multiplexed gene editing in eukaryotic cells. **Methods.** To assess alternative formats of synthetic crRNA 3 variants were synthesized: unmodified RNA; flanked with poly-rU with 3 terminal rUs substituted with 2-O-Me-Us linked by phosphorothioate bonds; flanked by complementary to the target sequence DNA bases. All synthetic crRNAs were synthesized by TBDMS-phosphoramidite chemistry and purified by DMT-on RP SPE. A set of plasmid constructs expressing pre-validated crRNAs targeted to CyOFP1 under control of the U6 promoter were assembled to test 2 crRNA array configurations: 1 — with two crRNAs expressed from separate PolIII expression cassettes with 300bp spacer in between; 2 — single expression cassette with crRNAs placed in "head-to-tail". Also 2 alternative DRs were used: full-DR and hairpin-only DR (trDR). Separate plasmid vectors with PolIII cassettes for each crRNA with trDR and their co-transfection were used as controls. MAD7 nuclease was expressed from a plasmid vector driven by mCMV promoter for both arrays. CHO DG44 stable cell line expressing both EGFP or CyOFP1 had been generated previously. Tested sets were electroporated and at passage 3 post-transfection cells were analyzed by flow cytometry to determine the fraction of cells with inactivated reporter (referred as "editing efficacy" below). The experiment was performed in three replicates. **Results:** For the alternative synthetic crRNA forms we obtained the highest editing efficacy for 2-O-Me/PS-modified crRNA (23.9%), potentially attributing to increased crRNA stability *in situ*. The lowest efficiency was for DNA-flanked form — 12,7%, with unmodified crRNA resulting in 18,5%. The editing efficacy of arrayed crRNA systems were influenced by DR variant, favoring the first crRNA in arrays with trDR, if placed in single-cistron configuration, while having little positional effect with full-DR, highlighting necessity of 5`-terminal sequences to DR hairpin for efficient crRNA array processing. **Conclusions:** We demonstrated that the MAD7-based editing activity depends on synthetic crRNA modifications, favoring stabilizing modifications at both 5`- and 3`-ends of crRNA. Efficient crRNA array processing in single-cistron format requires 5`-terminal sequences from full-DR configuration. Further optimization of all components is required to increase system activity in mammalian cells.

Keywords: CRISPR-Cas, MAD7, synthetic crRNA, crRNA array.