



Medium-throughput *in vitro* detection of DNA cleavage by CRISPR-Cas12a

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ABSTRACT

Quantifying DNA cleavage by CRISPR-Cas nucleases is usually done by separating the cleaved products from the non-cleaved target by agarose gel electrophoresis. We devised a method that eliminates the quantification from band intensity on agarose gel, and uses a target with a fluorescent dye on the one end and a biotin on the other. Cleavage of the target will separate the dye from the biotin, and cause the dye to stay in solution when streptavidin beads are introduced. All non-cleaved target will be eliminated from solution and no longer contribute to detectable fluorescence. Cleavage will therefore increase the fluorescent signal. A control, which has no streptavidin treatment, is taken along to correct for any errors that might have been introduced by pipetting, inactivation of the fluorescent dye or release of the biotin during several steps of the procedure. With this method we were able to quantify the fraction of active Cas12a in a purification sample and assess the cleavage rate.

1. Overview

Quantifying DNA cleavage by CRISPR-Cas nucleases is key in studying their kinetics [1]. We found the need for a method that is more high-throughput than analysis by a commonly used method like agarose of polyacrylamide gel electrophoresis and subsequent quantification [2], and does not require expensive equipment like the Agilent Fragment Analyzer. The basic principle behind this technique is the separation of a fluorophore and a biotin upon cleavage of the nuclease. The target DNA features a fluorophore on the one end and a biotin on the other. After an appropriate incubation period with the nuclease, the biotin is bound to immobilised streptavidin on magnetic beads and removed from solution. Therefore, any DNA molecule that retains its biotin label will no longer contribute to the fluorescence of the solution. By comparing that fluorescence to the fluorescence when no streptavidin was added, one can estimate the fraction of DNA target that is cleaved by the nuclease. An overview of the principle is depicted in Fig. 1A and an overview of the workflow is described in Fig. 1B. While this method was developed for the assessment of CRISPR-Cas nucleases, it could be applied to other DNAses as well (argonaute [3,4], restriction enzymes), as long as it separates the fluorophore from the biotin. Note that the CRISPR-Cas nuclease cleavage activity observed in these assays may not resemble the *in vivo* situation perfectly, since more factors are involved *in vivo* (e.g. chromatin structure [5,6]).

2. Equipment

A microtiter plate reader that can measure fluorescence with the appropriate wavelengths for the fluorescent dye is required. We used a Biotek Synergy MX microplate reader. We made our own magnet plate to pull down the streptavidin beads. The rims were sawn off a flat-bottom 96 wells plate (Greiner). Neodymium disc magnets with a diameter of 6 mm and a height of 1 mm were used (<https://www.supermagnete.nl> (S-06-01-N)). The magnets on the bottom of the plate are held in place by another magnet inside the well (Fig. 2). Magnets in a horizontal or vertical line on the plate alternate their polarity, while all diagonal lines have the same magnetic polarity (like a chess board).

3. Target design and generation

Targets can be made either by “round the horn” overhang extension PCR and subsequent re-circularisation, or by ligating annealed oligos into a Golden Gate entry site. Alternatively, an oligo with a target flanked by two primer annealing sites (one for the biotin primer and the other for the Alexa Fluor primer) can be used as a template. A 770 bp amplicon that harbours the protospacer target was made by polymerase chain reaction (PCR), with a biotin on the 5'-end of one primer and the an Alexa Fluor 594 dye on the 5'-end of the other primer. In our experience, the Alexa Fluor 594 can withstand the PCR denaturation steps to a sufficient degree for detecting the DNA, but the integrity is somewhat compromised (by about 4%). Target PCRs were purified by

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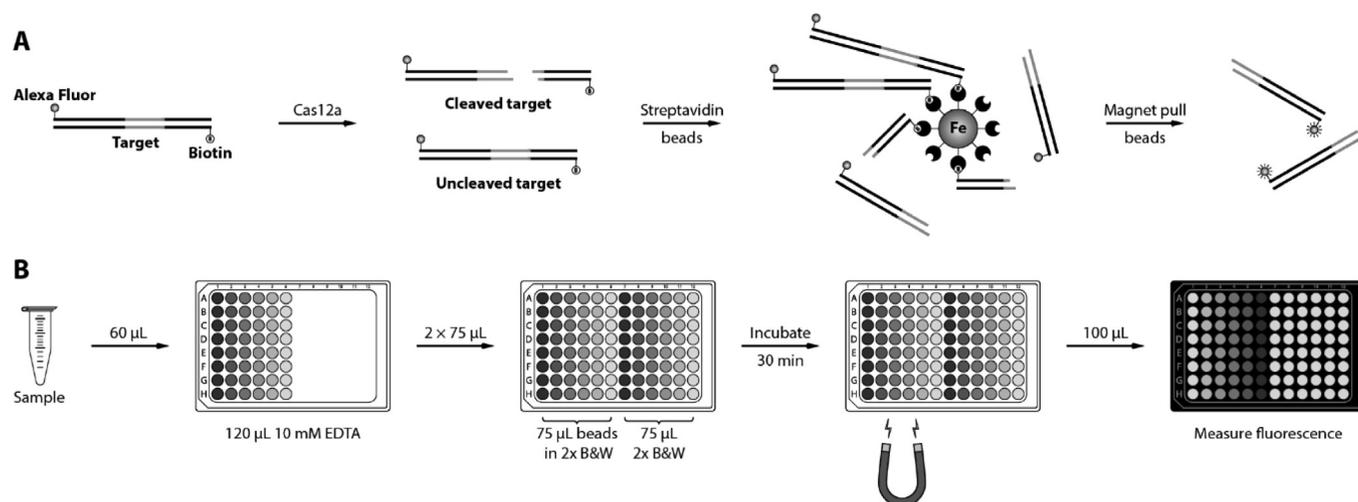


Fig. 1. Medium-throughput *in vitro* detection of DNA cleavage. (A) The principle of the here-described method is that the DNA nuclease of interest separates the biotin from the fluorophore, which results in different fluorescence intensities when the biotin-labelled target DNA molecules are removed from solution. (B) Practical overview of the technique. The colour intensity indicates the amount of cleaved target DNA, which can be caused by a higher concentration of Cas protein, or a longer reaction time. Half of the plate is dedicated to the controls. Control samples without beads do not have fluorescence removed, so show maximum fluorescence always.



Fig. 2. Magnet plate to pull down the streptavidin beads. Only half of the plate will contain beads, so there is no need for a full plate of magnets.

the Zymo clean and concentrator kit (Zymo Research) and quantified with a Qubit dsDNA BR Assay Kit (Thermo Fischer Scientific). The Qubit quantifies DNA mass, so the concentrations were recalculated to molar.

4. Guide design and *in vitro* transcription

A template oligonucleotide was designed for *in vitro* transcription (IVT). It is the reverse complement of the T7 promoter that is followed by a guanine for IVT efficiency, the mature repeat and the spacer derived part of the guide. The T7 RNA polymerase only requires the promoter to be dsDNA, so the non-template oligo only comprises the T7 promoter and no part of the actual transcript. IVT was performed with 1 mM rNTPs, 0.5 μ M T7 promoter sense oligo, 0.25 μ M template oligo and 10 U/ μ L home-made T7 polymerase in T7 buffer (NEB) at 37 $^{\circ}$ C for 1 h. The gRNAs were purified from denaturing polyacrylamide gel by the crush and soak method in 50 mM Tris-HCl, 1 mM EDTA and 10 mM DTT and subsequently concentrated and purified by ultrafiltration using a Amicon 3 k filter unit (Merck-Millipore) and washing with ultrapure water. gRNAs were quantified with the Qubit RNA BR Assay kit (Thermo Fischer Scientific).

5. Titrating the streptavidin beads

The target was titrated with Dynabeads MyOne Streptavidin C1 (Thermo Fischer Scientific) to determine their specific capacity. Target molecules may not carry the biotin label, while they do carry the Alexa Fluor label. These target molecules cannot be bound to the streptavidin beads and cause a minimum residual fluorescence after streptavidin binding the subsequent removal of bound target molecules. The concentration of beads at which the minimal residual fluorescence was observed was used in the cleavage assays. The size of the target will largely determine the binding capacity of the beads, where larger DNA

molecules reduce the binding capacity. This means that for other target sizes, the titration should be performed again. The following was used: 50 μ L of a 1 ng/ μ L solution of 770 bp target was bound to different amounts of Dynabeads[®] MyOne[™] Streptavidin C1 that was diluted 40 \times in 1 \times bind and wash solution.

6. Nuclease titration

Protein quantification assays like the Bradford assay can estimate the total amount of protein, but cannot distinguish between active and inactive protein. As a test case, we used the Cas12a nuclease (formerly known as Cpf1 [7]) from *Francisella novicida* U112. The binding of Cas12a is not easily reversed, so it will stay bound to the target, making it a single-turnover enzyme [1]. This makes the activity quantification different from a multi-turnover enzyme, as the concentration of Cas12a needs to be in the same range as the target concentration for full cleavage to occur.

10 nM of target was cleaved by different amounts of Cas12a in the presence of high amounts of gRNA (> 100 nM) in NEBuffer4 to determine the concentration of Cas12a after purification. Full cleavage can be observed when the amount of Cas12a is equal or higher than the amount of target, but since the cleavage rate slows down when the target is depleted, the best estimates can be derived from reactions where 5–8 nM of target is cleaved.

7. Cleavage assay

The target DNA solution was diluted to 8 nM in ultrapure water (equals 2 \times concentrated). The 2 \times RNP solution was composed of 6 nM Cas12a protein and 18 nM gRNA in 2 \times NEBuffer4 (NEB). The 2 \times target and 2 \times RNP solutions were pre-warmed at 37 $^{\circ}$ C in a climate chamber. The solutions were mixed 1:1 while leaving enough sample for a non-cleaved control and incubated for up to 30 min at 37 $^{\circ}$ C. The cleavage reactions were quenched by pipetting 60 μ L of cleaved sample in 120 μ L of 10 mM EDTA. The non-cleaved control was made by pipetting 30 μ L of 2 \times target solution in the 120 μ L of 10 mM EDTA first and then adding 30 μ L of 2 \times RNP solution. The quenched reactions were cooled down to room temperature for further processing.

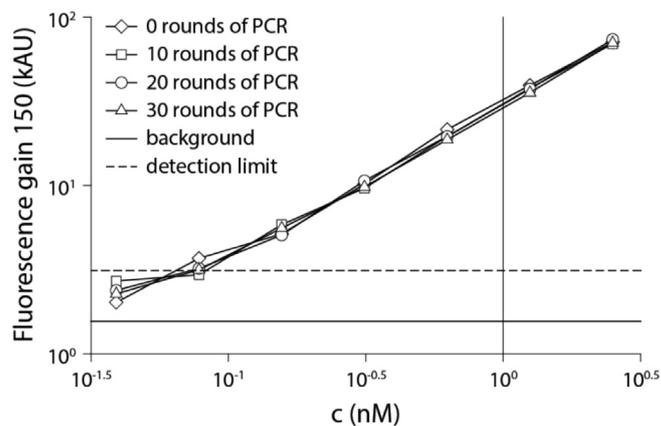


Fig. 3. Thermostability and detection limit of the Alexa Fluor 594. The solid line indicates the basal fluorescence of the buffer without any fluorescent dye, while the dotted line is two times that value.

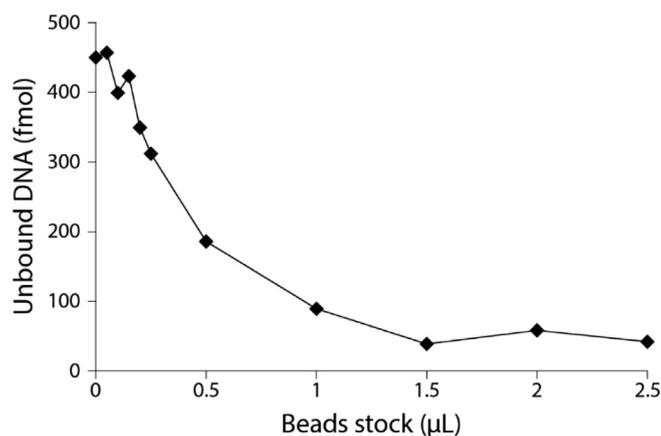


Fig. 4. Streptavidin beads titration. Without beads, the fluorescence is at its maximum. Maximum binding is reached at 1.5 μL streptavidin beads stock suspension.

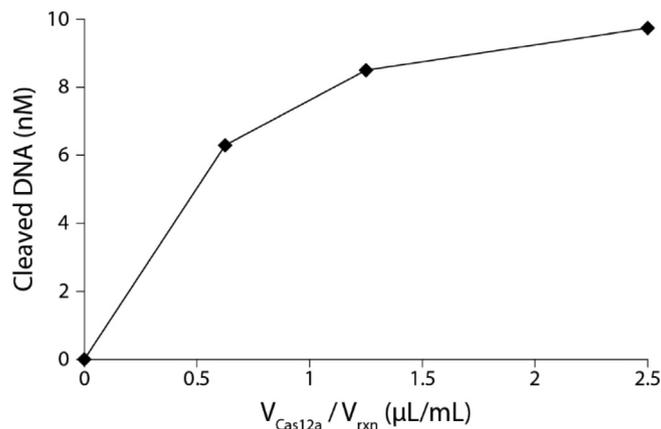


Fig. 5. Titration of FnCas12a. On the y-axis is the amount of cleaved DNA, while the inverse dilution factor is on the X-axis.

8. Streptavidin treatment

75 μL of quenched reaction was pipetted into 75 μL $2\times$ B&W buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl) and another 75 μL was pipetted into 75 μL $2\times$ B&W buffer containing 2 μL of streptavidin beads. The samples were then incubated at room temperature whilst shaking mildly for 45 min. The beads were pulled down by a magnet and 100 μL of sample was transferred to a black 96 wells plate for fluorescence measurement at 585 nm with a bandwidth of 20 nm as excitation wavelength and 626 nm with a bandwidth of 20 nm as emission wavelength.

9. Calculating the cleavage

The cleavage can be estimated by the liberation of fluorescent dye. Provided that all targets are biotinylated, no fluorescence should be observed without cleavage. However, in practice, there is always a fraction of primers that have no biotin on the 5'-end. Therefore, it is important to take that fraction into account, when accurately determining the cleavage.

Each cleavage assay is comprised of a dsDNA that is targeted by Cas12a ("cleaved") and a non-targeted control ("uncleaved"). Both of these are split in two; one is treated with streptavidin ("strep") and the

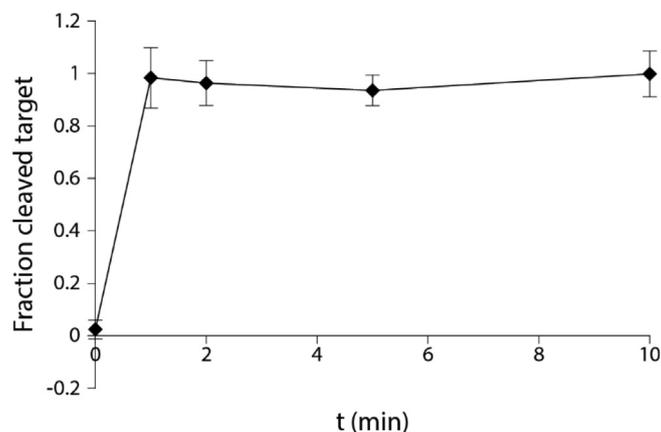


Fig. 6. Kinetic assay. A 770 bp target was cleaved by FnCas12a in the presence of corresponding gRNA. The error bars indicate the SD for 6 replicates.

other is not (“total”). The fluorescence (FI) of all samples is measured.

Estimating the fraction of non-biotinylated targets is done by

$$f_{nonBtm} = \frac{FI_{uncleaved_strep}}{FI_{uncleaved_total}}$$

We assumed that the biotinylation of the target does not influence the cleavage rate. However, the fraction that is not biotinylated does not lose fluorescence upon streptavidin treatment. The fluorescence in the cleaved sample that is still observed after streptavidin treatment can either be derived from target that is biotinylated and cleaved, or target that is not biotinylated.

$$f_{FI} = \frac{FI_{cleaved_strep}}{FI_{cleaved_total}} = f_{cleaved} \cdot (1 - f_{nonBtm}) + f_{nonBtm}$$

Rewriting this formula gives:

$$f_{cleaved} = \frac{f_{FI} - f_{nonBtm}}{1 - f_{nonBtm}}$$

Or in its expanded form:

$$f_{cleaved} = \frac{\frac{FI_{cleaved_strep}}{FI_{cleaved_total}} - \frac{FI_{uncleaved_strep}}{FI_{uncleaved_total}}}{1 - \frac{FI_{uncleaved_strep}}{FI_{uncleaved_total}}}$$

Appendix A Equipment and supply list

Equipment

Microplate reader that can measure fluorescence
96 wells magnet plate
Multichannel pipettes

Supplies

96 wells plates
Black 96 wells plates for fluorescence
Dynabeads® MyOne™ Streptavidin C1
DNA oligo with Alexa Fluor 594 attached to the 5' end
DNA oligo with biotin attached to the 5' end

Appendix B Assay setup

Alexa Fluor 594 heat stability and detection limit

100 nM of the oligonucleotide with the Alexa Fluor 594 attached to it was subjected to several rounds of PCR (98 °C 20 s, 60 °C 30 s, 72 °C 30 s) after an initial 98 °C 60 s step. The reaction was carried out in 1 × Q5 master mix (NEB). The reactions were then serially diluted by a factor 2 starting at 10 nM and 100 μL was measured in a Biotek Synergy MX microplate reader (Fig. 3). The Alexa dye is at about 96% of its initial fluorescence after 30 rounds of PCR. The detection limit of this fluorescent dye is about 0.1 nM.

10. Tips and tricks

- While pipetting accuracy is always important, from the step where the quenched sample is split into a streptavidin treated part and an untreated part and onwards, accurate pipetting is paramount. Using reverse pipetting is a good way to keep the volumes exactly the same.
- Air displacement pipettes are sensitive to differences in temperature. In a climate chamber this will not be an issue when all equipment is acclimatised.
- Alexa Fluor dyes may be slightly light sensitive, so keep everything in the dark.

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Streptavidin bead titration

457 fmol of a 770 bp linear DNA fragment with on one side a biotin and on the other side an Alexa Fluor 594 was incubated with different amounts of streptavidin beads. After magnetic pulldown, supernatant fluorescence was measured and recalculated to unbound DNA (Fig. 4). The minimum fluorescence is reached at about 1.5 μ L of streptavidin beads stock suspension. From this experiment, the amount of non-biotinylated DNA is estimated at about 8%.

Cas12a titration

FnCas12a was purified following an established protocol [8]. The final concentration was about 200 μ M as estimated by Roti nanoquant (Roth) analysis. 10 nM of DNA target was digested with different amounts of FnCas12a at 37 °C for 30 min (Fig. 5). Since this procedure is carried out to estimate the amount of active FnCas12a in the batch, a significant fraction of target must be cleaved, while the non-cleaved DNA is in excess. The reaction of FnCas12a with its target lowers both of their concentrations and will slow down the reaction. This is prevented by an excess of target. Just over 6 nM of DNA is cleaved at a factor 1600 dilution of the stock (0.625 μ L/mL), which indicates that the amount of active protein in the batch is actually quite low: about 10 μ M.

Kinetic assay

3 nM (final concentration) of FnCas12a was mixed with 9 nM crRNA (final concentration), equilibrated at 37 °C and mixed with 4 nM target (final concentration). Samples were taken at 1, 2, 5, 10 and 30 min and analysed (Fig. 6). The cleaved fraction was then normalised for the maximum cleaved fraction at 30 min. In this case, cleavage is quite fast and the reaction is already over after only 1 min.

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