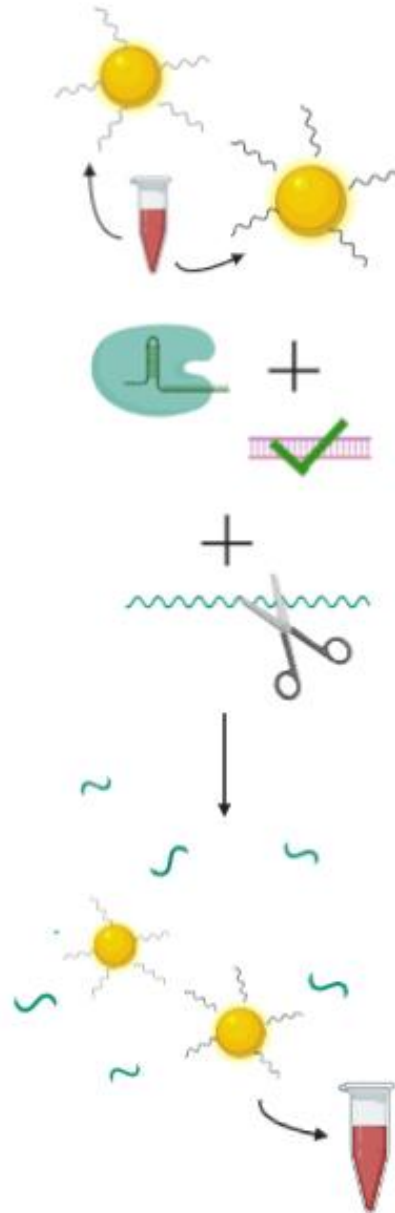


## Combining CRISPR/Cas12a and gold nanoparticles to detect low sample concentrations of nucleic acids



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## Abstract

As the pressure of healthcare in hospitals increases, a new and easy detection method for nucleic acids could bring the diagnostic part hospitals have to perform to a general practitioner or even patients' homes. This new diagnostic assay could be gold nanoparticles combined with CRISPR/Cas12a. Gold nanoparticles (AuNPs) can be functionalized with DNA to detect nucleic acids already. However, they lack sensitivity measuring low sample concentrations. CRISPR/Cas12a could be used to amplify the signal. Here, the color change of solutions with AuNPs and a linker ssDNA is analyzed. But, naked-eye detection was challenging as the changes in color were only 'visible' from absorbance values. Furthermore, the enzymatic activity of CRISPR/Cas12a is analyzed. This showed that, after target dsDNA and the CRISPR/Cas12a RNP complex were incubated for 1 hour, the cutting of CRISPR/Cas12a can be followed with a Michaelis-Menten analysis and the maximum velocity of the cutting was 2072 M/s. Also, by varying the reporter and target dsDNA concentration in the CRISPR assay and measuring the fluorescence, the relation between these was determined. A calibration curve showed the target dsDNA concentration and percentage of cut ssDNA reporters by CRISPR. From this, it can be concluded that adding a [ssDNA linker] of 444,44 nM to ssDNA functionalized AuNPs should provide a fast and sensitive colorimetric assay readable with the naked-eye. Further research could be done on optimizing the functionalizing of the AuNPs with DNA, in fact using the recommended ssDNA linker concentration and using other Cas enzymes. Also, creating a one-pot assay could provide even more advantages in detecting low sample concentrations of nucleic acids.

## Introduction

The pressure of healthcare in hospitals increases as new variants of viruses, such as COVID-19, increase. A new tool to detect such viruses could relieve the pressure of the diagnostic part hospitals have to perform and move the detection of diseases by their nucleic acids (DNA/RNA) to a general practitioner or even to patients' homes.[1] This research investigates a tool combining existing techniques to provide a simple and sensitive detecting assay.[2]

Gold nanoparticles (AuNPs) could provide a solution to create a readout assay that is easy to perform. AuNPs have excellent chemical and physical properties. Furthermore, Li et al. found that their chemical stability and extinction coefficients are high.[3] They can also be functionalized with, for example, ssDNA.[4][3] The attached ssDNA on the surfaces of the NPs can hybridize with complementary ssDNA in a solution. It, therefore, can help to provide a tool to read out signals with the naked eye. When both ends of the ssDNA in the solution attach to ends of ssDNA attached on different AuNPs, the NPs will aggregate. The aggregation of the AuNPs makes the solution turn into a different color, which can be seen with the naked eye (see Figure 1).[2][5][6][7][8][8][12] This detection method seems very usable but lacks sensitivity when measuring low sample concentrations. As, the limit of detection for most AuNPs is reported, for example by Diaz et al., to be between 10-20 nM and up to 0,5 nM after six hours.[9] They showed that this detection limit means that around  $9.6 \cdot 10^7$  copies/mL of the virus RNA need to be present.[9] Frithiof et al. found that for example SARS-CoV-2's presence in urine is on average 1200 copies/mL.[10] Thus, AuNPs are only an applicable detection method when using samples with a high concentration of target.

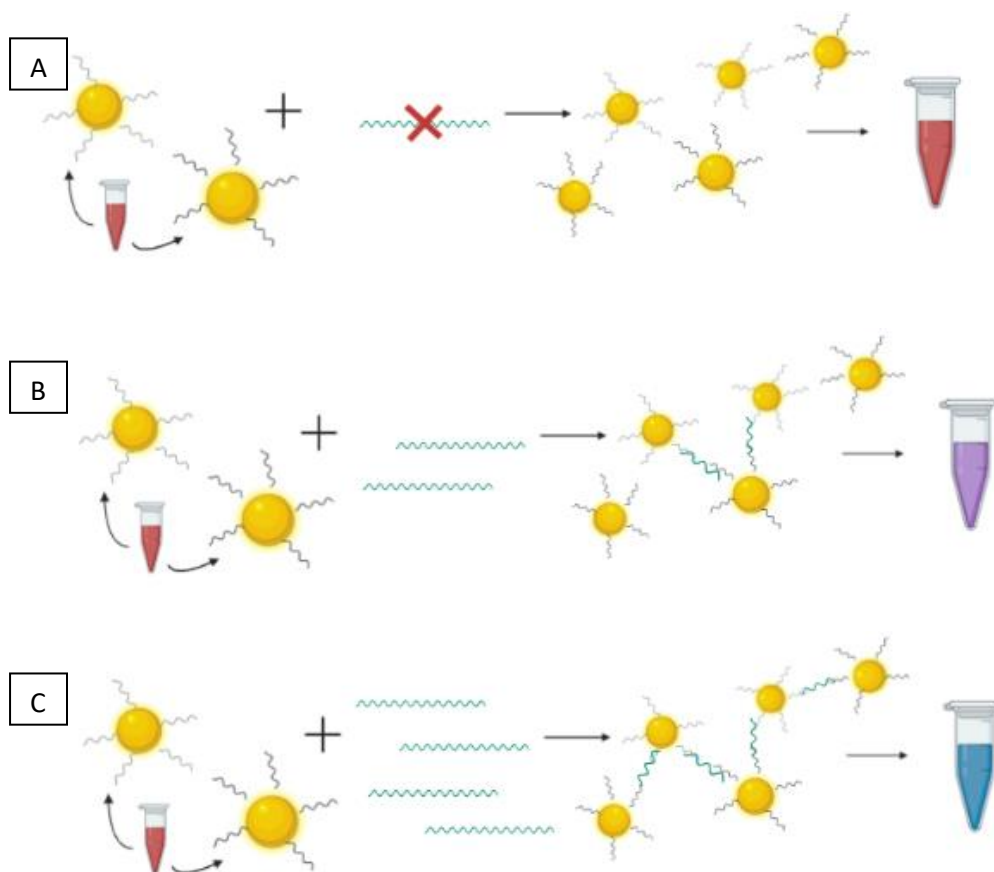


Figure 1: Working principle of ssDNA functionalized AuNPs with addition of ssDNA linker showing aggregation and color change of solutions. A: AuNPs without complementary linker addition show no color change. B: AuNPs with low concentration of linker, show purple color change. C: AuNPs with high concentration linker added show a lot of aggregation and blue colored solution.

Combining Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) proteins with AuNP sensing could provide a method to amplify the colorimetric signal of the DNA-functionalized AuNPs and make the detection of low sample concentrations possible. The CRISPR effector protein can form a surveillance complex from a ribonucleoprotein (RNP) associating with a guide RNA sequence (gRNA) complementary to the detectable genes.[2] If Cas12a-gRNA RNP binds to the designed complementary target dsDNA, the enzymatic activity of Cas12a is activated.[5] This then cuts ssDNA by *trans*-cleavage.

A CRISPR assay is often done with an ssDNA fluorophore-quencher pair in a solution.[2][11][5] When CRISPR is active, it cleaves ssDNA. For the ssDNA, a reporter ssDNA (such as the fluorophore-quencher pair) can be used, the fluorophore will be released, and the fluorescence signal of the solution increases (see Figure 2).[12][13] This detection method requires a readout of the fluorescence. The fluorescence measurement will provide information about the target binding. Reading out the fluorescence makes this detection method difficult at the point of care. Therefore, this research aims to correlate the color of an AuNP solution to the target dsDNA concentration present in that solution.

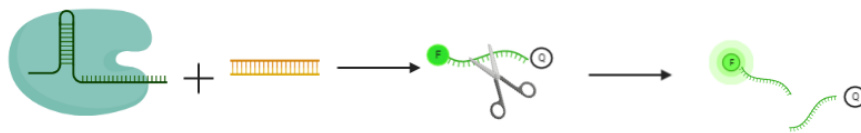


Figure 2: Illustration of CRISPR/Cas12a *trans*-cleavage activity assay with a target dsDNA and fluorophore-quencher ssDNA sequence in solution.

To combine CRISPR and AuNPs, CRISPR will be used with a crRNA and a target double-stranded DNA (dsDNA) (complementary to the crRNA). A free ssDNA crosslinker, which can hybridize to ssDNA on different NPs, resulting in the AuNPs' aggregation, is added to the solution containing the CRISPR and the dsDNA target sequence. The ssDNA crosslinker will be cleaved into fragments if the target sequence is recognized. If this solution is added to the AuNPs, they will stay away from each other as the ssDNA cannot act as a linker between them, which will be seen as a red solution (Figure 3).

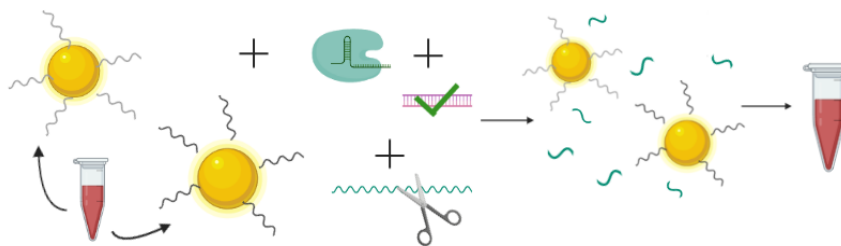


Figure 3: Illustration of AuNPs based colorimetric assay detecting target dsDNA which does not activate CRISPR/Cas12a using a ssDNA linker which gets cut by CRISPR/Cas12a and causes the color of the NP solution to stay red. (CRISPR/Cas12a assay will be performed first, and after the reporter is cut or not and CRISPR is done cutting, the solution will be added to the AuNPs)

However, if the target sequence is not, it can act as a linker between the AuNPs, as the sequences at the end of this ssDNA molecule are complementary to the end of the ssDNA molecules attached to the NPs. This aggregation is detectable regarding a color change (see Figure 3 and 4).[1][5][6][8][14][15][16]

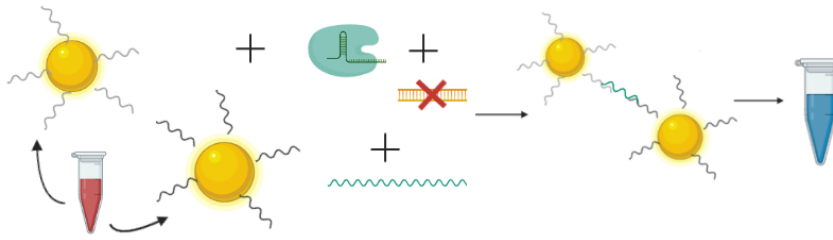


Figure 4: Illustration of AuNPs based colorimetric assay detecting target dsDNA which activates CRISPR/Cas12a using a ssDNA linker which causes the color of the NP solution to change from red to blue.

This research has two subgoals. The first is to determine the cutting activity of CRISPR/Cas12a in relation to the amount of target dsDNA and linker ssDNA present. The second goal is to determine how much ssDNA in the solution is needed to detect the red-to-blue color change caused by the aggregation of the AuNPs. The result will be a graph showing the relation between the dsDNA concentration (target) and the red:blue color ratio of the AuNP solution. Expected is that as the concentration of the dsDNA increases, the red:blue ratio increases.[17][14][15][7][17][16][14][7][4][5][6][2]

## Theoretical background

### AuNPs

As mentioned before, AuNPs have excellent properties. The main property used in this research is that functionalizing them with ssDNA enables them to link other ssDNA in a solution and then aggregate which causes a color shift of the solution they are in.[4][3] To properly functionalize the AuNPs with DNA, different techniques can be used.

The AuNPs can be covered with mPEG first, and Tween20 can be used in the solution to provide protected and stable NPs.[3][6] The mPEG packs the AuNPs with a protective layer against steric hindrance, and Tween20 assists in the reaction. To allow dense DNA loading on the particles, NaCl is used.[3]

Functionalizing the AuNPs with mPEG and Tween20 is a new rapid method. The method mostly used before, set up by Mirkin et al., was the salt-aging method.[18] This method uses the spontaneous absorption of thiol groups on gold surfaces. However, it must be done in several steps to prevent aggregation of the AuNPs and maintain stable NPs, which takes at least two days. Adding salt to the AuNPs is therefore done in steps. As the AuNPs absorb DNA by adding salt (NaCl), they can absorb more DNA and tolerate the salt better. Also, the salt reduces the repulsion between DNA sequences on the AuNPs. Therefore, the AuNPs become stable, and a high density of DNA on the AuNPs can be obtained.[18]

Li et al. proved that when using a bigger mPEG-SH, a smaller concentration of the mPEG-SH was needed to stabilize the NPs. The bigger mPEG-SH also increased the DNA packing ability of the NPs. [3] Another factor influencing the packing ability and density on the AuNPs surface is the size of the NPs. AuNP sizes currently used in research by Zhang et al., Kasputis et al., and Nourisaeid et al. differ from 10 to 80 nm. Some use the same-sized AuNPs for colorimetric detection, while others use different sizes of AuNPs combined.[7][6][16][19] Nourisaeid et al. also proved that bigger AuNPs can provide more attachment of ssDNA on the surface, resulting in more aggregation of the NPs and bigger changes in color. And they showed that attaching longer ssDNA on the surface can cause a bigger distance between the AuNPs resulting in less aggregation.[16] Thus, it is important to specifically determine the size and amount of the ssDNA you want to use on the AuNPs.

The surface probe density on the NPs and the steric hindrance of the attached ssDNA influence the efficiency and sensitivity of the colorimetric shift detectable with surface plasmon resonance

(SPR).[8][6][16][14][20][8] Absorbance measurements of the NPs should be done in the colors of the solutions. The amount of light absorbed by the AuNP solutions will be measured. This way, the change in color of a non-aggregated and aggregated AuNP solution becomes clear. Since the solutions are expected to stay red or turn blue, absorbance is mostly measured at 520 nm (red) and 610 nm (blue).[14][8]

For the AuNPs to aggregate, an ssDNA linker must also be present in the solution. Both ends of the ssDNA sequence should complement the ssDNA on the surface of the nanoparticles to make the binding and aggregation possible. The effect of adding different concentrations of the linker to the AuNPs has been analyzed by Yuan et al. This showed that the color change in the AuNP solutions with the linker kept happening regardless of the concentration of the linker used. They showed that the distance between the AuNPs had the most significant effect on the intensity of the color change.[8]

### CRISPR/Cas12a

In this research, Cas12a will be used. Cas12a is a type V effector protein that recognizes target dsDNA and cleaves ssDNA.[5][2] Cas12a needs to be combined with a crRNA to form an RNP complex. Target dsDNA should be complementary to the crRNA to activate CRISPR enzymatic activity. The target DNA is added to RNP-RNA complexes through collateral cleavage. This makes it possible to measure low sample concentrations without the need to amplify the signal caused by the cutting activity of CRISPR.[21]

The fluorescence signal of the quencher-labeled reporter ssDNA depends on the amount of activated RNP. It, therefore, relies on the amount of CRISPR and the amount of target dsDNA concentration used, as the target dsDNA can activate the RNP complex.[21][22] The effect of different concentrations of RNP and target dsDNA on the CRISPR activity and, therefore, the fluorescent signal have been researched in some papers. The most common ratio of Cas12a:crRNA used by for example, Cao et al. and Li et al. is 1:2.[17][15][12]

When the target dsDNA is recognized, CRISPR will be active and can cut the reporter ssDNA in the solution. The reporter ssDNA is only cleaved in the presence of the RNP complex, making it very useful to detect nucleic acids and analyze the CRISPR enzyme's trans-cleavage activity.[23][11] When a fluorophore-quencher pair reporter gets cut by the Cas12a enzyme, a fluorescent signal induced by the fluorophore can be detected (see Figure 1).[21][13][24]

The enzymatic activity of CRISPR/Cas12a varies with the buffer and crRNA sequence used. To obtain information about the enzymatic activity of Cas12a, a Michaelis-Menten analysis can be done by analyzing the reaction in Figure 5. This assay will provide information about the cutting efficiency of CRISPR. It can help find the proper concentrations and times that need to be used to perform the most sensitive and fast detection method.[25]

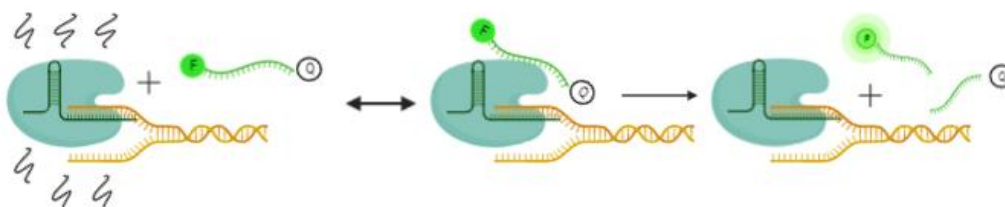


Figure 5: Reaction which can be analyzed with Michaelis-Menten. Activated CRISPR/Cas12a will be present with crRNA in a RNP complex and target dsDNA is bound, a fluorophore-quencher is added. This reporter can be trans-cleaved and then cut by the enzymatic activity of Cas12a.

With the Michaelis-Menten analysis, the reaction velocity and the turnover rate of the RNP can be determined.[26] The Michaelis-Menten equation is:

$$v \left( \frac{M}{s} \right) = \frac{v_{max} * [\text{reporter}]}{K_m + [\text{reporter}]}$$

$v$  is the reaction velocity,  $K_m$  represents the affinity of the analyzed enzyme to the substrate, and  $v_{max}$  is the maximum reaction velocity where all enzymes react.[26][27] These values differ per enzyme, but  $k_{cat}/K_m$  is mostly in orders of  $10^5$ - $10^6$  /Ms.[28] [26][27]Avaro and Santiago recommended, after researching the errors in Michaelis-Menten's analysis of Cas enzymes, that using the  $k_{cat}/K_m$  as a measure for the enzymatic activity of Cas enzymes leads to the most precise estimation compared to only reporting  $k_{cat}$  and  $K_m$ .[25]

To obtain these values, the following calculations can be done based on the article of Ramachandran et al.[26]

$$F(t) = F_{cl}(t) + F_{ucl}(t)$$

In this equation,  $F_{cl}(t)$  and  $F_{ucl}(t)$  are obtained by measuring the fluorescence signal of different concentrations of a fluorescent reporter. Then, mass conservation shows that  $C_{cl}(t) + C_{ucl}(t) = C_0$ . And  $C_0$  is the initial uncleaved reporter concentration. With this formula, the equation of  $F(t)$  can be rewritten.

Then, the reaction velocity can be obtained by differentiating the equation over time ( $a$  is the slope of the  $F(t)$  formula).

$$\frac{dc_{cl}}{dt} (nM/s) = \frac{1}{a} * \frac{dF}{dt}$$

Also, the concentration of cleaved reporter over time can be determined:[26]

$$C_{cl}(t)(nM) = \frac{F(t) - F_{ucl}(C_0)}{a}$$



## Materials and Methods

Here, the exact materials used in all experiments can be found. Also, the performed experiments are explained.

### Materials

#### *AuNP experiments*

##### Functionalizing with mPEG and Tween20

Gold nanoparticles with a diameter of 20 nm were purchased from BBI solutions. ssDNA with -SH modification was purchased from Eurofins genomics; the sequences are listed in Figure S1. DNA1 was a forward primer, DNA2 was the reverse primer. Furthermore, AuNPs were functionalized with mPEG, the stock solution contained 100  $\mu$ M of ~6kD mPEG in MilliQ water. 1 wt% Tween in a solution of MilliQ water and a 5M NaCl (from Sigma-Aldrich) solution in MilliQ water were used, as well as PBST (pH 7.4 and filtered containing 0.01% Tween 20).

##### Functionalizing with SDS and NaCl in solution (salt-aging method)

The same 20 nm AuNPs and DNA sequences were used as for the first functionalizing method. AuNPs were mixed with 1% sodium dodecyl sulfate and 1 M NaCl, both dispersed in MilliQ water and purchased from Sigma-Aldrich. Also, PBST (pH 7.4 and filtered containing 0.01% Tween 20) was used.

#### *CRISPR/Cas12a trans-cleavage experiments*

For the trans-cleavage experiments of Cas12a, the following materials were used. AsCas12a was purchased from Integrated DNA technologies. The ssDNA reporter, crRNA and target dsDNA sequences are listed in Figure S1. The ssDNA reporter was obtained from Eurofins Genomics, the crRNA (Mal2sh) from integrated DNA technologies and targets dsDNA (Mal195 and Mal190) was obtained from Eurofins Genomics at concentrations of respectively 100  $\mu$ M and 45  $\mu$ M. The advantage of the Cas12a system is that it can target sequences rich in T's.[20] Thus, a target sequence rich of T's is used. The target dsDNA was used as an activator to measure the trans-cleavage of Cas12a with a fluorophore-quencher ssDNA which acted as a reporter. 1x NEBuffer 2.1 containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub> and 100  $\mu$ g/mL Recombinant Albumin at a pH of 7.9 was purchased from New England Biolabs.

All the data obtained by the experiments were analyzed with Microsoft Excel (Office 365). For measurements, a Biorad T100 Thermocycler was used, as well as a SpectraMax iD3 plate reader, a transparent 96-wells plate and a Geiner 384-wells plate. The centrifuge used was the Eppendorf 5430. Furthermore, all samples were pipetted in Eppendorf vials of different volumes.

### Methods

#### *Relationship between ssDNA linker concentration and AuNP aggregation*

##### AuNP properties

The concentration of the 20 nm AuNPs in the stock solution was  $1.16 \cdot 10^{-9}$  M. Surface area of the particles was determined to be  $1.26 \cdot 10^3$  nm<sup>2</sup>.

##### AuNP functionalization

##### Functionalizing using mPEG and Tween20

Two solutions of 20 nm AuNPs were functionalized with different sequences of ssDNA. The following steps were performed for both solutions. First, 10  $\mu$ L of a 1 wt% Tween 20 solution was added to 1000  $\mu$ L of AuNPs. Then 10  $\mu$ L of 100  $\mu$ M mPEG was added. This was incubated for 20 minutes at room temperature. Then, 20  $\mu$ L of 100  $\mu$ M DNA and 400  $\mu$ L 5M NaCl were added. This

was done for AuNP solution with DNA1 and with DNA2. The solution was mixed and incubated for 1 hour. Finally, the AuNP solutions were centrifuged for 10 minutes at 10000 RCF, then 800  $\mu$ L of the solution was removed, and 800  $\mu$ L PBST (PBS with 0.01% Tween and pH 7.4) was added. These washing steps were performed seven times.

#### Functionalizing using SDS and NaCl in solution (salt-aging method)

Two solutions of AuNPs were functionalized with different sequences of ssDNA. The following steps were performed to both solutions. First, to 1000  $\mu$ L AuNPs, 40  $\mu$ L of ssDNA1 or ssDNA2 was added. These samples were incubated for 24 hours at room temperature. Then 5  $\mu$ L of 1% sodium dodecyl sulfate (SDS dispersed in MilliQ water) was added, and samples were incubated for 1 hour at room temperature. Finally, 100  $\mu$ L of 1 M NaCl (also dispersed in MilliQ water) was added in steps of 10  $\mu$ L. After every addition, samples were vortexed. The final solutions were incubated for 24 hours at room temperature. The solutions were then washed with filtered PBST three times by adding and removing 1000  $\mu$ L PBST and first centrifuging at 12000 rpm for 15 minutes, then at 11500 rpm for 12 minutes and then at 12000 rpm for 15 minutes again.

#### ssDNA linker addition to functionalized AuNPs

##### AuNPs functionalized with mPEG and Tween20

In a total reaction volume of 90  $\mu$ L, 40  $\mu$ L 5 nM AuNPs functionalized with ssDNA1 solution and 40  $\mu$ L of 5 nM AuNPs functionalized with ssDNA2 solution as well as 10  $\mu$ L of ssDNA linker solutions (with concentrations: 0, 20, 40, 80 and 100 nM, to determine aggregation) were mixed. The solutions were incubated at room temperature for 3 minutes and then centrifuged at 4000 rpm for 3 minutes.

##### AuNPs functionalized with SDS and NaCl in solution (salt-aging method)

In a total reaction volume of 90  $\mu$ L, 40  $\mu$ L 1.16 nM AuNPs functionalized with ssDNA1 solution and 40  $\mu$ L of 1.16 nM AuNPs functionalized with ssDNA2 solution as well as 10  $\mu$ L of ssDNA linker solutions (with concentrations: 0, 20, 40, 80 and 100 nM, to determine aggregation) were mixed. For the 0 nM concentration, 10  $\mu$ L of filtered PBST was used. The solutions were incubated at room temperature for 3 minutes and then incubated in the Thermal cycler for 15 minutes at 56 degrees Celsius. After this, the samples were centrifuged at 4000 rpm for 3 minutes.

#### Absorbance measurement

##### AuNPs functionalized with mPEG and Tween20

To determine the aggregation of the AuNPs due to the addition of the ssDNA linker, the absorbance of the supernatant of the centrifuged solutions was measured with a SpectraMax iD3 plate reader at room temperature. Also, the absorbance of the AuNP solutions without the addition of the ssDNA linker was measured. Each concentration of ssDNA linker was measured in triplicate. Absorbance was measured at 520 (red) and 610 nm (blue) with 80  $\mu$ L of sample using a transparent Geiner 96-wells plate. Spectrum measurements were done as well. The spectrum measurement was done from 450 to 700 nm with steps of 5 nm. Also, photos of the Eppendorf vials were taken.

##### AuNPs functionalized with SDS and NaCl in solution (salt-aging method)

For AuNPs made using the salt-aging method, absorbance was measured, before washing, with 75  $\mu$ L of the supernatant of the centrifuged solutions with a SpectraMax iD3 plate reader at room temperature. Also, the absorbance of the AuNP solutions without adding the ssDNA linker was measured. Each concentration of ssDNA linker was measured in duplo. Absorbance was measured at 520 (red) and 610 nm (blue) using a transparent Geiner 96-wells plate. Spectrum measurements were done as well. The spectrum measurement was done from 450 to 700 nm with steps of 10 nm. These same absorbance measurements were done with 20  $\mu$ L of the supernatant using a transparent 385-wells plate after washing two and three times.

### *Calibration curve of trans-cleavage activity of Cas12a*

#### *Trans-cleavage experiment*

This first experiment is based on the principle in Figure 1. First, the RNP complex was formed. This was done with the Cas12a enzyme at a concentration of 62  $\mu\text{M}$ . The starting concentration of crRNA was 100  $\mu\text{M}$ . The RNP complex was prepared with a total volume of 30  $\mu\text{L}$ , crRNA and Cas12a enzyme were mixed in a 2:1 ratio, respectively, and this was incubated for 30 minutes at room temperature. An excess of CRISPR will be used to ensure that all the fluorophore-quencher present in the solution will be cut. The RNP complex was diluted 1000x. Also, both the target dsDNA and the reporter sequence were diluted to better pipette the wished concentrations. To activate Cas12a, target dsDNA was added. To measure Cas12a and reporter trans-cleavage activity, a solution with a total volume of 70  $\mu\text{L}$  was prepared, containing 100 nM RNP complex, 200 nM target dsDNA, 10x NEBuffer 2.1, MilliQ water, and different concentrations of reporter ssDNA. The tested reporter ssDNA concentrations were 0, 7.8, 15.6, 31.3, 62.5, 125, 500, and 1000 nM. Also, 70  $\mu\text{L}$  solutions containing only the reporter sequence (in the same varying concentrations), 10x NEBuffer 2.1, and MilliQ water were made. All solutions were pipetted into 0.1 mL Eppendorf tubes. Each condition was made and measured in triplicate.

The Eppendorf tubes were placed in a Biorad T100 Thermocycler for 18 hours at 39 degrees Celsius. After 18 hours, the tubes were vortexed, and the solutions were moved to a Greiner 384-wells plate for fluorescence measurements. The fluorescence of every condition was measured in triplicate with 20  $\mu\text{L}$  sample at  $\lambda_{\text{exc}} = 485$  nm and  $\lambda_{\text{em}} = 535$  nm and reading height (previously optimized) 0.98 mm with a SpectraMax iD3 plate reader.

From the fluorescence measurements, three values of the uncleaved reporter at [reporter] of 250 nM were not considered, as these diverged a lot from all other values from the same concentration.

#### *Michaelis-Menten analysis*

To measure CRISPR activity over time and obtain the reaction velocity of the cutting of the reporter by CRISPR, the same experiment as mentioned above was done, but with fluorescence measurements over time. 70  $\mu\text{L}$  samples contained 1 nM of RNP complex, 10 nM dsDNA, 10x NEBuffer 2.1, MilliQ water, and different reporter concentrations (0, 31.25, 62.5, 125, 250, 500, 1000, 1500 and 2000 nM). The fluorescence of samples was measured in triplicate with 20  $\mu\text{L}$  sample at  $\lambda_{\text{exc}} = 485$  nm and  $\lambda_{\text{em}} = 535$  nm and reading height 0.98 mm every minute for 1 hour at 39 degrees Celsius with a SpectraMax iD3 plate reader.

A non-linear fit of the fluorescence data was done using the Excel Solver add-in to obtain Michaelis-Menten curves.

#### *Influence of target dsDNA concentration on the trans-cleavage activity of Cas12a*

The same materials were used as in the previously explained experiment. Again, first the RNP complex was formed. This was done with 62  $\mu\text{M}$  Cas12a enzyme and crRNA of 100  $\mu\text{M}$ . The RNP complex was prepared with a total volume of 30  $\mu\text{L}$ , crRNA and Cas12a enzyme were mixed in a 2:1 ratio respectively, this was incubated for 30 minutes at room temperature. The RNP complex was diluted 1000x. Also, both the target dsDNA and the reporter sequence were diluted to better pipette the wished concentrations. To measure the Cas12a trans-cleavage activity, a solution with a total volume of 70  $\mu\text{L}$  was prepared, containing 100 nM RNP complex, 1000 nM reporter ssDNA, 10x NEBuffer 2.1 and MilliQ water. Then, different concentrations of dsDNA (0, 0.0125, 0.025, 0.005, 0.1, 0.25, 0.5, 1 nM) were added. Also, 70  $\mu\text{L}$  solutions containing only the reporter sequence (in the same varying concentrations), 10x NEBuffer 2.1, MilliQ water and different concentrations of target dsDNA (0, 0.0125, 0.025, 0.005, 0.1, 0.25, 0.5, 1 nM) were made. All solutions were pipetted into 0.1 mL Eppendorf tubes. Each condition was made in triplicate.

From the moment the target dsDNA was added, CRISPR could be activated, so fluorescence measurements were done over time to determine the trans-cleavage activity. The fluorescence measurement was performed every 5 minutes for 2 hours. The tubes were vortexed, and solutions were moved to a Greiner 384-wells plate for the fluorescence measurements. The fluorescence of every condition and sample was measured in triplicate with 20  $\mu$ L sample at  $\lambda_{\text{exc}} = 485$  nm and  $\lambda_{\text{em}} = 535$  nm and reading height 0.98 mm at 39 degrees Celsius with a SpectraMax iD3 plate reader.

## Results and discussion

In this section, the obtained results from the different experiments can be seen. First, the results showing the relation between the color change of the solutions of AuNPs and ssDNA linker by varying the ssDNA linker concentration can be found. Then, the results obtained by varying the reporter concentration in the CRISPR/Cas12a assay showing the trans-cleavage activity of Cas12a can be seen, including the Michaelis-Menten analysis. And lastly, the results obtained by varying the dsDNA target concentration performing the same CRISPR/Cas12a assay can be seen.

### Relationship between ssDNA linker concentration and AuNP aggregation

This section shows the results which will give information about the color change of the ssDNA functionalized AuNP solutions when adding different ssDNA linker concentrations. From these results, a conclusion can be made about how much ssDNA linker needs to be present in the solution to detect a color change and thus to detect the presence of a nucleic acid is in the examined sample.

#### *Naked-eye detection*

Detecting the aggregated AuNPs in terms of a color change of the solution was not really possible with the naked eye. For example, Figure S2 shows a photo of Eppendorf vials with the ssDNA functionalized AuNPs in solution with different concentrations of linker ssDNA. It was expected that when more linker ssDNA would be available, the color of the solution would turn more into blue/purple, because of the aggregation of the AuNPs.[3] This has not happened.

AuNPs functionalized with ssDNA using the salt-aging method did not have visible changes in color either. Even after incubating for 15 minutes at 56 degrees Celsius. The color change was expected to be visible since Kasputis et al. performed the same protocol and saw a color change.[7]

#### *Absorbance measurements*

Absorbance measurements of supernatant of AuNP solutions functionalized using mPEG and Tween20 can be seen in Supplementary Figure S3 and S4. There was no clear relation seen between the ssDNA linker concentration and the red:blue ratio samples.

Yuan et al. reported that when the distance between the AuNPs is less than the diameter of the particles, a clear color change of the solution after AuNP aggregation was visible. They used 13 nm AuNPs functionalized with DNA and added ssDNA linker in concentrations from 0 to 60 nM. They observed a color change after short low-speed centrifuging of their samples. The absorbance at 520 decreased as the linker ssDNA concentration increased. Centrifuging was very important, before the centrifuging step, the absorbance was almost the same for every linker ssDNA concentration.[8]

In this research, the protocol was based on the research of Yuan et al. so this centrifuging step was performed.[8] Our solutions did not have big differences in absorbance even after centrifuging. This research used the same sequences to functionalize the AuNPs and from the ssDNA linker (see S1) as Kasputis et al. used. They observed the absorbance of their NP solutions with linker ssDNA concentrations varying from 0 to 200 nM. The color of the solutions did not change after adding the ssDNA linker. The absorbance of their samples did show minor differences, a shift of the peak of the absorbance was visible in the spectrum with a higher concentration of linker, and the  $A_{560/520}$  increased as the concentration of linker increased. Their NPs were functionalized with the salt-aging method.[7]

Absorbance measurements of AuNPs functionalized with the salt-aging method can be seen in Figure 6 and 7. AuNPs were also functionalized with this method since functionalizing with mPEG and Tween20 did not show clear results, see Figure S2, S3 and S4. The salt aging method could provide a way for the AuNPs to be stable without mPEG.[18] The mPEG could disturb the binding of the ssDNA on the surface of the AuNPs and therefore hinder the linker from binding and the AuNPs

aggregation. For this method, an excess of ssDNA for on the particles is used and salt (such as NaCl) is added in steps.[18][29]

The same thing can be observed in the spectra as the AuNPs functionalized with the other method. There is no clear shift in the absorbance with different concentrations linker ssDNA added. The peak of the spectrum of all samples is around 525 nm. Furthermore, a linker concentration of 20 nM shows the highest absorbance, and the 100 nM concentrations shows the lowest absorbance values. This is actually in line with the hypothesis, a higher linker concentration should result in more aggregation of the AuNPs and therefore a bluer colored solution, which has a lower absorbance than the initial red solution. A weird shift in the absorbance values can be seen at 680 nm. This can be caused by the well in which the samples were pipetted.

Even though no clear conclusion or shift can be seen in the spectrum, the spectrum is different than the spectrum of just AuNPs in a solution. Thus, it can be concluded that something is happening when the ssDNA is attached on the AuNPs and the ssDNA linker is added. As the AuNP sample only contained AuNPs in MilliQ water (see also Figure S5). The addition of the ssDNA both on the AuNPs and the ssDNA linker in the solution causes absorbance values to be much lower than those of ‘naked’ AuNPs. Zhang et al. observed this as well.[29] They also observed a shift in the peak to a higher wavelength compared to the ‘naked’ AuNPs.[29] This is not clearly visible in the spectrum we measured.

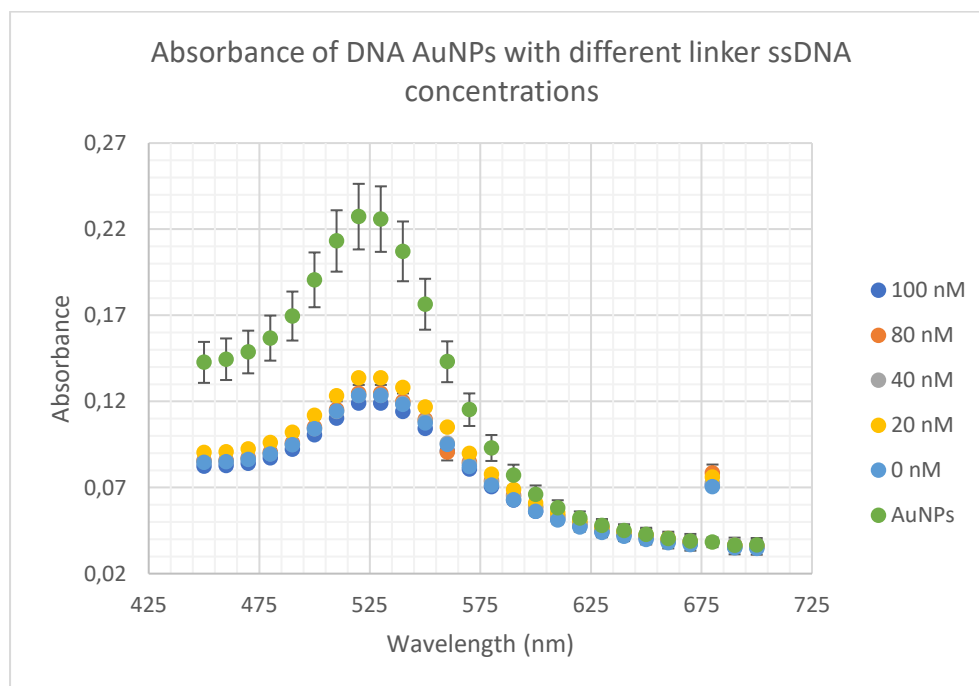


Figure 6: Absorbance measurements of DNA functionalized AuNP solutions with SDS and NaCl (salt-aging method) with different linker DNA concentrations ( $n=2$ ) and non-functionalized AuNPs ( $n=3$ ) between 450 and 700 nm measured with a plate reader.

Also, the absorbance ratio red:blue (see Figure 7) is in line with the hypothesis. As the ssDNA linker concentration decreases, the absorbance increases, because the AuNPs will be less aggregated and the solution will have a more red color.[3]

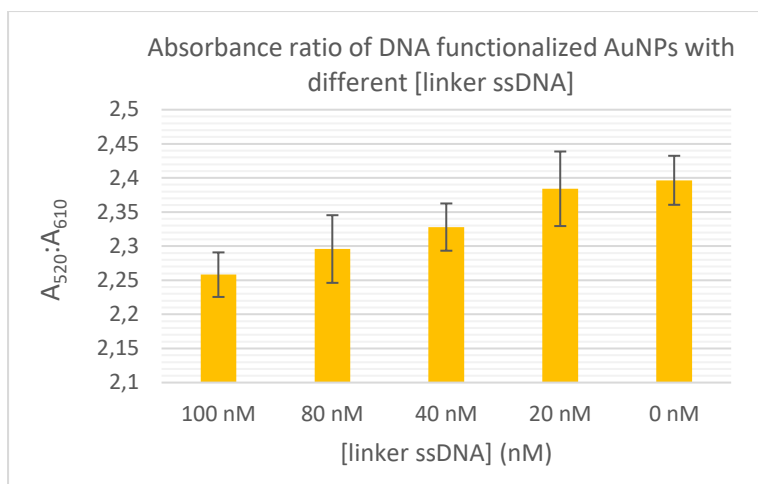


Figure 7: Absorbance ratio at 520 and 610 nm of DNA functionalized AuNPs with different [linker DNA] in solution with SDS and NaCl (salt-aging method). (n=2)

It must be noted that the spectra and values obtained in Figure 6 and 7 were obtained with the supernatant of AuNP solutions that were not washed. Not washing the ssDNA functionalized AuNPs means that some of the ssDNA added which did not attach on the surface of the AuNPs was still in the solution and could thus also be measured in the absorbance. Absorbance ratios of AuNP solutions which were washed two and three times can be seen in Figure S5.

### Trans-cleavage activity of Cas12a

Here, the results of experiments performed with CRISPR/Cas12a, different reporter concentrations and different dsDNA target concentrations are presented. These results will mainly provide information about the enzymatic activity of CRISPR/Cas12a.

### Fluorescence signal per cleaved reporter percentage

Calibration curves of cleaved and uncleaved reporter concentrations were made by varying the reporter concentration in the CRISPR/Cas12a assay and measuring the fluorescent signal. These calibration curves and standard deviations are visible in Figure 8.

Both figures make clear that the intensity of the fluorescent signal increases linearly as the concentration of the reporter increases. The uncleaved reporter will still have a fluorescent signal, but not as much as the cleaved reporter since the cleaved reporter emits more light.

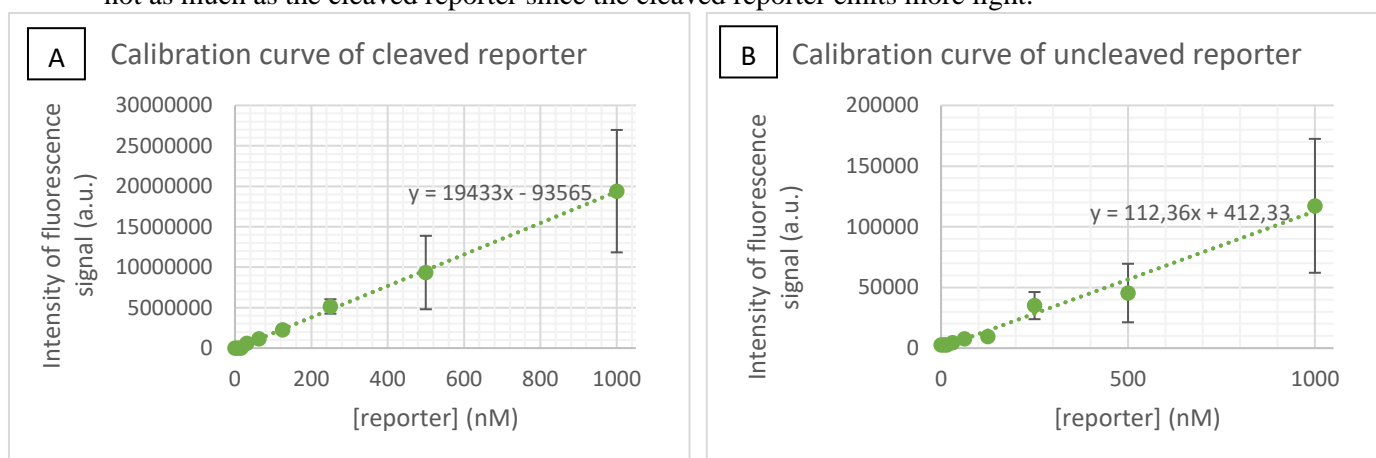


Figure 8: Calibration curves. A: Calibration curve of trans-cleavage activity of Cas12a, obtained by measuring different reporter concentrations fluorescence after incubating. B: Calibration curve of fluorescent signal of different reporter concentrations after incubating. (n=3)



### Michaelis-Menten analysis of Cas12a

The following Michaelis-Menten analysis was done using the calculations proposed in the Theoretical background. The total formula for the fluorescence was obtained by substituting the formulas from the curves with the CRISPR/Cas12a cleavage activity in Figure 8.[26] Thus,  $F(t) = 19320,64C_{cl}(t) + 112,36C_0 - 93152,67$ . By differentiating this, the reaction velocity was obtained:

$$\frac{dc_{cl}}{dt} (nM) = \frac{1}{19320,64} * \frac{dF}{dt}$$

The reaction velocity was determined when using different reporter concentrations. For each concentration, the slope from Figure S7 (fluorescence measurements over time) was determined and used to calculate the reaction velocity. Then, the Michaelis-Menten plot was plotted. This can be seen in Figure 9. In the plot the reaction velocity increases until a reporter concentration of 1500 nM is used. Then, only a slight increase in the reaction velocity is visible. This means that a certain level of the reporter concentration is reached, the reaction can not be performed any faster and every CRISPR/Cas12a enzyme is occupied or has already cut the reporter. However, the curve in Figure 9 does not look like how a Michaelis-Menten curve usually looks. The relation does not look logarithmic.

The relation between the reaction velocity and the reporter concentration looks more sigmoidal. This raises questions if the Cas12a enzyme is a sigmoid enzyme. A sigmoid enzyme is known to have allosteric activity. This means that there should be cooperative binding of targets to the enzyme. For Cas12a, first the target dsDNA binds and then the second target, the fluorophore-quencher, pair can cleave Cas12a as well.[31][32] However, most research such as that from Ramachandran et al. suggest that Cas12a activity is not per se sigmoidal.[26] But, research from East et al. has also shown that for example Cas9 enzymes do have allosteric activity.[33]

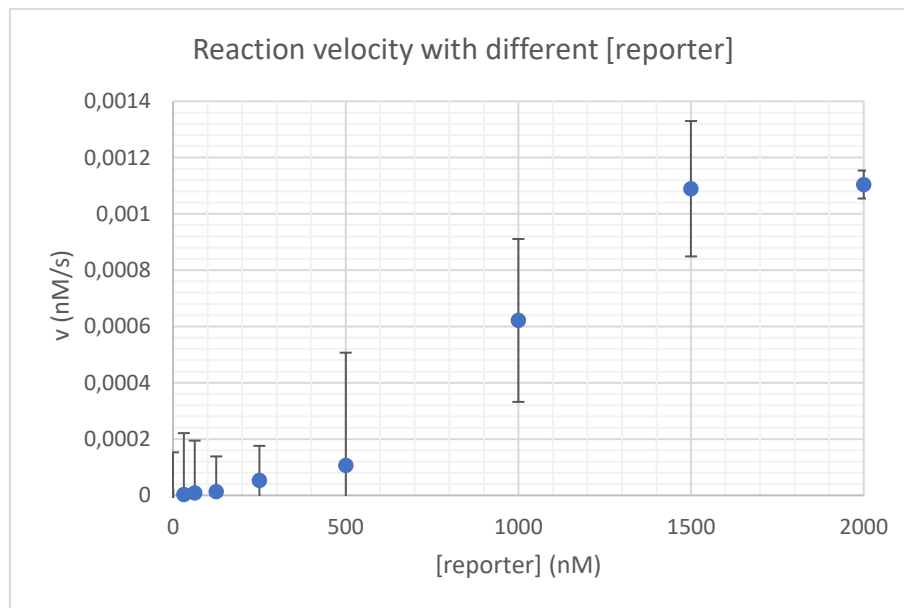


Figure 9: Michaelis-Menten plot showing the reaction velocity of CRISPR/Cas12a when adding different reporter concentrations.

Thus, to make the trans-cleavage activity of Cas12a clearer, a new Michaelis-Menten experiment was done with a higher concentration of target dsDNA in the solution. Since this will make sure that first all Cas12a enzymes are activated and can then cut the fluorophore-quencher pair. Also, in this experiment, the RNP complex and dsDNA were first mixed and incubated for 1 hour to ensure all target dsDNA was bound to CRISPR/Cas12a first. After 1 hour, the solutions were mixed with the different reporter concentrations. The results of this Michaelis-Menten experiment can be seen in



Figure 10. These results show that the Michaelis-Menten curve does look logarithmic and not sigmoidal. Thus, the enzymatic activity of Cas12a can be described with Michaelis-Menten. The reason for the sigmoidal curve in Figure 9 was that not all dsDNA target was already bound to the RNP complex. Figure 10 shows that after incubating for 1 hour all dsDNA target is bound and Cas12a is fully active in cutting the reporter.

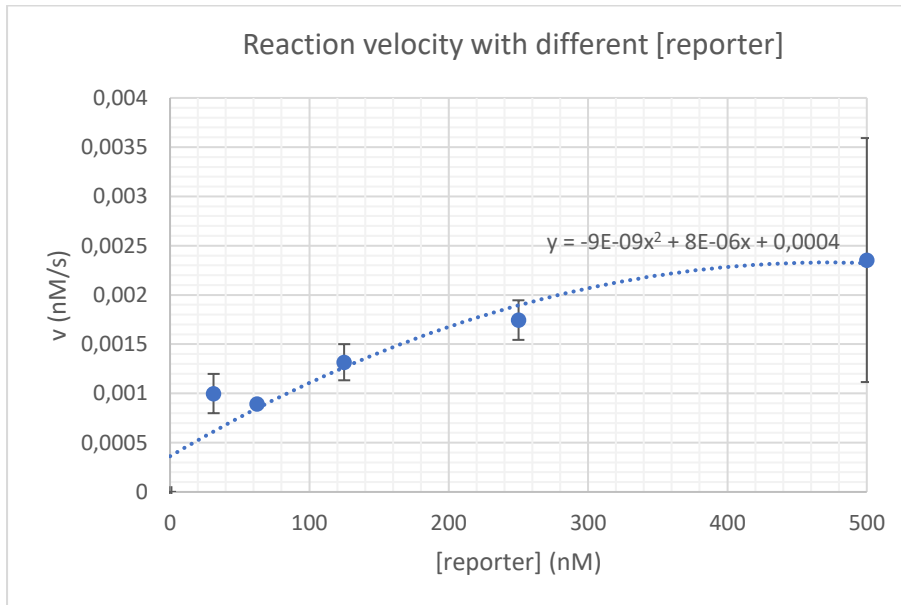


Figure 10: Michaelis-Menten plot showing the reaction velocity when using different reporter concentrations, after first incubating the RNP complex with the dsDNA target for 1 hour and then adding the reporter.

Using Excel Solver, the formula of Michaelis-Menten ( $v = \frac{v_{\max} * [\text{reporter}]}{K_m + [\text{reporter}]}$ ) and  $\frac{dc_{cl}}{dt} (nM) = \frac{1}{19320,64} * \frac{dF}{dt}$ , the  $v_{\max}$  and  $K_m$  of the reaction of Cas12a with the fluorescent reporter was obtained.  $v_{\max}$  was 2072 M/s and  $K_m$  was 6244 M.

The obtained values for  $v_{\max}$  and  $K_m$  are high compared to Cas12a enzymes analyzed in other research. A comparison of the obtained values from this experiment compared to other research can be seen in Figure 11.[28][26]

As said before our calculations are based on the calculations Ramachandran et al. did. They also performed a so called back-of-the-envelope check to verify the obtained Michaelis-Menten values.[26] The calculations of these checks can be seen in the Supplementary information. From the checks it can be concluded that our reported values for  $v_{\max}$  and  $K_m$  are in line with the checks and thus should be valid. In Figure S11 the curve obtained by calculating the reaction velocity using the Michaelis-Menten formula and the formula obtained by using the calculations of Ramachandran et al. can be seen.[26] The curves are very similar.

Ramachandran et al. performed various measurements to determine the trans-cleavage activity of CRISPR using different Cas enzymes and different target activators. They showed that the enzyme activity of Cas is influenced by the sequence of the target and the type of DNA/RNA used. However, their measurements showed much lower values for  $k_{\text{cat}}$  and  $K_m$  than other papers.[26]

Nalefski et al. found that increasing the target DNA concentration caused a decrease in the  $k_{\text{cat}}$  of the reaction. They also found that the fluorescence decreased as the reporter concentration decreased. This is in line with the reported data in Figure S7, S8, S9 and S10 of this research.[30]

As said before in the theoretical background, Avaro and Santiago proposed that looking at the  $k_{cat}/K_m$  is the best way to analyze Cas enzyme kinetics with the least errors.[25] So, let's look at the reported values of  $k_{cat}/K_m$  from different papers stated in Figure 11. We can conclude that the found value of  $3,31 \cdot 10^8 / \text{Ms}$  in our Michaelis-Menten experiment is high. Higher than the range of  $10^5 - 10^6$  which is normally reported using Cas12 enzymes.[28]

The high values for  $k_{cat}$ ,  $K_m$  and  $k_{cat}/K_m$  could be caused by the type of Cas12a enzyme which was used. As stated by the supplier, Integrated DNA Technologies, our AsCas12a enzyme is an Ultra enzyme which provides specific and rapid activity.[34]

CRISPR enzyme	Activator	$k_{cat}$ (/s)	$K_m$ (M)	$k_{cat}/K_m$ (/Ms)	Reference
AsCas12a	dsDNA	2072	6244	$3,31 \cdot 10^8$	-
AsCas12a1	dsDNA	0,46	$280 \cdot 10^{-9}$	$1,6 \cdot 10^6$	[28]
AsCas12a2	dsDNA	0,90	$1100 \cdot 10^{-9}$	$8,2 \cdot 10^5$	[28]
AsCas12a	dsDNA	0,6	$2,7 \cdot 10^{-6}$	$2,4 \cdot 10^5$	[26]
LbCas12a	dsDNA	1250	$7,25 \cdot 10^{-7}$	$1,7 \cdot 10^9$	[26]
Cas12a	ssDNA	2,6	$31 \cdot 10^{-9}$	$8,4 \cdot 10^7$	[30]
Cas12a	dsDNA	0,04-0,05	$13-16 \cdot 10^{-9}$	$3,3-3,5 \cdot 10^6$	[30]
AsCas12a	dsDNA	-	-	$4,2 \cdot 10^5$	[27]

Figure 11: Table showing values of  $V_{max}$  and  $K_m$  obtained in this research compared to other research using a Michaelis-Menten analysis.

### Target dsDNA influence on trans-cleavage activity of Cas12a

In Figure 12 a calibration curve obtained by combining the calibration curves of Figure 8 can be seen. The curve shows the relation between the percentage of cleaved reporter and the intensity of the fluorescent signal measured with the SpectraMax iD3 plate reader at  $\lambda_{exc} = 485 \text{ nm}$  and  $\lambda_{em} = 535 \text{ nm}$ . The curve was obtained by first calculating what concentration of the reporter would be cleaved at every percentage. Then, these values were substituted in the formulas of the trendlines (see Figure 8) to determine the intensity of the fluorescent signal at every percentage of cleaved and uncleaved reporter concentration. The total intensity of the fluorescent signal at every percentage of the cleaved reporter was calculated by the sum of the fluorescent signals at every percentage of the cleaved and uncleaved reporter.

The final calibration curve shows that the intensity of the fluorescent signal increases linearly as the percentage of cleaved reporter increases. Thus, this shows that as the trans-cleavage activity of Cas12a increases, the intensity of the fluorescence signal increases linearly along the line  $y = 96548x + 34213$ .

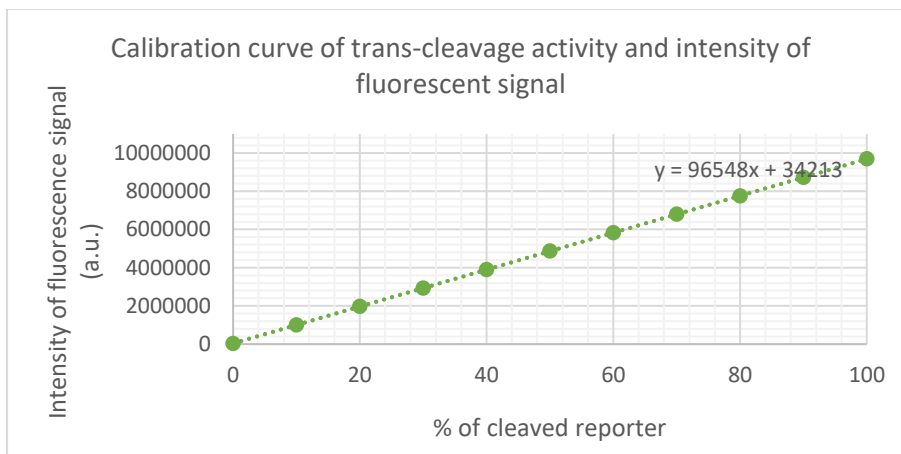


Figure 12: Total calibration curve combining fluorescence values from Figure 8, showing trans-cleavage activity of Cas12a by the percentage of cleaved reporter and the intensity of the fluorescent signal.

In Figure 13 the calibration curve obtained from the experiments done for Figure 12 and the measurements of the experiment varying the dsDNA target concentration done in Figure S13 are combined. This results in a curve showing the relation between target dsDNA concentration and the percentage of cleaved reporter. It can be seen that as the target dsDNA concentration increases the % of reporter which is cut increases as well. However, the slope of the graph decreases as the [target dsDNA] increases. These results are in line with the hypothesis and with what has been seen in earlier research. For example, in the research of Urbaitis et al. They observed the same relation between [target dsDNA] and percentage of cleaved reporter.[27]

This curve can be used to determine how active CRISPR is when the fluorescence signal is known. From this, when the percentage of cleaved reporter is known, the concentration of target dsDNA in the analyzed sample can be determined. And it can be diagnosed how much of the DNA from a virus or disease you want to detect is present in the tested sample.

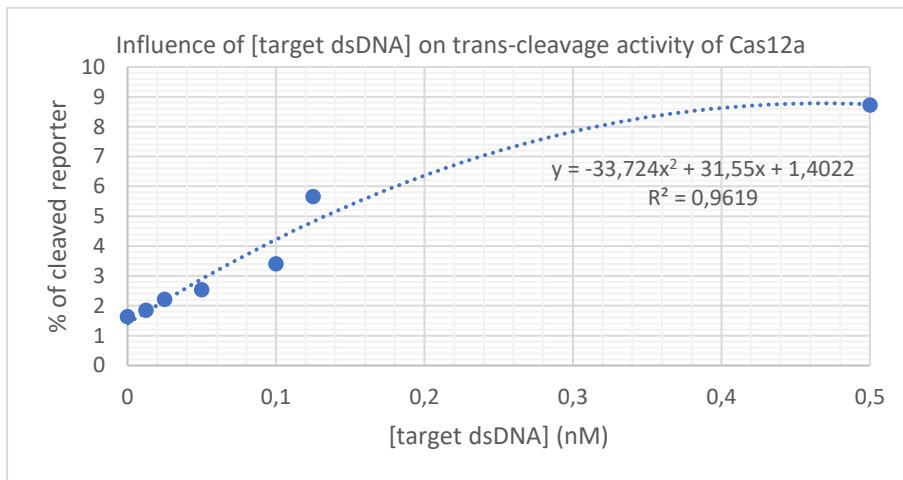


Figure 13: Combination of results obtained in Figure 6 and 7 showing the influence of [target dsDNA] on trans-cleavage activity of fluorescent reporter to Cas12a.

If we zoom in to the most linear part of the curve, this seems to be between [target dsDNA] of 0.025 and 0.1 nM. Target dsDNA concentrations between these concentrations give a good representation of the CRISPR cutting activity.

## Conclusions

Varying linker ssDNA concentrations and adding these to ssDNA functionalized AuNP solutions made clear that detecting a color change with the naked eye was not yet possible. Especially, AuNPs functionalized with mPEG and Tween20 in solution did not show a clear relation between the linker concentrations and aggregation of the AuNPs. Absorbance measurements of AuNPs functionalized with the salt-aging method did show that an increase in ssDNA linker concentration showed an increase in aggregation of the AuNPs and bluer solutions. However, this method is very time consuming and after washing the AuNPs the relation between aggregation and ssDNA linker addition was no longer visible.

As a result, the detection limit of the AuNPs to see a change in absorbance when using a ssDNA linker could not be determined. Research from Yuan et al. showed that adding 40 nM of ssDNA linker was optimal.[8]

Besides that, CRISPR/Cas12a assays and fluorescence measurements showed that increasing the reporter concentration caused more trans-cleavage and more cutting activity of the Cas12a enzyme. The  $v_{\max}$  of the cutting was 2072 M/s and can be followed by Michaelis-Menten. First, the RNP complex and the target dsDNA need to be incubated for 1 hour and then the reporter ssDNA can be added. This also should be kept in mind when combining the CRISPR assay with the AuNPs. In Figure 10, the reaction velocity of the trans-cleavage reaction of the ssDNA reporter and CRISPR/Cas12a does not increase anymore after a certain reporter concentration. The concentration at  $v_{\max}$  was obtained to be 444,44 nM (by differentiating the equation of the curve in Figure 10). Thus, using this concentration of ssDNA (as reporter now, but when combining with AuNPs as ssDNA linker) would provide the most fast and efficient read-out assay of the signal.

However, our final goal is to be able to determine the concentration target dsDNA in a sample. For this, it is recommended to use a [reporter] below  $K_m$ . [35] Since, this will cause an increase in both the enzymatic activity and concentration of cleaved reporter. The more the reporter is cleaved, and thus the more (when combining with AuNPs) the ssDNA linker is cut, the more sensitive the colorimetric assay will be. As, when the optimal concentration of ssDNA linker which can be cut is used (when there is a target dsDNA recognized by Cas12a), the less the AuNPs will be able to aggregate and there will be no color change in the solution. When a certain concentration of ssDNA linker is cut, because the Cas12a enzyme does not cleave at its optimal ability, the color of the AuNP solution will be gradiented to purple/blue. So, it can be concluded that to obtain the most sensitive assay a concentration of ssDNA linker must be used below  $K_m$ , so [ssDNA linker] < 6244 nM.

Combining the concentration of the ssDNA linker with the limit of detection of the AuNPs, it can be concluded that, to the total system of AuNPs and CRISPR/Cas12a, a concentration of ssDNA linker of 40 nM must be added to obtain a sensitive colorimetric assay (reported by Yuan et al.) and [ssDNA linker] < 6244 nM and around 444,44 nM will provide a fast and efficient assay.[8] Hence, the recommended concentration of ssDNA linker which should be added to the system is 444,44 nM, since this is within the detection limit of the AuNPs color change and the concentration at which Cas12a's cutting is efficient.

However, this assay can not yet be read out with the naked eye. For this, further research should be done especially in optimizing the ssDNA functionalizing of the AuNPs in the solution. Also, AuNPs aggregation tests were not done using a [ssDNA linker] this high (max was 100 nM) yet. So, this should also be done to see if the aggregation of the ssDNA functionalized AuNPs in the presence of this linker ssDNA concentration is still possible. It could be that using a concentration this high will reduce the signal-to-noise ratio and therefore cause the AuNPs solution to have a clearer visible color change.[5]

In addition to that, a calibration curve of the percentage of cleaved reporter by CRISPR and fluorescent signal showed a linear relation. From now on, this curve can be used to determine how active CRISPR is when the fluorescence signal is known. From this, when the percentage of cleaved reporter is known, the concentration of target dsDNA in the analyzed sample can be determined. And it can be diagnosed how much of the DNA from a virus or disease you want to detect is present in the tested sample.

## Outlooks

In this research, 20 nm AuNPs are used functionalized using the salt-aging method. However, with this method takes at least two days to obtain stable DNA functionalized AuNPs. Functionalizing the AuNPs with mPEG and Tween20 did not show a clear color change of the solution when the ssDNA linker was added. In next research the method of functionalizing with mPEG and Tween20 can be improved. For example, the concentration of mPEG could be optimized, to make sure the ssDNA on the surface of the AuNPs can properly bind and will not cause hindrance with the ssDNA linker in the solution. Also, the amount washing steps can be optimized. It is thought that washing the AuNPs seven times clears out the free DNA in the solution which did not bind on the AuNP surface yet. Optimizing the washing steps will improve the signal-to-noise ratio of the colorimetric signal.[2][5]

Also, Cas12a is used. However, there are multiple types of CRISPR/Cas systems currently available.[2] Further research can also be done about applying these Cas enzymes to this sensing method. As other Cas enzymes can recognize other types of targets. For example, Cas13a can cleave ssRNA and Cas9 cleaves only dsDNA targets. Cas9 is known to be very sensitive and effective, also being able to see a color change in the solution with the naked eye. Cas14 should also be able to recognize target ssDNA and seems to be more sensitive than Cas12a.[20] Additionally, Cas14 can also perform trans-cleavage of ssDNA.[36]

Another research that could be done would be to implement the findings of this research to develop a system in which the CRISPR and AuNPs sensing assay could be performed without the need to transfer the distinct reactions.[20] This will reduce contamination risk and further simplify the detection of diseases.[37] For example, Fei Hu et al. developed a one-pot biosensor with CRISPR/Cas13a. This sensor basically had one tube in another, allowing the first reaction to take place in the inner tube and the next in the outer one after centrifugation.[38] Also, in this research, the CRISPR/Cas12a assay with target dsDNA and ssDNA (later used for AuNP linkage) is now performed before adding this to the AuNPs. It would be interesting to analyze what happens when everything is added together to see if CRISPR/Cas12a will cut the ssDNA attached to the AuNPs. If this is not the case, a one-pot assay would be easier to design.

## Acknowledgments

Illustrations in were made with Biorender.

Thankyou Nienke, for the daily supervision and answering any of my doubts. Learning and teaching me the confidence to do research.

## Supplementary data

### DNA sequences

usage	type	Purchased from	Sequence
crRNA	RNA	Eurofins Genomics	Complementary to Mal195 and Mal190 gene
Target dsDNA	dsDNA	Eurofins Genomics	Mal195 gene and Mal190 gene
Fluorophore-quencher pair	ssDNA	Eurofins Genomics	5'-[AF488]TTTTT-[BHQ1]-3'
Functionalizing AuNPs1	ssDNA	Eurofins Genomics	5'-[TH I]AAAAAAAAACACACACTCACAC-3'
Functionalizing AuNPs2	ssDNA	Eurofins Genomics	5'-[TH I]AAAAAAAAACCTCACCACCAACAC-3'
Linker for AuNPs	ssDNA	Eurofins Genomics	GTGTGAGTGTGTGTGATAGTGTGGTGGTGAGG

Figure S1: Table showing RNA and DNA sequence used.

### Naked-eye detection of aggregated AuNPs

#### Functionalizing using mPEG and Tween20

In Figure S2 a photo with solutions of 90  $\mu$ L including 5 nM AuNPs functionalized with DNA1, 5 nM AuNPs functionalized with DNA2 and different ssDNA linker concentrations are seen. It can be seen that the colors of the solutions are a lot like each other and almost no distinctions can be seen with the naked-eye. It can be seen that the solution containing a linker concentration of 100 nM looks slightly less bright red/pink than the solution with 0 nM linker.

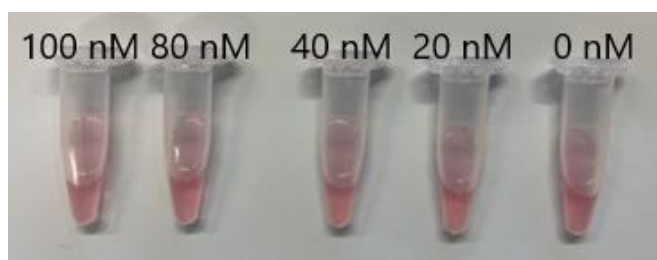


Figure S2: Photo of part of Eppendorf vials with solutions containing 5 nM AuNPs functionalized with DNA1 and 5 nM AuNPs functionalized with DNA2 and different concentrations of linker DNA.

### Absorbance measurements of AuNPs

Absorbance of the supernatant of all solutions was measured between 450 and 700 nm with UV-Vis spectroscopy. The obtained spectra can be seen in Figure S3.

The spectrum of the solution with 0 nM of ssDNA linker added to the AuNPs has the highest absorbance with a peak around 525 nm. Actually, the peak of the absorbance of all solutions is at 525 nm. This corresponds to a red color, the color of the solutions was indeed red. This can also be seen in the photo in Figure S2. No shift of the peak can be seen. Also, the spectra show that the solution with 80 nM (seen underneath 20 nM) of linker had the lowest absorbance values. It was expected that the solutions with a high concentration of linker DNA would turn in to a bluer color and therefore have a lower absorbance value.[5][7] Results like this also appeared in the research of Lopez-Valls et al.[5] An explanation for the minor differences in absorbance of the different concentrations could be that the tested concentrations are too low. It causes a low signal-to-noise ratio and makes the color change not clearly visible.[5]

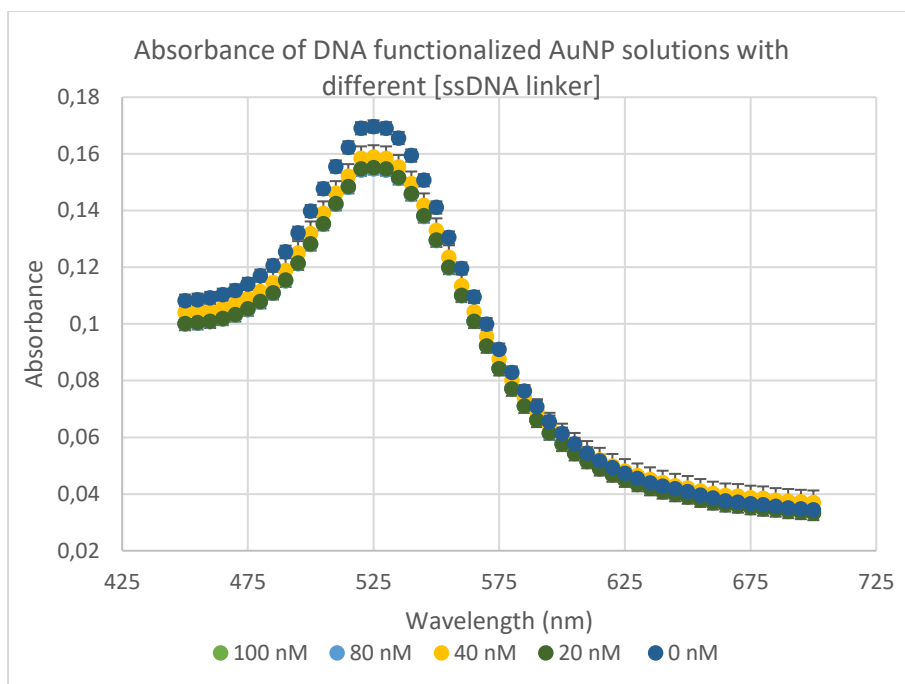


Figure S3: Absorbance measurements of mPEG, Tween20 and DNA functionalized AuNP solutions with different linker DNA concentrations between 450 and 700 nm measured with a plate reader. (n=3)

A plot of the red:blue absorbance ratio ( $A_{520}:A_{610}$ ) in relation with the concentration of ssDNA linker can be seen in Figure S4. It can be seen that as the concentration of the ssDNA linker decreases the red:blue ratio increases. This is in line with what was expected. As the expectation was that when more linker DNA would be available (so a higher concentration in the solution), more aggregation of AuNPs would take place causing a less red and more blue solution and thus a lower red:blue ( $A_{520}:A_{610}$ ) ratio.[7] Kasputis et al. observed that 40 nM was the ideal concentration of crosslinker DNA to add to the AuNPs and detect the best color shift.[7] However, in the Figure it can be seen that the linker concentration of 80 nM and especially 100 nM do not have absorbance values in accordance to the hypothesis. As these red:blue ratios are higher than the lower concentrations.

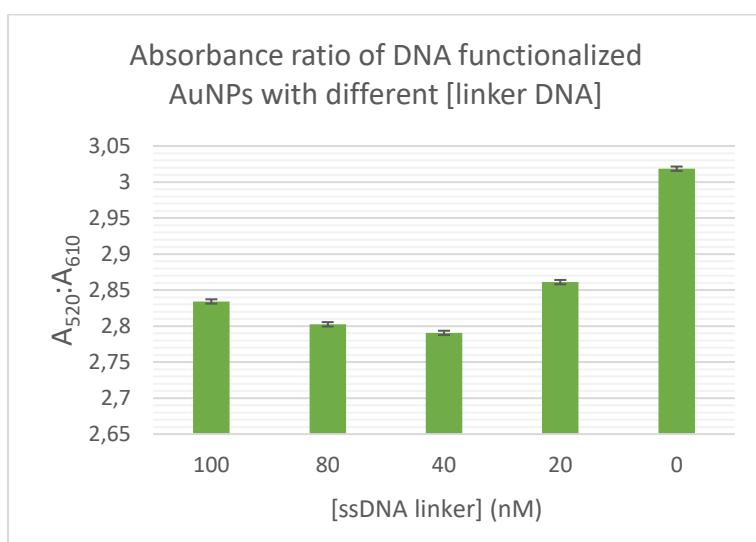


Figure S4: Absorbance ratio at 520 and 610 nm of mPEG, Tween20 and DNA functionalized AuNPs with different [linker DNA] in solution. (n=3)

Also, absorbance measurements of AuNPs functionalized with the salt-aging method and then washed two and three times with filtered PBST can be seen in Figure S5. The measurements were done using



a transparent 384-wells plate and 20  $\mu\text{L}$  of sample. The 2x washing samples were measured in duplo and the 3x washing samples were measured in quintuple. These results show that washing causes the absorbance of the samples to be lower. The results however, do not provide a clear relation between the addition of the ssDNA linker and the red:blue ratio and thus the aggregation of the AuNPs. In fact, the relation observed here is the opposite as the hypothesis. It was thought that a higher linker concentration would cause more aggregation of AuNPs and therefore a lower red:blue ratio, as more aggregation would mean a bluer solution. In Figure S5 however, the opposite can be seen. It is unclear why this is happening.

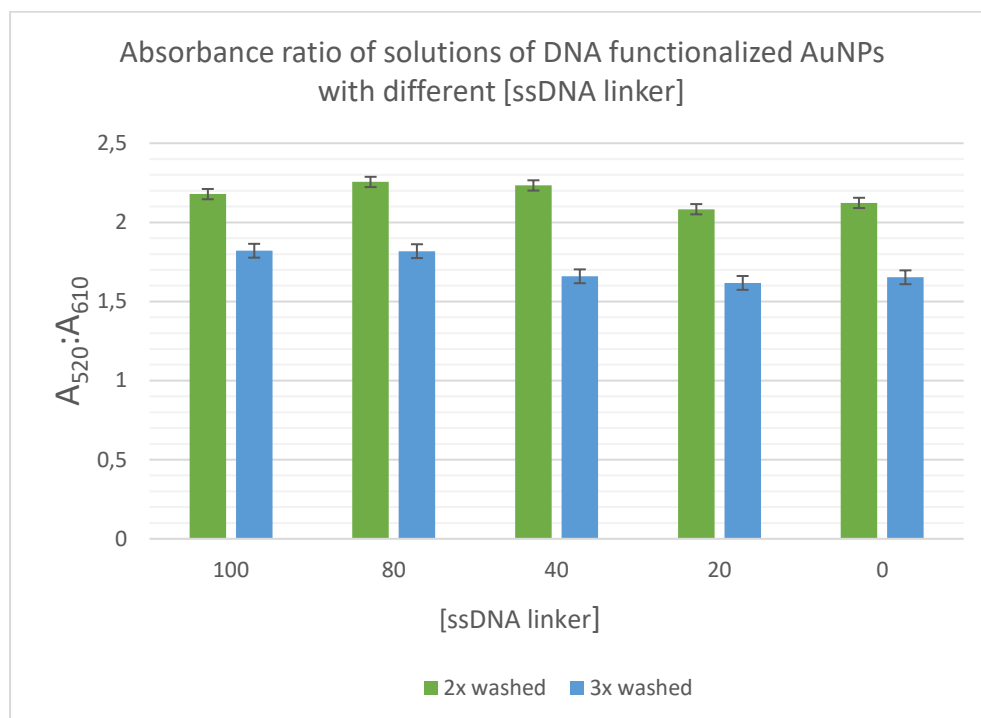


Figure S5: Absorbance ratio at 520 and 610 nm of supernatant of ssDNA functionalized AuNPs with ssDNA linker in solution after washing two and three times with filtered PBST. ( $n=2$  and  $n=5$  respectively)

#### Absorbance of non-functionalized AuNPs

In Figure S6 the absorbance spectrum of AuNPs not functionalized with DNA can be seen. For these measurements, 80  $\mu\text{L}$  of 1,16 nM AuNPs (20 nm) was mixed with 10  $\mu\text{L}$  MilliQ water. Absorbance was measured in a transparent 384-wells plate with 20  $\mu\text{L}$  of sample in triplo between 450 and 700 nm with steps of 10 nm.

It can be seen that AuNPs not functionalized with ssDNA have their absorbance peak around 525 nm. Compared to the spectrum of AuNPs functionalized with ssDNA and in the presence of a ssDNA linker in the solution, the absorbance values of the ‘naked’ AuNPs are higher. Zhang et al. reported results like this as well.[29]

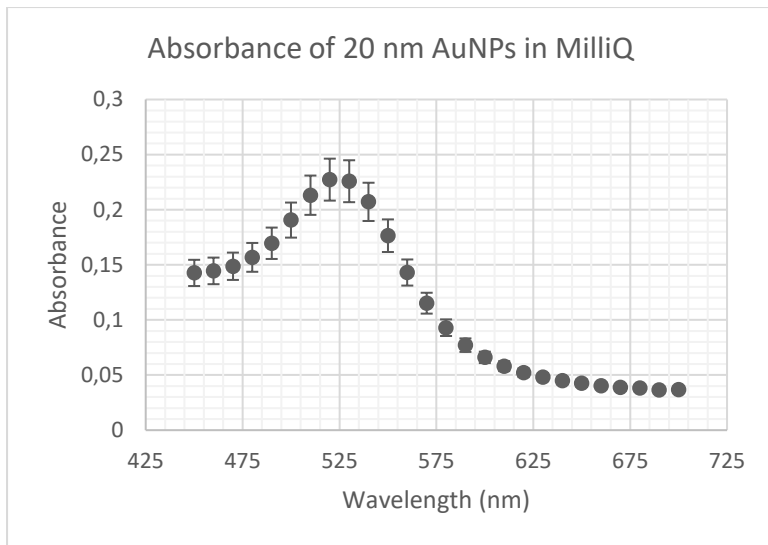


Figure S6: Absorbance spectrum of 20 nm AuNPs in a solution with MilliQ water. ( $n=3$ )

### Fluorescence signal of reporter over time

Figure S7, S8, S9 and S10 show the fluorescence measurements over time when varying the concentration of the reporter. In Figure S9 the same fluorescence measurements as in Figure S8 can be seen, but a bit more zoomed in on the lower concentrations. It can be seen that as the reporter concentrations decreases, the intensity of the fluorescence signal decreases as well.[30] This was expected, since there is more reporter available for CRISPR to cut, the reporter will emit more light and thus a higher fluorescence is measured.

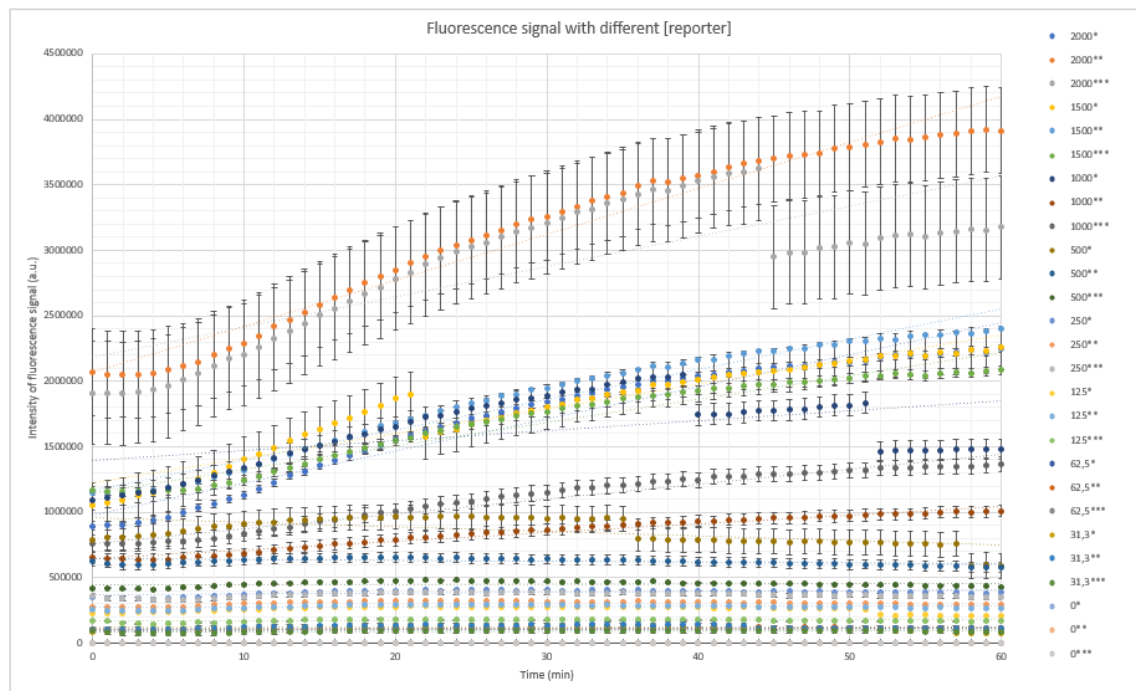


Figure S7: Fluorescence measurements for 1 hour, measured every minute showing CRISPR/Cas12a activity with different fluorescent reporter concentrations. Error bars show standard deviations per data set. Wit plate-reader settings on low. ( $n=3$ )

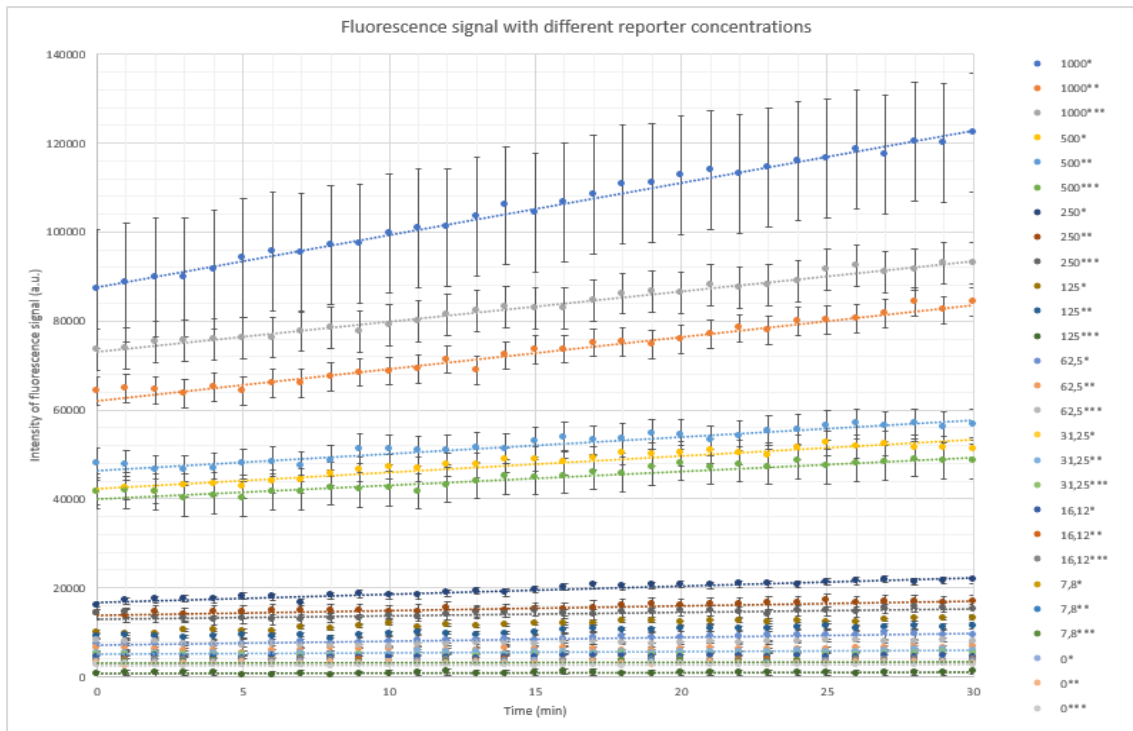


Figure S8: Fluorescence measurements for 1 hour, measured every minute showing CRISPR/Cas12a activity with different fluorescent reporter concentrations. Error bars show standard deviations per data set. With plate-reader settings on high. (n=3)

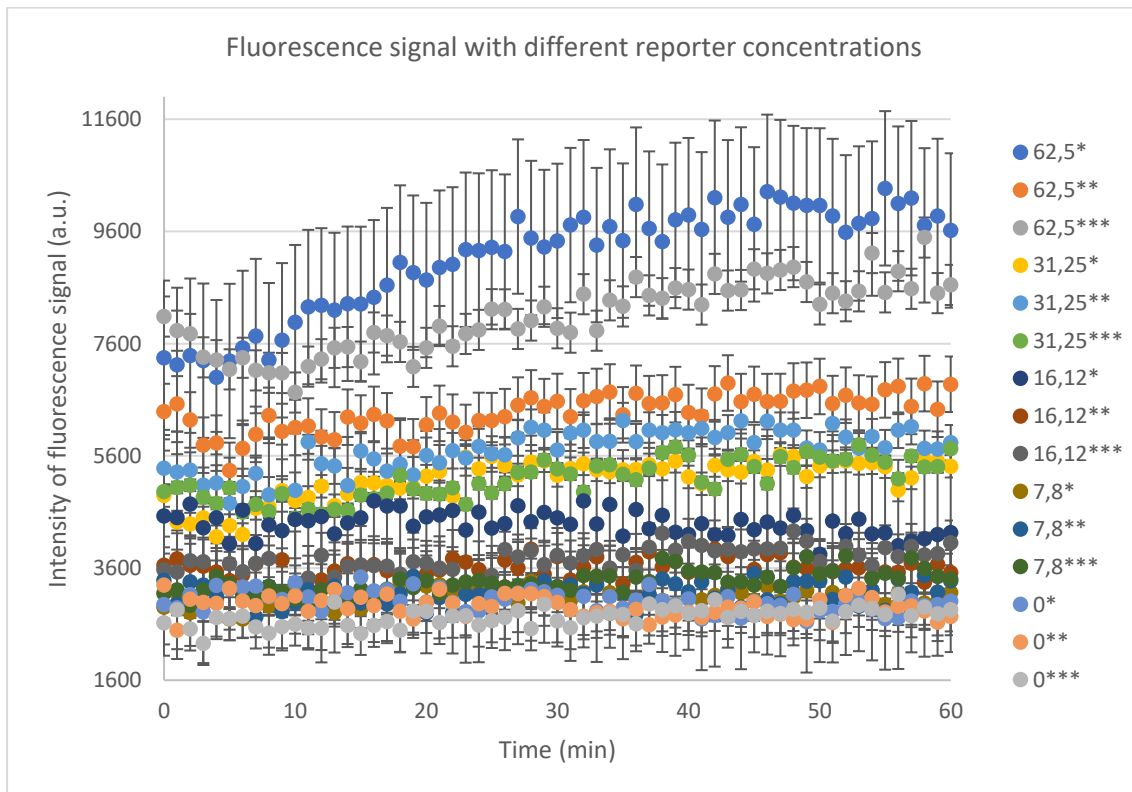


Figure S9: Fluorescence measurements during 1 hour zoomed in from Figure 7, measured every minute showing CRISPR/Cas12a activity with different fluorescent reporter concentrations. Error bars show standard deviations per data set. With plate reader settings on high. (n=3)

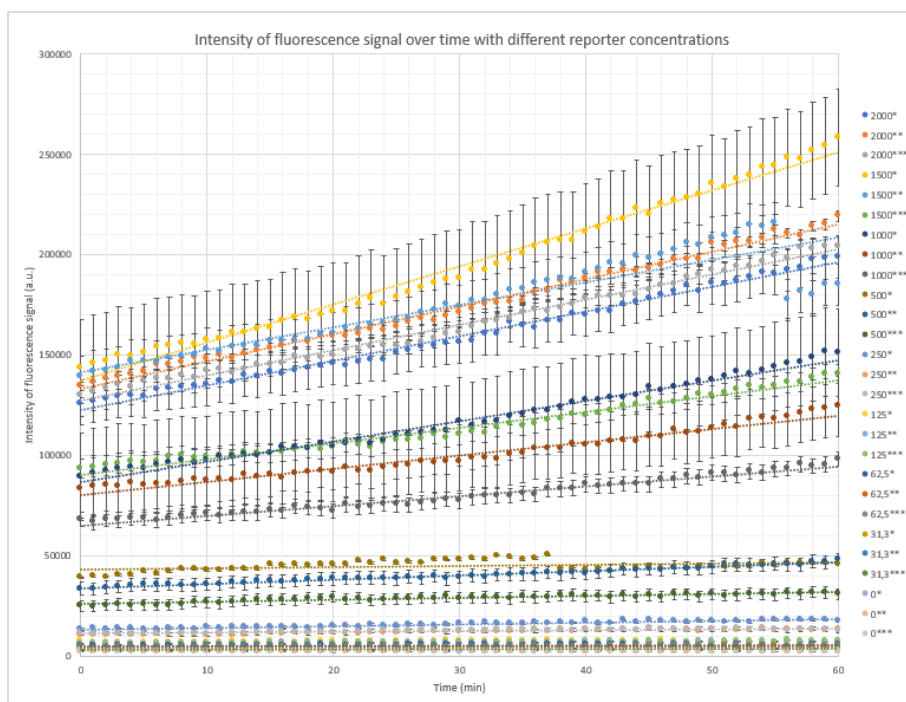


Figure S10: Fluorescence measurements during 1 hour, measured every minute showing CRISPR/Cas12a activity with different fluorescent reporter concentrations. Error bars show standard deviations per data set. With plate-reader settings on high. (n=3)

### Additional Michaelis-Menten curves

In Figure S11 a comparison between the reaction velocity using the calculations from Ramachandran et al. and our own fluorescence measurements and the reaction velocity according to the Michaelis-Menten equation can be seen.[26] It can be seen that the curves are very similar.

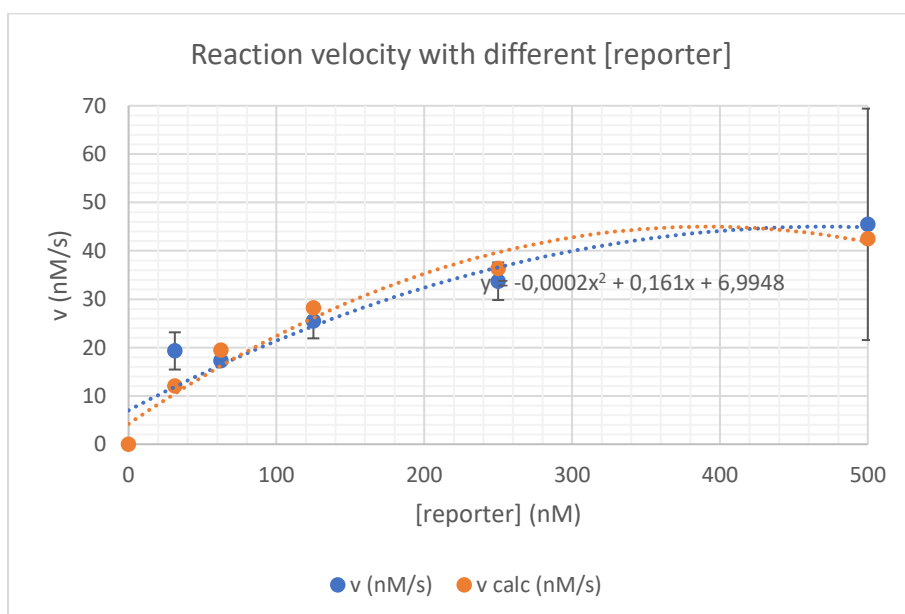


Figure S11: Michaelis-Menten curve comparing the reaction velocity using the calculations from Ramachandran et al. and our own fluorescence measurements and the reaction velocity according to the Michaelis-Menten equation.[26]

Below, in Figure S12, a Michaelis-Menten plot can be seen. This plot was obtained with the plate-reader settings on high instead of low. So no values for  $K_m$  and  $v_{max}$  were obtained. However, the curve does show that the Cas12a enzymatic activity is logarithmic and follows Michaelis-Menten.

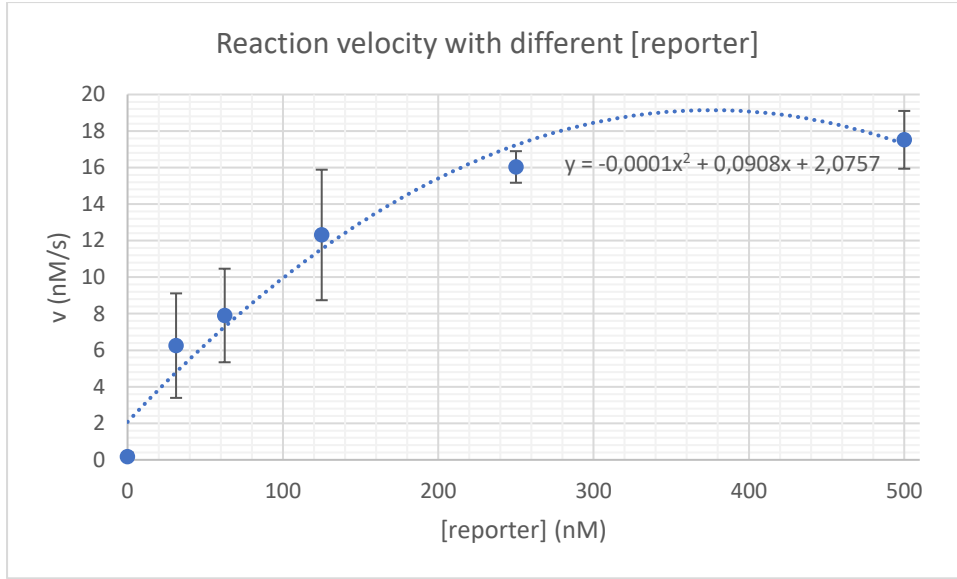


Figure S12: Michaelis-Menten curve obtained with plate-reader settings on high instead of low.

### Back-of-the-envelope checks of Michaelis-Menten values

Back-of-the-envelope checks on the reporter Michaelis-Menten values were done using calculations from Ramachandran et al.[26]

The first check that can be performed is the following equation:

$$\alpha = \frac{vt_{lin}}{S_0} < 1$$

Where  $v$  (nM/s) is the velocity of the reaction,  $\alpha$  is a parameter for the part of total product formed at  $t_{lin}$  and  $S_0$  is the initial concentration of uncleaved reporters. The conservation of species rule limits the equation to  $< 1$ .

From Figure 10,  $t_{lin}$  was defined to be between [reporter] of 50 and 100 nM (from Figure S10), 10 and 20 minutes. So  $t_{lin} = 10 \text{ min} = 600 \text{ s}$ ,  $S_0 = 50 \text{ nM}$  and  $v = 0,00078 \text{ nM/s}$  (obtained from filling in the formula of the trendline in Figure 10). With these values  $\alpha = 0,00933$ . So  $\alpha < 1$ , this check is correct for our Michaelis-Menten analysis.

Then, a check regarding the maximum velocity of the reaction can be done. Michaelis-Menten states that when the  $[S]$  is much higher than  $K_m$ ,  $v_{max} = k_{cat}E_0$ . This can be converted to the following formula:

$$\beta = \frac{v}{v_{max}} = \frac{v}{k_{cat}E_0} \leq 1$$

In our research,  $E_0 = 1 \text{ nM}$ ,  $k_{cat} = 2072 \text{ M/s}$  and a random reported value for  $v = 0,00132 \text{ nM/s}$  (at [reporter] = 125 nM). This gives  $\beta = 6,36 \cdot 10^{-7}$ . So also,  $\beta < 1$ , makes also this check correct.

Lastly, a check regarding the linear portion of the Michaelis-Menten plot and the time can be done.  $t_{lin}$  should be the same magnitude as the total reaction time scale  $\tau$ . The following equation can be obtained:[26]

$$\gamma = \frac{t_{lin}}{\tau} = \frac{t_{lin}k_{cat}E_0}{K_m} \leq O(1)$$

From Figure S9  $t_{lin} = 10 \text{ min} = 600 \text{ s}$ ,  $E_0 = 1 \text{ nM}$ ,  $k_{cat} = 2072 \text{ M/s}$  and  $K_m = 6244 \text{ M}$ . Then,  $\gamma = 199,1$  which is indeed in the order of magnitude of the  $[S]$ , since  $[S]$  was between 50 and 100 nM during  $t_{lin}$ .

### Fluorescence signal of different target dsDNA concentrations

In Figure S13 the intensity of the fluorescence signal over time using different concentration of target dsDNA can be seen. It can be seen that the intensity of the fluorescent signal increases as the concentration of the target dsDNA increases. However, the slope of the curves decreases as the time increases. The distribution of the data is in line with the hypothesis, because it was thought that when more target dsDNA is available, more CRISPR will be activated and thus more of the initial concentration of reporter will be cut and emit more light, showing a higher fluorescent signal.

The target dsDNA concentration of 1 nM was not taken into account, as this concentration was not pipetted with the right volume making the concentration not representative.

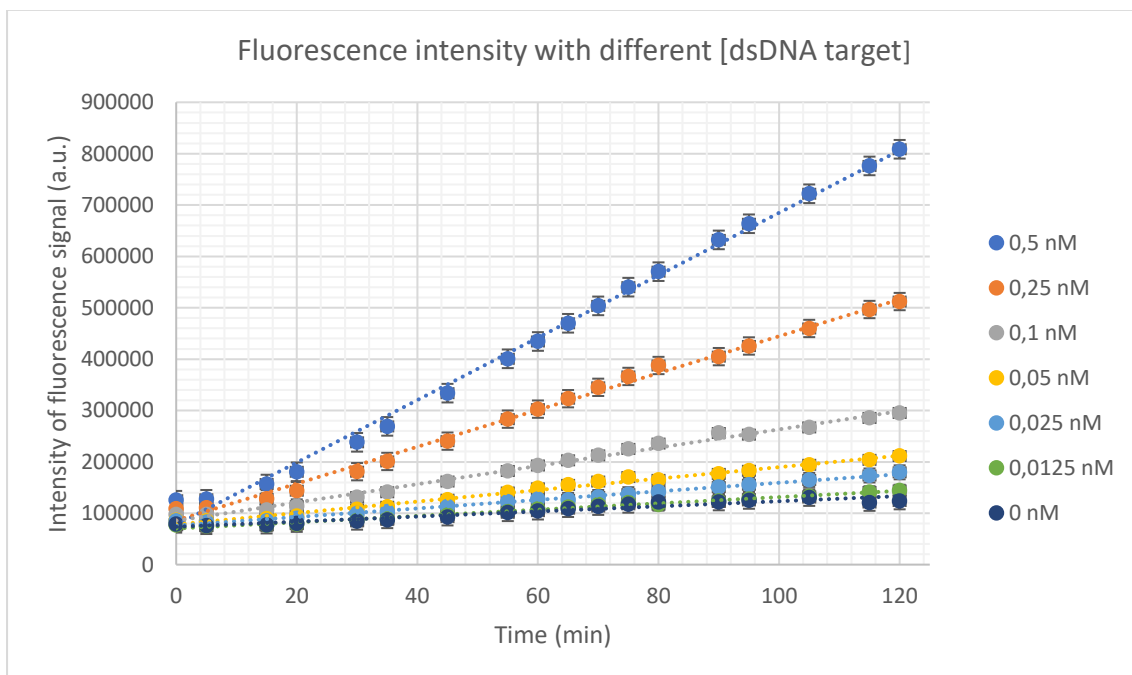


Figure S13: Intensity of fluorescence signal over time using different concentrations of target dsDNA including one standard deviation. (n=3)

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