Implementation of an autocatalytic signal amplifying protocol in digital droplet assays.

Kevin Lampe, January 2025

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Abstract

This thesis is a first attempt to implement AutoCAR-1 in a digital droplet CRISPR assay, a novel attempt to lower the limit of detection (LoD) in Cas-based DNA detection. AutoCAR-1 amplifies the signal instead of the target by introducing a secondary target, a linearized circular mediator. Difficulties in synthesizing the circular mediator meant that experimental work shifted towards troubleshooting the synthesis protocol. No conclusive solution was found, so we conclude with recommendations for further research into mediator synthesis.

Introduction and context

In all self-replicating biological systems, genetic information is stored in and passed on through a long biopolymer called deoxyribonucleic acid, or DNA. In its basic form, it presents as two intertwined or connected strands, each a sequence of individual nucleic acids, or nucleotides. Only four different types of building blocks code for the entirety of the genome, simply by varying sequences. In the last few decades, humanity has been able to increasingly faster and more accurately determine the exact sequence an organism has.

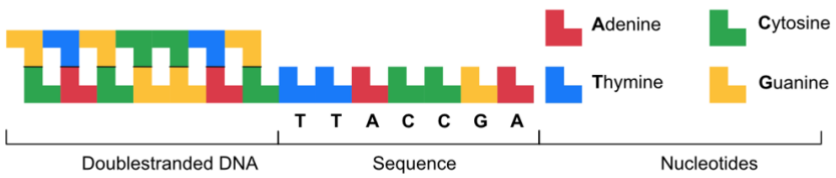

Figure 1: Structure of DNA, with the sequence determined by four types of nucleotides. Nucleotides form two complementary pairs: adenine and thymine, and cytosine and guanine.

Knowing the exact DNA sequence can yield interesting insights on the functioning of metabolism, disease or other characteristics. By taking a sample from a body, assays can determine the specific mutations of a tumor, the variant of a virus or a genetic defect. Samples can either be solid biopsies (pieces of tissues or tumors), or liquid biopsies.

Liquid biopsies can be blood, saliva, urine or other bodily fluids and the sample collection is a much more patient-friendly, non-invasive procedure compared to solid biopsies.

Despite the advantages of liquid biopsies, including their non-invasive nature and ease of sample collection, they come with certain limitations. Liquid biopsies often contain only minute quantities of DNA, such as circulating tumor DNA (ctDNA) or cell-free DNA (cfDNA), which are usually fragmented and present amidst a large background of normal DNA (Linthorst et al., 2021). This low abundance of target DNA poses a challenge for detection and accurate analysis.

A practical situation where low concentration DNA poses a challenge is found in testing for ultralow concentration markers for dangerous viruses. Since 2017, Dutch women are offered non-invasive pregnancy testing, or NIPT. NIPT takes a blood sample from the mother and checks for a wide range of potentially dangerous biomarkers. A recent study on a large dataset of NIPT results showed that human cytomegalovirus (hCMV) can be detected in early stages of pregnancy (Linthorst et al., 2023). hCMV is a virus carried by the mother that can have serious consequences on the successful carriage. While internationally deemed untreatable, recent research has shown that hCMV can be suppressed and strongly improve the chances of a successful pregnancy (Faas et al., 2024). While these treatments are yet to be internationally recognized, their implementation would benefit from accurate testing in clinical settings, highlighting a need for fast, accurate, ultra-low concentration nucleotide sensing.

CRISPR/Cas sensing

A relatively young technique for highly specific nucleotide detection is found in the field of CRISPR/Cas sensing. CRISPR (Clustered Regularly Interspaced Palindromic Repeats) are a repeating sequence in bacterial DNA. Discovered in 1987, the repeating sequences have been linked to bacterial immune response, mediated by CRISPR associated (Cas) enzymes (Gostimskaya, 2022). Cas enzymes "patrol" the cell, looking for non-self DNA. When they encounter such DNA, the Cas hybridizes and checks the DNA sequence against its "library of dangerous bits", a large DNA library made of DNA, encoded by CRISPR sequences. Upon finding a matching entry, Cas enzymes activate and trigger a process that could result in cell death (Gostimskaya, 2022).

The complete range of those Cas-mediated processes stretches far beyond this, as native behavior of Cas proteins can vary per Cas variant. Cas variants can be classified on several physical properties. One of them is the type of target it binds to, being single-stranded DNA (ssDNA), double-stranded DNA (dsDNA) or the slightly different genetic structure ribonucleic acid, RNA. Other properties can be the length of possibly

required spacing of a target sequence and the CRISPR; the specific spacer sequence; cleavage behavior and its type; or, notably, the possibility of the Cas to exhibit collateral cleavage (Son, 2024; van Dongen et al., 2020). In recent years, the particular behaviors have been exploited for nucleotide sensing.

A commonly used Cas-variant in biosensing is Cas12a. Cas12a is relatively unique in that it can target both ssDNA and dsDNA. It can be primed with a piece of guiding RNA (gRNA), after which Cas12a forms a ribonucleoprotein (RNP). While roaming the cell, Cas12a remains inactive until it finds a piece of DNA complementary to its gRNA – the target. Upon target recognition, Cas12a not only exhibits cleaving of the target DNA (*cis*-cleavage), but also activates a secondary cleaving mechanism, resulting in collateral cleavage, or *trans*-cleavage, in ssDNA molecules distant from the target site. In nucleotide sensing, this *trans*-cleavage can be exploited to generate a fluorescent signal by cleaving fluophore-quencher pairs, coupled by ssDNA. While *cis*-cleavage is done once, a singular activation can result in thousands of *trans*-cleaving instances. Figure 2 gives a general overview of the process. The signal can be read by exposing the sample to a wavelength of light, and measuring the resulting excitation signal (van Dongen et al., 2020).

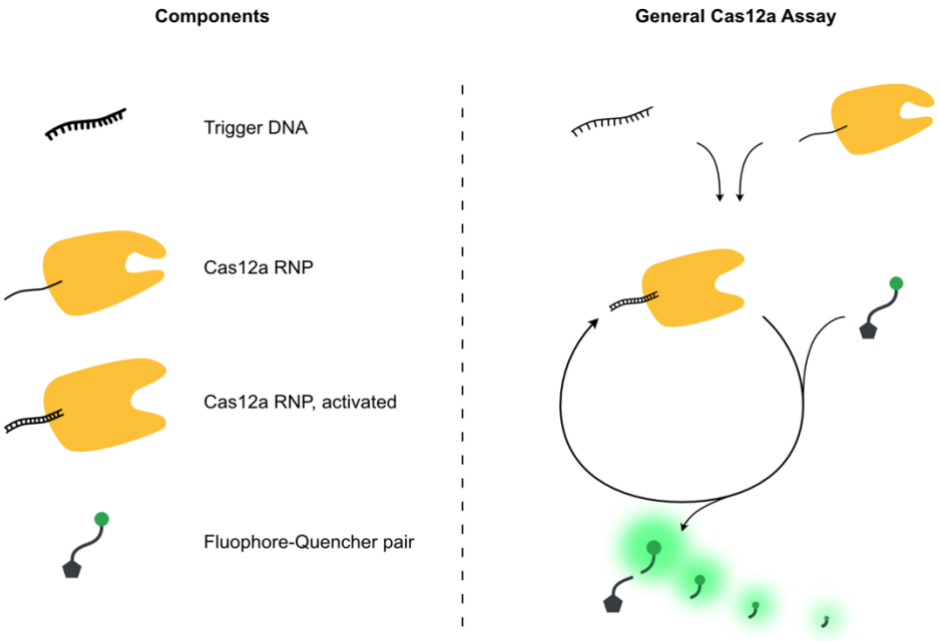

Figure 2: Schematic overview of the reaction scheme in a fluorescence-based Cas12a assay. A target molecule, the trigger DNA, hybridizes with an inactive RNP to activate it. The activated RNP cleaves FQ-pairs, resulting in a fluorescent signal from excitable fluorophores.

Limit of Detection?

Analysis on ultra-low concentrations of DNA, such as in the case of hCMV, are difficult due to an inherent limitation in most assays, the limit of detection (LoD). When a signal generated from the DNA is below the LoD, the signal cannot be interpreted. This is problematic in ultra-low concentration samples, as it is difficult to generate a signal from sometimes only a single molecule in a relatively large bulk fluid. To compensate, considering that concentration is the ratio of targets over volume, two methods can improve the LoD: amplification of the target molecule, or partitioning to lower the volume.

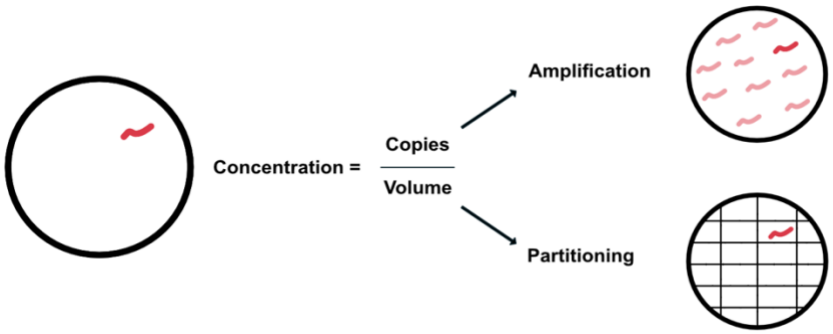

Figure 3: Schematic overview of two basic approaches to lower the limit of detection. LoD is dependent on concentration of target. Concentration can be improved by amplifying the target and bypartitioning the volume the target is in.

For decades, the field of nucleic acid has been dominated by nucleid acid amplification tests, reliant on target amplification method. The most popular being polymerase chain reaction (PCR), these methods are often still employed in CRISPR/Cas assays to boost assay duration or LoD (Politza et al., 2023). PCR is a technique used in nucleic acid amplification tests (NAATs) to amplify the target DNA. Other amplification methods include RPA and LAMP, but the common denominator is that they are often used as pre-amplification method, after which the nucleic acid is further analyzed assays, such as blotting or sequencing.

Microfluidic partitioning methods offer an alternative method to improve LoD by dividing a bulk over many small droplets (10^5-10^6), a technique often called digitalization. Quantitative data is retained in the ratio of positive/negative samples and droplet size, while local concentrations in target-containing droplets are much higher, which increases reaction kinetics. A Figure of Merit analysis highlights the effect digitalization has had on CRISPR diagnostics, naming the digitalization of Cas assays as a comparable merit to that of amplification methods (Nouri et al., 2022). However, the authors note that digital integrated amplification methods span the crown regarding low LoD in short assay durations.

Deng et al. published a a novel method to amplify the fluorescent signal from a normal Cas12a assay (2024). By introducing a piece of circular DNA as a mediator and fake target, their two-step AutoCAR-1 system is supposed to increase the reaction kinetics and form an autocatalytic reaction. This would potentially enable attomolar (10^{-18}) target concentration results within a short time span. This is a comparable result to established methods, but in a shorter time span. While Deng et al. demonstrated the potential in several sensor methods, no attempt at digitalization has been made. Considering the significant effect of digitalization on the merit of target-amplification methods, AutoCAR-1 implementation in a digital assay shows high potential for high-throughput, fast and ultrasensitive nucleotide detection.

This thesis attempts to assess the implementation potential of AutoCAR-1 in digital droplet assays. By implementing autocatalytic signal amplification in digital partitioning techniques, the aim is to obtain ultra-high DNA sensing sensitivity, while simultaneously allowing for high throughput, low-cost and fast DNA diagnostics. Initially, three goals are defined.

Goal 1: Replicate the AutoCAR-1 protocol

Goal 2: Adapt the AutoCAR-1 protocol to use organic targets related to the CMV-virus

Goal 3: Characterize the reactions selectivity and sensitivity and assess implementation of the adapted AutoCAR-1 protocol in digital droplet assays.

Theoretical framework

Exploiting *trans*-cleavage by Cas12a

Cas12a's *trans*-cleavage can be exploited in nucleic acid detection methods. Cas12a is primed to recognize the target sequence by hybridizing unbound Cas12a enzymes with specifically designed gRNA, complementary to the target DNA sequence, forming an RNP. Exposure of RNP to a target-containing sample then allows the formation of an RNP-target complex, activating *trans*-cleaving of nearby ssDNA. In the case of biosensing, this activation can be translated into a fluorescent signal by introducing specially designed fluorophore-quencher pairs connected by ssDNA to the mix. Cleavage of these fluorophore-quencher pairs results in free fluorophores, which can be excited in a fluorescence plate reader. *Trans*-cleavage will continue until the RNP-target duplex dissociates, but low dissociation constants allow for modelling the reaction as continuous (Deng et al., 2024). The resulting signal is measured and interpreted in arbitrary fluorescence units (AFU).

Last March, Deng et al. introduced a novel way to amplify a fluorescent signal in Cas enhanced nucleotide detection (2024), visualized in Figure 4. They proposed an autocatalytic signal amplifier that does not increase the amount of genetic material, but instead introduces a secondary signal mediated by circular units (Cir-Mediators of partially double stranded DNA. A first Cas, recognizing the primary target sequence, expresses *trans*-cleavage and cleaves both fluorophore-quencher pairs, but also cleaves single-stranded bits of the circular mediators, after which the DNA linearizes and forms a target for a second RNP. This second RNP will then also cleave fluorophore-quencher pairs, ánd more Cir-Meds, and so on.

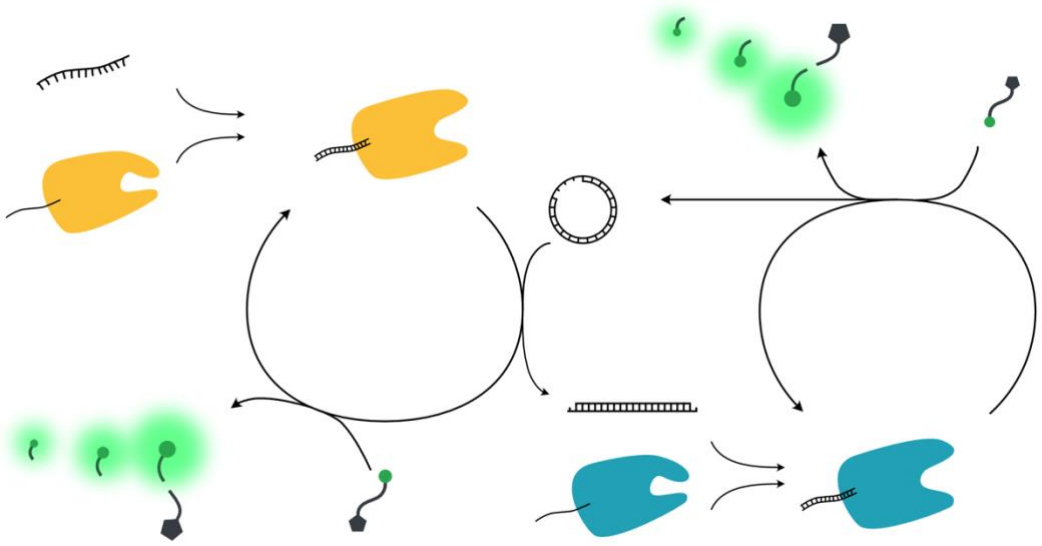

Figure 4: Schematic representation of AutoCAR-1. The first RNP1 (yellow) has a similar schematic to that of a general Cas12a assay. The difference is the introduction of Cir-Mediators, which, upon linearisation, form a target for RNP2 (blue).

By a single RNP1 activation, multiple Cir-Mediators can be linearized. Every linearization of a Cir-Mediator provides a new target for RNP2, activating more RNP, which linearizes more Cir-Meds. All the while, each RNP also cleaves fluorophore-quencher pairs. This means that the signal will behave differently from the general Cas12a assay. In a normal Cas12a assay, the signal will initially increase exponentially, until all RNP1 is activated, or all target DNA hybridized. When saturated, the signal is linear, until it reaches a plateau caused by a lowering concentration of fluorophore-quencher pairs. When all pairs are cleaved, the signal would ideally remain steady, but bleaching of fluorophores causes it to decline. The difference can be observed in Figure 5.

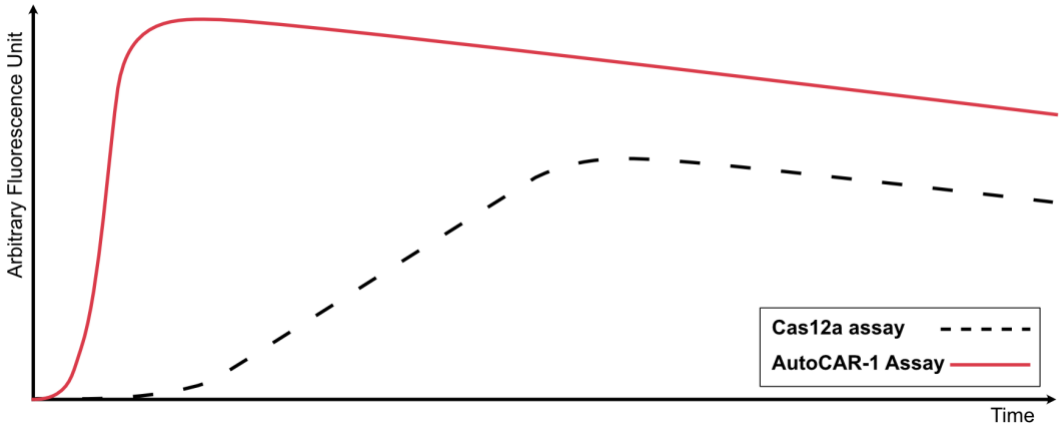

Figure 5: The difference between signal from a classical CRISPR assay and an AutoCAR assay. AutoCAR-1's autocatalysis means the signal will reach the plateau much faster; making fast readouts easier, and increasing the maximum signal due to less bleaching occurin

Amplification and partitioning

Currently, DNA analysis often relies on a technique called Polymerase Chain Reaction (PCR). This technique uses polymerase enzyme and sometimes sequence-specific primers to replicate either all or a specific DNA, often referred to as amplification. Amplification makes samples with low DNA concentrations suitable for further analysis. In clinical DNA analysis, PCR-based methods are often used to analyze presence of specific DNA mutations, or bacterial, viral or tumor DNA.

For decades, PCR-based methods have been the most common method in nucleotide sensing. PCR can specifically amplify target DNA to meet the LoD, after which blotting or sequencing provides insight into DNA presence or structure (Xiang et al., 2022). The disadvantage of PCR is in its thermocyclic nature, which relies on large machinery and skilled workers being needed to use PCRs and subsequent assays (van Dongen et al., 2020). In recent years, isothermal amplification methods have been presented, such as RPA and LAMP. While these do not require the machinery that PCR does, they are less specific and more prone to false positives and false negatives than PCR (Politza et al., 2023).

An alternative to amplification is partitioning, where LoD is improved by increasing local concentrations. A significant improvement in lowering LoD is made in digital CRISPR assays, where microfluidic techniques are used to partition a bulk into millions of partitions. Assuming the location of targets in the sample is random and that the partitions are sized such that it is statistically likely that there is either 1 or 0 target molecule per partition, Poisson statistics apply. The target concentration of the bulk can be found in the ratio of positive versus negative signals from partitions (Basu, 2017).

Depending on the application, partitioning can be done in wells or in droplets (Basu, 2017). When done in wells, assays can be done on 384- or 1536-well plates, or when scaling down, on chips with miniaturized, etched chambers. It is possible to freeze-dry reagents such as DNA or probes in the chambers, and deposit sample droplets in the chambers for optical readout. A second method is to create droplet emulsions in an oil. Microfluidic channels and junctions can be used to create droplets from multiple inlets, allowing reagents to mix in the droplet. Surfactant is added to the droplet ingredients to stabilize droplets in oil. Partition size is controlled by flow from both fluids and/or limited by chip dimensions, but also by expected target concentration. Both systems have particular benefits, but droplet systems offer higher scalability as droplet size (and number) are more easily adjusted (Basu, 2017).

Amplification and partitioning are not mutually exclusive. Integrations of digitalization and amplification have become mainstream with several examples on the market. PCR, LAMP and RPA have all been integrated with digital droplet (dd) or chamber-based digital (cd) techniques (Xiang et al., 2022).

Challenges in CRISPR assays

Although the CRISPR sensing field is still in relative infancy, established CRISPR techniques show promise in competing with established biomolecular sensing fields. SHERLOCK, HOLMES and DETECTR allow for sensitive nucleotide detection with Cas13 or Cas12 (Son, 2024; van Dongen et al., 2020) and a suitable amplification method; HOLMESv2 combines the specificity of Cas enzymes with a streamlined isothermal amplification method, eliminating the need for complex thermal cycling as seen in traditional PCR.

In their 2020 review, Van Dongen et al. (2020) defined five challenges for existing CRISPR sensing systems. Quantitative analysis, measuring the target concentration, is an important aspect of diagnostics as a marker of disease progression. They note that established CRISPR sensing techniques such as SHERLOCK or HOLMES can not provide quantitative data, as they rely on pre-amplification. HOLMESv2 does provide quantitative insights, but requires large machines (van Dongen et al., 2020).

Besides quantitative analysis, target amplification can be a challenge in developing CRISPR assays, especially considering point-of-care applications (van Dongen et al., 2020). As mentioned before, target amplification is used to increase the LoD by replicating the target, but essential epigenetic information is lost in the process (van Dongen et al., 2021).

The AutoCAR-1 protocol initially presents itself as an alternative for other isothermal amplification methods like RPA or LOOP. In bulk assays, low concentration DNA

sensing is demonstrated and achieving attomolar (approximately 1 DNA copy per µL) resolution for DNA detection in a sample is possible (Deng et al., 2024). The exploitation of topological activation restriction for an autocatalytic process is novel and promising. The possibility of implementing AutoCAR-1 in digital assays could push the sensitivity of nucleotide sensing to new highs and opens even more possibilities for ultra-high sensitivity systems.

Results, discussion

To replicate the AutoCAR-1 protocol, the first step was to replicate a few intermediate tests from the Deng et al. publication. For this, Cir-Mediators are required, which need to be synthesized first. A list of all used materials and methods can be found in Appendix I and II.

Forming Circular Mediators

Cir-Mediators consist of an outer ring of circular ssDNA, annealed with a partial inner ring, the complimentary DNA (cDNA). They are formed by circularizing the ssDNA with linker DNA and using ligase enzyme to close the loop. The linker is removed through exonuclease with ExoIII, after which cDNA is added to anneal to the now circular ssDNA, as illustrated by Figure 6.

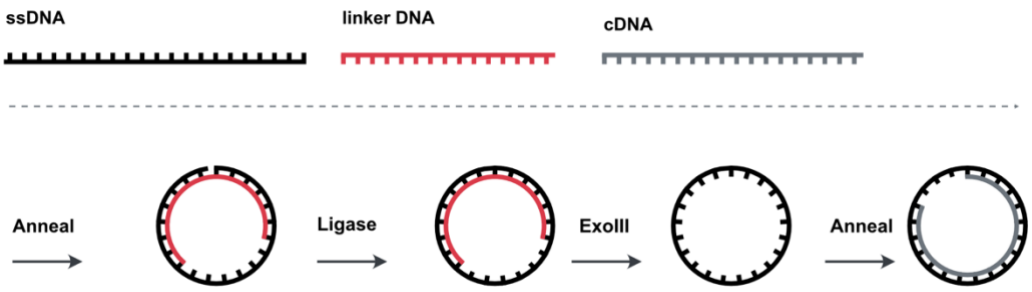

Figure 6: Overview of the DNA transformations in Cir-Med synthesis. First ssDNA and linker anneal, after which ligase circularizes the ssDNA ring. ExoIII removes the linker, after which cDNA is added and anneals with the ssDNA, forming the final Cir-Mediator.

First, Cir-Mediators were formed through Method 1. As an induced method of Cir-Mediator confirmation, a relinearisation and RNP2 activation assay was ran. Method 4 was used to make relinearise Cir-Mediators , with a No Template Control by omitting the trigger ssDNA. The linearization product and NTC was used as input in an activation assay with RNP2 following Method 5.

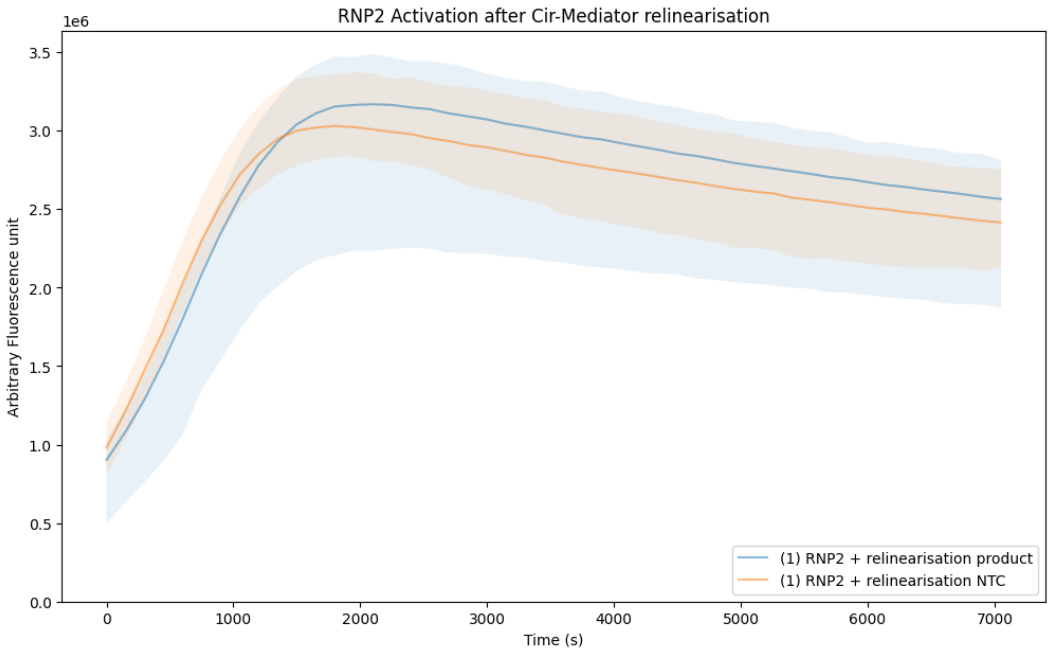

Figure 7: Graph of the fluorescent signal from RNP2 activation by relinearisation product and an NTC for relinearisation.

The result of the relinearisation experiment in Figure 7 are somewhat surprising. In the control, trigger is omitted. This should lead to a lack of signal, as there should be no linearization of Cir-Meds and hence no activation of RNP2, as illustrated by Figure 8.

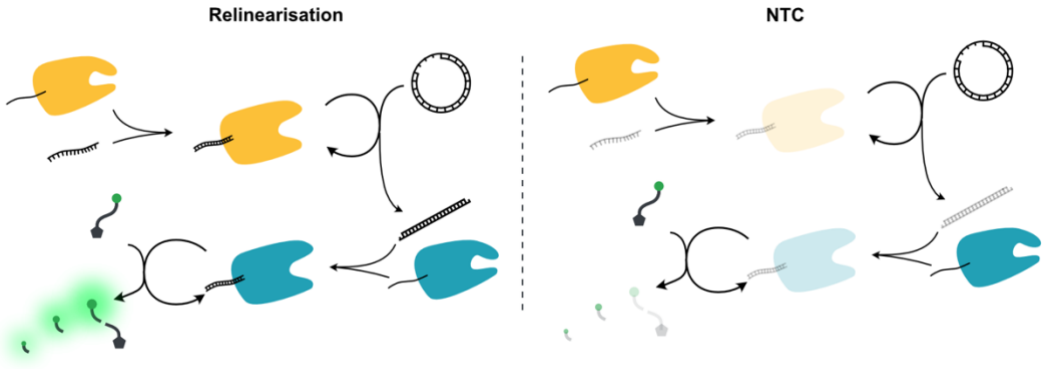

Figure 8: Schematic overview of the relinearisation and the NTC, in which omitting trigger DNA should lead to a lack of signal.

To troubleshoot the result, each point of failure was extensively considered in a decision-tree style point-of-failure analysis. After, several hypotheses were formed. RNP2 contamination could have caused a signal from residual trigger DNA. On the other hand, a combination of mistakes in Cir-Mediator synthesis could lead to similar results as well. To troubleshoot the first hypothesis, possible faulty RNP functioning, each RNP was used in an activation assay.

RNP activation assays

For both RNP's, an activation assay was set up using Method 5, such as in the scheme of Figure 2. For RNP1, 1 nM trigger ssDNA was used as target. For RNP2, 1nM linear dsDNA was used as target, made by first annealing ssDNA and cDNA in a 1:1 ratio, and diluting accordingly.

Figure 9 shows the results of the two RNP activation assays. It should be noted that there is a stark difference in the intensity of the signals in the positive replicates. It would be fair to address this to human errors in making the mix. Yet, in both replicates, a stark difference is observed between the activation in the positive replicates and the negative replicates, indicating that the RNPs work as expected.

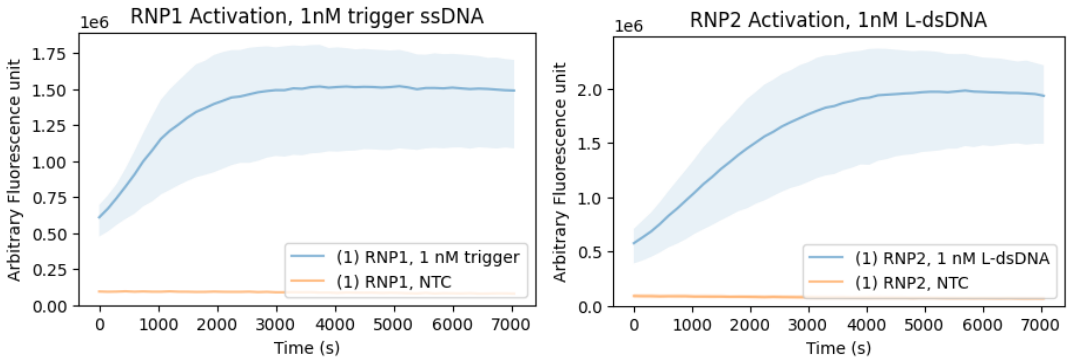

Figure 9: Fluorescence readings of RNP activation assays. In both assays, a clear signal

With the activation assay results assuring us the RNPs work as suspected, it must be deduced that there is something wrong with the Cir-Mediator product. The product used leads to activation of RNP2, regardless of cleavage by RNP1, as seen in Figure 7. This should not happen after successful Cir-Mediators synthesis, so the suspicion that a different product is made arises. This implies a different product at one or more of the three synthesis steps (ligation, exonuclease, annealing) while retaining an intact sequence for RNP2 activation. An initial hypothesis could be that ligation does not take place. In that case, the product of Cir-Mediator synthesis would be linear dsDNA. A first insight into the product of each step can be found by running using Qubit assays, to analyse the presence of dsDNA. Qubit assays make use of an intercalating fluorescent probe. The probe intercalates on dsDNA and by calibrating the readout device with two standards, the concentration of dsDNA is measured.

Qubit Assays

Cir-Mediators were produced following Method 1 and linear dsDNA was made by annealing ssDNA and cDNA in a 1:1 ratio and diluting accordingly. A deviation was made from Method 1 in the annealing step: annealing was done at room temperature, for 1 hour, as done in the method described by Deng et al. (Deng et al., 2024). A Qubit

assay was ran to compare the annealing efficiency of Cir-Mediator product and controlled with an equal annealing protocol. This yielded the results found in Table 1.

Qubit assay result	Cir-Mediator	Linear dsDNA
Concentration	5,59 ng/mL	181 ng/mL
Yield[1]	0,95%	30,7%

Table 1: Result from Qubit assay comparing Cir-Mediator product with linear dsDNA in identical theoretical concentrations. [1] Yield is calculated comparing measured weight to the theoretical maximum double-stranded DNA fraction's weight.

It becomes evident that something is going wrong in the synthesis of Cir-Mediators, as very little dsDNA is formed. A secondary observation is that the yield of linear dsDNA with the annealing protocol as described by Deng et al. (2024) is lower compared to more typical annealing protocol yields when using a more general annealing protocol.

To determine which step of Cir-Mediator synthesis performs unexpectedly, two further Qubit assays were ran. The first of those assessed the ability of Exonuclease III to digest ssDNA, as literature hinted that this might be the case – a finding not taken into account by Deng et al. (2024). ssDNA was taken through a exonuclease step such as in Method 1, after which it was annealed following Method 7. In the No Enzyme Control (NEC), no exonuclease was added. The final concentrations of the product were similar to the theoretical molarity of Cir-Mediator product. The results are found below in Table 2.

Qubit assay result	Exonucleaseproduct	NEC
Concentration	55,3 ng/mL	405 ng/mL
Yield[1]	9,40 %	68,2%

Table 2: Results from Qubit assays to asses the effect of Exonuclease III on ssDNA. [1] Yield is calculated comparing measured weight to the theoretical maximum double-stranded DNA fraction's weight.

The yield of the NEC was significantly higher. This indicates that at the concentrations in Cir-Med synthesis, as described by Deng et al. (2024), Exonuclease III can efficiently digest blunt ssDNA. This is a surprising find, as the documentation of the used Exonuclease III (ExoIII), supplied by NEB, states ExoIII to be dsDNA specific. However, literature indicates that ssDNA digestion by ExoIII is highly efficient when concentrations of ExoIII exceed 0,1 unit/µL (Shen et al., 2023). In the Cir-Mediator synthesis described by Deng et al. and consequently our Method 1, ExoIII is used at 4 unit/µL.

A last Qubit assay was ran with a new Cir-Mediator synthesis. Here, the Cir-Mediator formation was divided in three steps, following Method 1: ligase, exonuclease and annealing. The results are found below in Table 3.

Qubit assay result	Ligase	Exonuclease	Annealing
Concentration	110 ng/mL	6,51 ng/mL	11,4 ng/mL

Yield[1]	22,2 %	1,03 %	1,94 %

Table 3: Result from Qubit assays comparing intermediate and final products in Cir-Mediator synthesis. [1] Yield is calculated comparing measured weight to the theoretical maximum double-stranded DNA fraction's weight.

Comparing the dsDNA yield of the ligase step to the annealing step, it is again evident something is going wrong. It appears that ssDNA and linker do hybridize in the ligation step, forming a linker-ssDNA double-stranded duplex. However, after exonuclease, there is little hybridization of cDNA and ssDNA. One explanation could be that during ligase, no ligation takes place. This would mean that when the linker, hybridized after ligation, is removed by exonuclease, ssDNA opens to its linear form, as demonstrated in Figure 10.

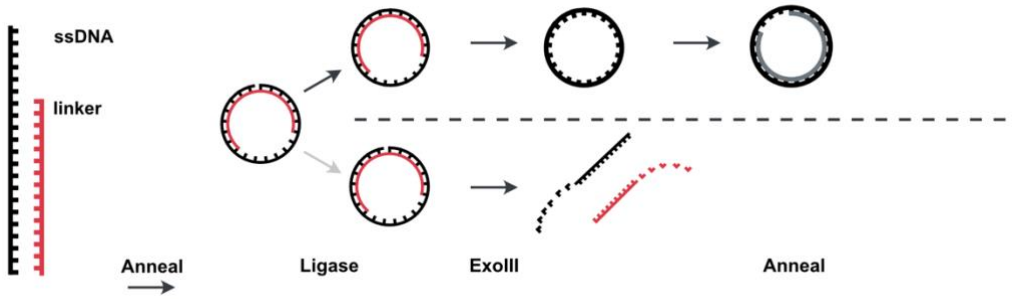

Figure 10: Schematic overview of the reaction when ligase works, or does not work.

Now knowing ExoIII efficiently cleaves ssDNA at the used concentration, as concluded from Table 3, it is not unreasonable for most of this now open, linear ssDNA to be broken down. It is clear that the result of the Cir-Mediator activates RNP2, regardless of relinearisation. Together with Table 2 we can conclude that this is likely to stem from single stranded DNA molecules. Question remains what the topology of those ssDNA molecules would be.

One option could be that no circularization takes place at all. Instead, linker and ssDNA crosslink to form long strands of ssDNA/linker duplexes, oligomerization (see . The conditions in which oligomerization or circularization takes place is well documented by Paluzzi et al. (2023). They establish that in concentrations of ssDNA and linker of around 100µL, circularization is optimal and can have a yield over 90%. In higher concentrations, they note oligomerization to be more likely. In the synthesis described by Deng et al. (2024), circularization is claimed to happen at a molarity of 4000 nM.

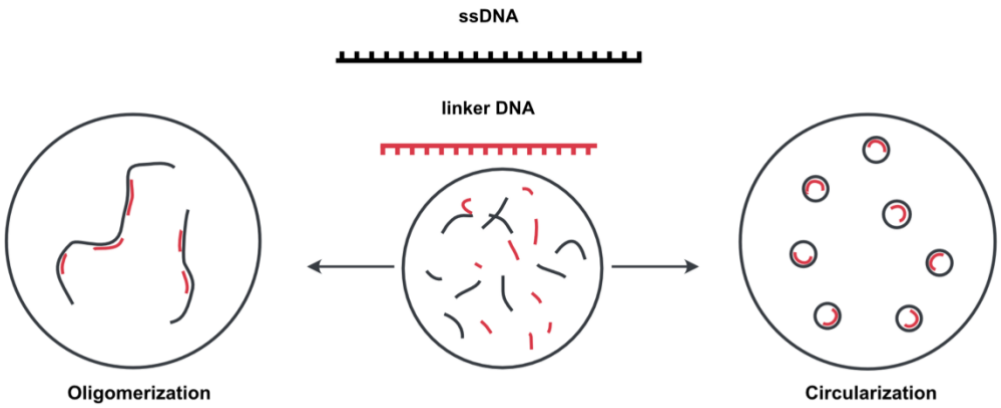

Figure 11: The difference between oligomerization and circularization, illustrated.

Oligomerization provides an explanation for the lack of circular products. It would be a resistant to ExoIII digestion as well, as ExoIII digests from the 3′ ending and only a nucleotide at a time. Longer strands would take long to digest and have multiple target sequences embedded. However, the question remains why a low increase in dsDNA is observed after the annealing step in Table 3. Unless strong secondary structures form on the oligomerized ssDNA, cDNA should be able to anneal to the corresponding sections.

To address the question of oligomerization, a last experiment was set up with a gel electrophoresis setup following Method 2. Gel electrophoresis separates DNA on size and weight, by pulling DNA fragments through a gel matrix with a current. The gel used, a 4-15% PAGE gel, has a gradient, making it suitable for a wide range of DNA fragment size separation. Wells were loaded with a range of different Cir-Mediator synthesis conditions, but unfortunately, none of the wells showed significant bands, as visible in Figure 12.

In Figure 12, we observe the image obtained from the gel electrophoresis. In the first two wells, we see the ladder. The corresponding standard has been placed next to it. In the twelfth well, a second ladder was placed, but this has spilled over into neighboring wells. Besides that, we see no bands in the between wells – where ligase product samples were placed. This means no conclusion can be made on the possibility of oligomerization yet.

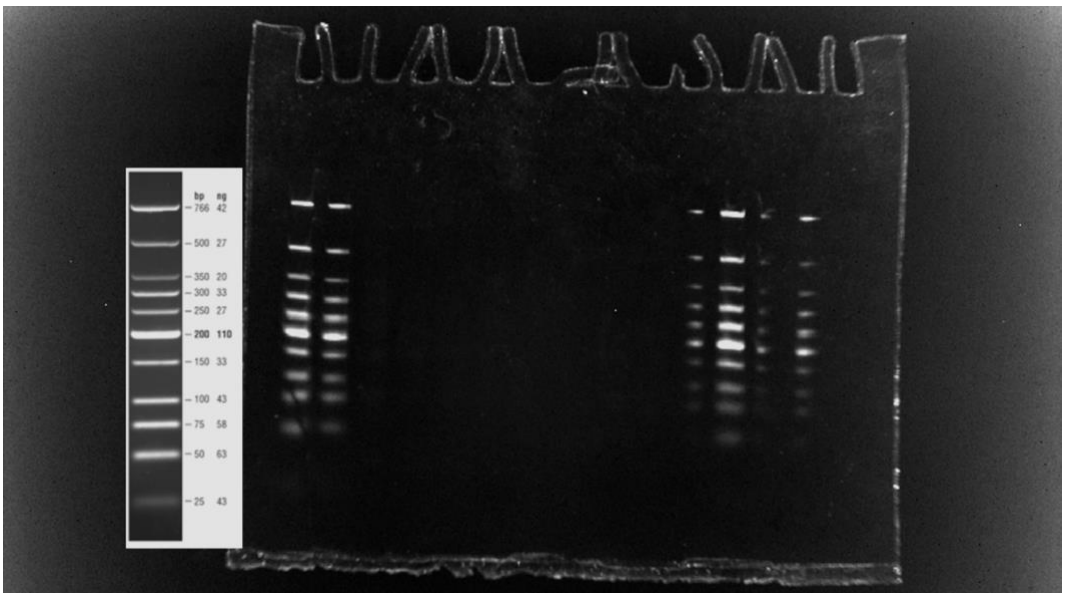

Figure 12: Image from a gel electrophoresis with multiple ligase products from Cir-Mediator synthesis.

Conclusion

This thesis started off with three goals: to replicate, adapt and implement the AutoCAR-1 protocol. However, issues in the synthesis of Cir-Mediators meant that the experimental work did not proceed past the replication phase. We did, however, extensively analyze, discuss and troubleshoot the synthesis of Cir-Mediators. Therefore, only limited conclusions can be drawn.

First, it is clear that the Cir-Mediator synthesis as described in its pioneering paper requires extra attention. Consequently to our troubles, the results of this paper have been handed to the corresponding author of the AutoCAR-1 paper. We are awaiting an answer, in the hopes that they can provide clarification on possible misunderstandings or extra information on the synthesis.

An attempt at adapting AutoCAR-1 to organic targets can only be made once successful Cir-Mediator synthesis is dialed in. This means no conclusions can yet be made on the adaptation of AutoCAR-1 to other targets. However, from literature we know that the specificity of Cas enzymes to the PAM sequence on targets can highly influence the RNP-target binding potential.

Lastly, the implementation of AutoCAR-1 in digital droplet assays was topic of investigation. We've been unable to successfully recreate AutoCAR-1. A thorough, evidenced judgement on implementation potential should be made in future research, but for now, a few findings can be shared to aid.

For instance, CRISPR assay kinetics are influenced by many factors. Temperature and concentration are potentially the two most impactful. Different Cas enzymes have different optimal operating temperatures but will display activity outside of that

optimal range as well. We used AsCas12a Ultra in our experiments. This strain works optimally around 37 °C, but will show activity in room temperature as well, albeit less. This means that as soon as a reaction is pipetted together, AsCas12a Ultra can start *trans*-cleavage. This is evident in Figure 7 and Figure 9; at the start of the assay, the activation signal is already above with a positive incline.

Implementing this in a digital droplet assay adds a few constraints to the chip. When partitioning, the bulk cannot contain the trigger already. Only at the last moment, right before reading, should trigger and RNP come together. One option would be to freeze-dry all AutoCAR ingredients into wells or a sensor plate, and partition droplets on the plate, an approach seen before in point-of-care applications (Yu et al., 2020).

A second, more adaptable option is a digital droplet generator with a double inlet, or a double-cross geometry. This allows to generate droplets with two separate fluid inlets and a continuous throughput. In such a setup, all AutoCAR-1 ingredients to only come together at the last moment. An example could be the below image of a chip currently sold on the market.

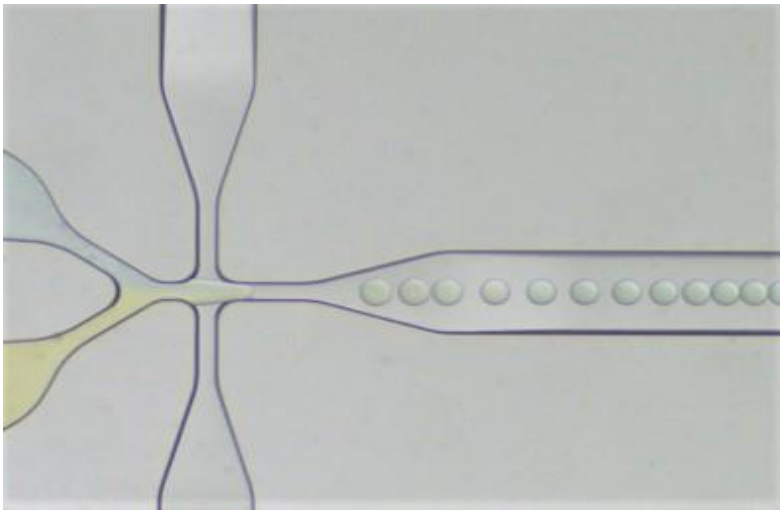

Figure 13: A double-cross geometry in a microfluidic chip, allowing partitioning of bubbles with two fluids at the same time (*Droplet Generator Chips - Double-Cross Geometry - Fluidic 1505 - Microfluidic ChipShop*, n.d.).

Future Research recommendation

1. Optimizing circularization

Literature suggests that DNA concentrations in the Cir-Mediator synthesis and described by Deng et al. (2024) (1000-4000 nM) are far from the optimal yield conditions for forming small circular DNA (Paluzzi et al., 2023). Reliable Cir-Mediator synthesis has

proven to be essential for research on AutoCAR implementation and was the bottleneck in this thesis.

1a. Alternative to linker in Cir-Mediator synthesis

An interesting idea could be to combine the linker with a complimentary DNA-function. After ligation, the outside ring does not have a distinguishable start or end anymore. The linker is removed through exonuclease, after which cDNA is added to form a duplex part. The current setup of Deng et al. (2024) keeps the complimentary PAM-sequence and target sequence in the middle of the ssDNA, sandwiched by spacer nucleotides. After exonuclease and annealing, the spacer nucleotides form the single stranded notch at which relinearisation is initiated. It could be interesting to place the spacer nucleotides in the center of the ssDNA, with the linker consisting of two interchanged halves of the PAM and target sequence. This could simplify Cir-Mediator production and improve total yield, while preventing activation from residual ssDNA as the sequence is no longer intact.

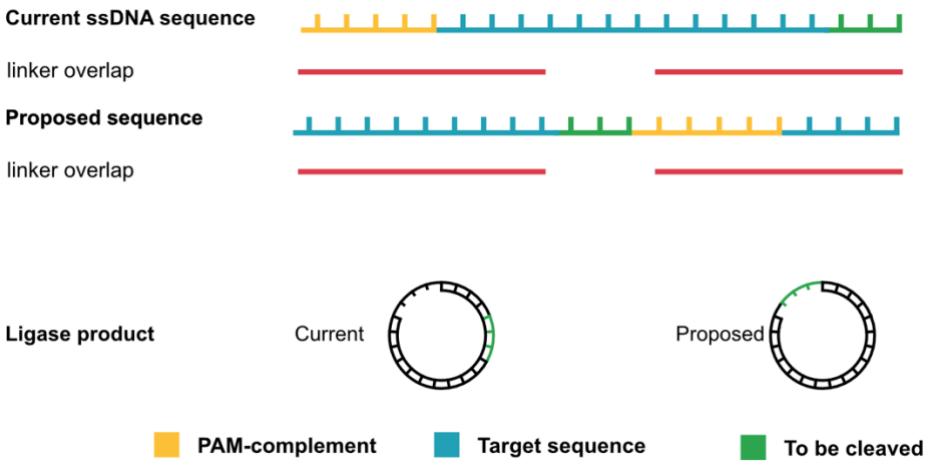

Figure 14: Proposed change in sequence for ssDNA. This allows the cDNA to fulfill the role of linker DNA.

2. Investigating ExoIII activity and residual nucleotide interference.

The documentation of Exonuclease III indicates that the enzyme digests only one nucleotide from the 3′ loose end of either ds- or ssDNA at a time, after which it dissociates (*Exonuclease III (E. Coli) | NEB*, n.d.). The protocol by Deng uses ExoIII to remove the linker from the circularized ssDNA. It is not unreasonable to suspect that this can result in residual nucleotides remaining on the circular ssDNA. These would then inhibit cDNA from annealing onto the ssDNA, hence a lack of Cir-Mediator synthesis. .This could also be the case in the hypothetical oligomerization scenario from

our previous discussion (p.14), which would lead to a very low ssDNA yield, but activation RNP2 would still hold, as AsCas12a Ultra can activate without a PAM sequence present.

3. Investigating integration of AutoCAR-1 in epigenetic-sensitive assays.

The unique proposition of AutoCAR-1 lies in its property of signal amplification, compared to target amplification. Keeping the original target intact and unique, metadata on the DNA such as methylation is kept intact. CRISPR/Cas systems can provide insight into the specific methylation of a DNA sample (van Dongen et al., 2021). Unfortunately, the methylation data is now coupled to the Cas12a activation levels, which would become ambiguous after signal amplification. Combining AutoCAR-1 with methylation-sensitive CRISPR/Cas systems ánd a digital droplet assay could provide a hypersensitive method of DNA detection and typing, if an alternative way to couple data and signal is developed.

Contextual exploration

This thesis is the last step within my bachelor program, ATLAS. Within ATLAS, we focus on interdisciplinarity and placing research in technical and societal context. While some of the context has already been mentioned in the introduction, I would like to take the opportunity in this last chapter of my thesis to more casually elaborate on this.

The research in my thesis was carried out within BIOS, the Lab-on-a-chip (LoC) research group. LoC is a growing interdisciplinary field, combining all sorts of disciplines. Electrical engineers work on new types of sensors, while biologists work on biocompatible applications and cell or DNA analysis methods. Physics comes into play in chip fabrication and mathematical models improve signal processing. The development of LoCs is, clearly, a multi- and interdisciplinary affair.

The societal context may be more interesting in this case. As mentioned in the introduction, the research that this thesis tries to contribute to is inspired by the publication of NIPT data and hCMV presence in the samples. NIPT in itself has revolutionized genetic screening and the detection of rare DNA variants like hCMV can aid in adequate treatment of the particular pathogen. This research tries to contribute to the development of methods to reliably and quickly do so. The AutoCAR-1 protocol uses relatively simple components and on paper allows for straightforward but fast and accurate data collection and processing. In the hypothetical scenario that an AutoCAR-1-based detection method is viable, it would be a safe bet to say that it would not necessarily have to be an expensive method.

This means AutoCAR-1 lends itself for point-of-care (PoC) applications. PoC DNA detection is something we're all too familiar with. Think about pregnancy tests, Covid tests or fatherhood tests. Instead of costly, labour-, material- and time-intensive laboratory testing, fast testing close to the PoC is preferred more and more in clinical settings as well (Murphy et al., 2021). The development this thesis contributes to ideally leads to a cheap, simple device where health care workers can quickly and accurately determine if a patient has a certain cancer or virus.

The societal impact of such devices can be great. Kosack et al. concluded that PoC devices generally adhere to the ASSURED framework, meaning they are Affordable, Sensitive, Specific, User-friendly, Robust, Equipment-free and Deliverable (2017). This means they can decrease workload on nurses in hospitals, as faster, more easily interpretable results reduce cognitive load. The devices can be operated by less-skilled workers, meaning less necessary training required for adequate diagnostics. Lastly, the shelve life and price of the devices mean that high-tech, specific diagnostic tools for even the rarest of deficiencies can be available for technically, infrastructurally or economically disadvantaged regions too. Faster, more accurate and cheaper diagnostics know almost no downsides.

A small difference might be the type of device we're talking about. Some of the first mainstream CRISPR assays allowed for very simple readouts, with the debuting paper already presenting proof-of-concepts for affordable, simple tests (Gootenberg et al., 2018). Incorporating CRISPR in digital assays, however, greatly increases the complexity of readouts. This introduces partitioning methods, readers and computers and that requires resources. Some devices have been designed to solve this problem using a smartphone or a smart chip ((Yu et al., 2020; Zhang et al., 2024). These devices use physical chips and chamber-based partitioning. While the research in this thesis was carried out with the idea of implementing in digital droplet-based assays, it can be extrapolated that the ingredients can be adapted for similar chamber-based partitioning devices as well.

While not having reached all the initially defined goals, this thesis has contributed a few specifics. For instance, the troubles encountered while trying to replicate the original AutoCAR-1 paper led to a letter addressed to the authors. While not necessarily groundbreaking, I see it as a contribution to the scientific community and their works, as it potentially opens a discussion on an interesting topic. Besides this, the recommendations for future research could streamline synthesis of the Cir-Mediators, or at least provide opportunity to learn more about circular DNA synthesis, oligomerization and enzymatic processes. In the end, this project provided a valuable contribution in ultra-low concentration nucleotide sequence detection, contributed to

a novel use of CRISPR/Cas sensing systems and a contribution to development of a digital Cas-enhanced PoC device, making high-tech sensing capabilities a bit more accessible for all.

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Appendix

I: Materials

Materials: Tabled

Item	Producer	Notes
DNA		
Trigger ssDNA	Eurofins	GAA GAC ACC CTA CCA ACC CCC CCC
gRNA, trigger	IDT	GGG GGG GGT TGG TAG GGT GTC
gRNA, Cir-Med	IDT	GAC ATA GCA CAT AGA CTG AGA CTG
ssDNA	IDT	TCT ATG TGC TAT GTC TAAA
linker	Eurofins	GCACATAGATTTTAG
cDNA	Eurofins	TTTAGACATAGCACATAG
FQ-pair		6C FAM probe. 56-FAM/CCCCCC/3IABkFQ/
Enzymes		
AsCas12a Ultra	IDT	
T4 Ligase	NEB	
Exonuclease III	NEB	

Buffers		
T4 Ligase buffer	NEB	
NEBuffer 2.1	Bioke	
Gels, Dyes and Qubit		
Invitrogen™ Novex™ TBE-Urea Gels, 4-15%	ThermoFischer	
Gel Loading Dye, Purple (6X)	NEB	B7024SVIAL
Low Molecular Weight ladder	NEB	
SYBR Safe DNA stain	ThermoFischer	
Qubit dsDNA HS Quantification Assay Kit	ThermoFischer	

II: Methods

The methods in this section are used in the experimental work contained in this thesis. Most was adapted from Deng et al. (2024), and from the product manuals contained with some of the used products.

Method 1: Synthesizing Cir-Mediators

Circular mediating pieces of dsDNA, or Cir-Mediators, form the core of Deng's AutoCAR system.

Materials: Cir-Med ssDNA, 100 µM; linker ssDNA, 100µM; Cir-med complementary ssDNA, 100µM; NEB T4 Ligase, 400 unit/mL; 10X NEB T4 Ligase buffer; NEBuffer 2.1 10X; NEB Exonuclease III, 100.000 unit/mL.

Steps: 2 µL ssDNA, 4 µL linker DNA, 2,5 µL T4 Ligase and 1µL Ligase Buffer are mixed with nuclease free water (NFW) to a total volume of 50 µL. Left at 16 °C for 12 hours, the ligase is inactivated at 65 °C for 20 minutes afterwards. To remove the linker DNA, 10µL of the mix is mixed with 4 µL of the 10X NEBuffer 2.1, 1,5 µL of exonuclease and NFW to a total volume of 40 µL, kept at 37 °C for 100 minutes, after which exonuclease is heat-deactivated at 75 °C for 30 minutes. To complement the ss-Cir-Meds, 1:1 molar ratio c-ssDNA is added and annealed by heating the product to 96 °C and cooled down to room temperature at a rate of 0,1 °C/s. In this case, 0,4 µL of the 100µM stock was added, resulting in an idealized yield of 990 nM Cir-Mediators.

Method 2: Gel Electrophoresis

To confirm the forming of circularized DNA, gel electrophoresis can be used to determine its presence. Gel electrophoresis separates DNA fragments on weight. By applying a current over a gel, samples are pulled through due to their charge. In circularizing, the structure of ssDNA changed and will change the hydrodynamic resistance experienced in gel-based electrophoresis compared to the linear homologue.

Materials: DNA samples; 4%-15% Invitrogen Novex TBE-Urea gel; Low Molecular Weight ladder; 6X Purple Loading Dye; TBE buffer; SYBR Safe DNA stain.

For electrophoresis steps, refer to the instruction manual provided with the gel and/or electrophoresis system. After electrophoresis, stain the gel with SYBR Safe and image.

Method 3: RNP Incubation

Used to prime Cas12a for activation on specific target. Used for all RNP's, differentiating between RNP1, RNP1b and RNP12 [TODO] by varying the gRNA for a different target,

Materials: Target gRNA, 100µM; LbCas12a, 64000µM; NFW.

Method: Enzyme and gRNA are mixed in a 1:2 volume ratio and left to incubate at room temperature for an hour. After, a 1:100 dilution is made in NFW.

Method 4: Linearisation of Cir-Mediators

Double-stranded Cir-Mediators have a single-stranded notch that can be cleaved to linearize, forming the linear L-Cir-Mediators.

Materials: Cir-Mediators; RNP1; Trigger ssDNA, 1µM; NEBuffer 2.1 10X; NFW;

Method: To a suitable volume, RNP1 is mixed to 25 nM; Trigger ssDNA to 100nM; and Cir-Mediators are mixed to 100nM, assuming a the maximum yield from Method 1. NEBuffer is mixed to 1X and NFW is added to come to a suitable volume. The mix is left to linearize Cir-Mediators at room temperature for an hour, after which RNP1 is heat-inactivated at 70 °C for ten minutes.

Method 5: Activation assay

To detect the presence of target, target-specific RNPs are used to cleave reporters. A fluorescent signal indicates RNP activation.

Materials: target DNA; RNP; NEBuffer 2.1 10X; FAM-BHQ ssDNA reporter probe; NFW; 396 well plate; Spectramax fluorescence reader.

Method: To a suitable volume are mixed: target dsDNA at 10nM; RNP at 25nM; reporter at 150 nM. NEBuffer 2.1 10X is added to a 0,1 total dilution and the volume is completed

with NFW. The total mix is pipetted onto a 396 well plate suitable for the Spectramax fluorescence reader.

Method 6: Qubit™ DNA quantification

To quantify the presence of double-stranded DNA, Qubit™ assays give a quick and easy answer.

Materials: dsDNA; Qubit™ dsDNA HS Assay kit; Qubit™ 2.0 Fluorometer.

Method: To a total volume of 200 µL, Qubit™ working fluid and dsDNA sample are mixed so that the theoretical dsDNA amount is within the detection range of the device. After two minutes of room temperature incubation, an assay is ran.

Method 7: General DNA Annealing

Mix an ssDNA sample 1:1 with its complementing DNA. Heat the sample at 95 °C for two minutes, let it cool at a rate of 0.1 °C/s until room temperature is reached.