RCA Engineering Success

Overview

1) **Design 1**: Lambert iGEM hand-built the miRNA-1 padlock. The padlock includes the 5’ and 3’ arms and a linear middle sequence. We decided to test with SYBR™ Safe to measure fluorescence in relation to the amount of rolling circle product (RCP).
2) **Build 1**: Our team arranged the arms and the middle sequence to facilitate the binding of the miRNA and ordered the padlock probe sequence from Integrated DNA Technologies (IDT).
3) **Test 1**: We ran rolling circle amplification (RCA) with the designed padlock probe, added SYBR™ Safe to the reaction, and measured the fluorescence with a plate reader.
4) **Learn 1**: There was no significant increase in fluorescence in the RCA reactions. Our team troubleshooted protocols and our padlock probe design where we realized that a 5’ phosphate modification was not added to the padlock probe, which prevented the ligation step from occurring.
5) **Design 2**: Our team redesigned the middle sequence for the padlock probe: we added a phosphate group to the 5’ end as we found that the SplintR ligase needs that phosphate to successfully ligate.
6) **Build 2**: We arranged the arms and middle sequence to facilitate the binding of miRNA to the padlock and ordered the sequence from IDT.
7) **Test 2**: We ran rolling circle amplification (RCA) with the designed padlock probe, added SYBR™ Safe to the reaction, and measured the fluorescence with a plate reader.
8) **Learn 2**: The readings showed no significant increase in fluorescence in the RCA reactions. However, the tubes containing these reactions appeared cloudy, so

we searched for other, more definite, ways to determine the success of the RCA process.

9) **Design 3**: We modified the mode of measurement from a plate reader fluorescence readout to a gel electrophoresis. Our goal changed from quantification to visualization of the RCP.
10) **Build 3**: We adjusted our protocols to view RCP on a gel and made different percent agarose gels to detect the short sequences of the padlock and miRNA.
11) **Test 3**: We ran RCA with the designed padlock probe, and the products on an agarose gel containing SYBR™ Safe.
12) **Learn 3**: The results of the gel showed a strong fluorescent band extremely close to the wells where RCA reactions were loaded, indicating that a long strand of DNA was formed by RCA.

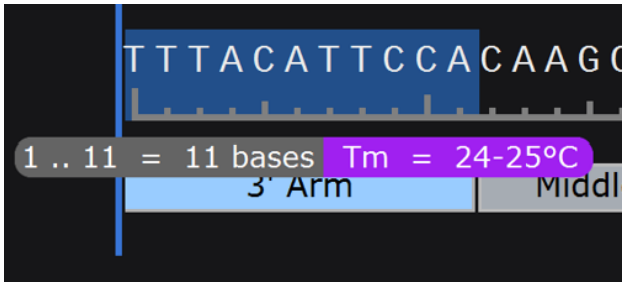
Round 1

Design 1

Lambert iGEM hand-built the initial version of the miRNA-1 padlock which included the 5' (BBa_K4245107) and 3' (BBa_K4245100) arms and a linear middle sequence. Also, we decided that we were going to test with SYBR™ Safe to measure fluorescence in relation to the amount of DNA products present.

For the padlock probe (PLP) design, part of the reverse complement of the microRNA (miRNA) makes up each end of the padlock probe. We determined the annealing temperatures to find where to split the arms. Moreover, to decide whether the reverse complement split properly, we figured out the melting temperatures of each arm through SnapGene (see Fig. 1). To allow successful hybridization of the miRNA and the padlock arms, the arms need to have a similar annealing temperature so that they can bind to the target sequence.

a)



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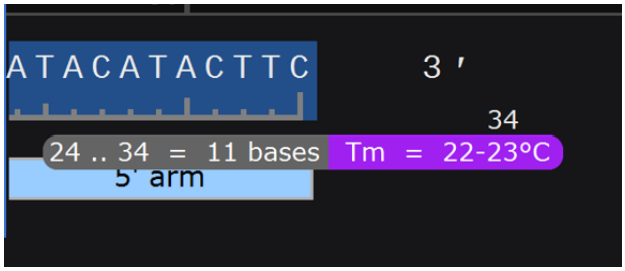


Figure 1. (a) Design and melting temperature of the 3' arm of the padlock probe complementary to half of miRNA-1. (b) Design and melting temperature of the 5' arm of the padlock probe complementary to the other half of miRNA-1. The design of the arms in this way allows for the annealing temperature of the two arms to be the most similar, enabling the success of hybridization.

In addition, we chose a linear DNA sequence from Wang et al.'s "Preparation of Small RNAs Using Rolling Circle Transcription and Site-Specific RNA Disconnection" (2015), to minimize folding within the padlock probe during rolling circle amplification (RCA) (see Fig. 2). The article used Aid-DNA which was a linear DNA sequence; therefore we adapted this sequence as our middle sequence.

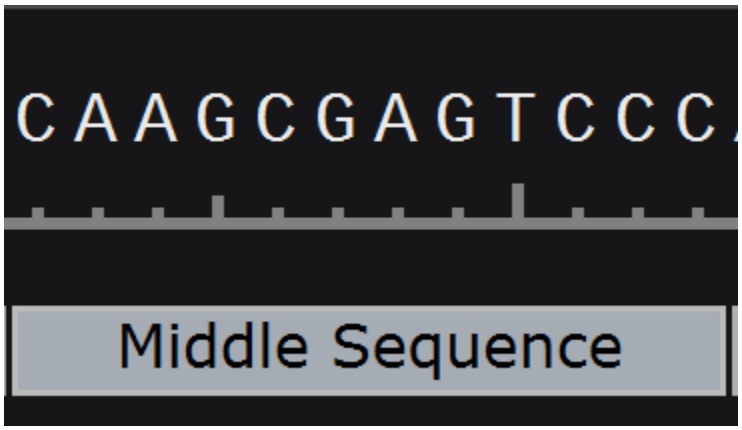


Figure 2. Design of the middle sequence used in the first padlock probe design.

Build 1

Additionally, the alignment of the 5’ and 3’ ends is essential to determine where each part of the padlock arms matches up the target miRNA (Liu et al., 2013). The miRNA strand hybridizes antiparallel to the padlock arms (see Fig. 3). Therefore, the 5’ end of the miRNA will end up overlapping the 5’ PLP arm, and the 3’ end of the miRNA will end up overlapping the 3’ PLP arm (Liu et al., 2013). Therefore, the complete design of the padlock probe includes the 3’ arm, the middle sequence, and the 5’ arm, respectively (see Fig. 4).

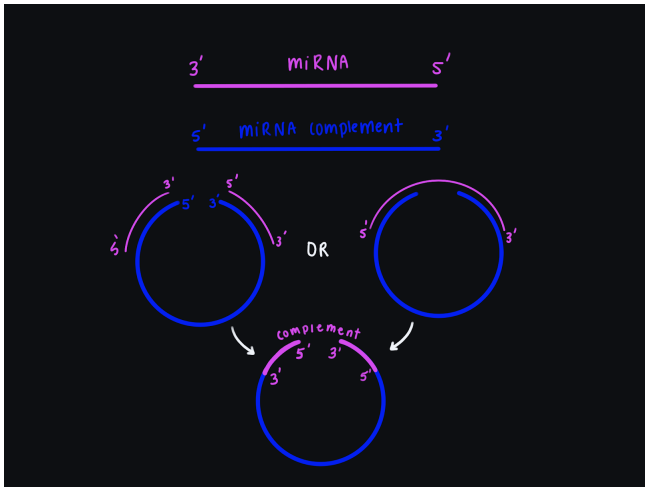


Figure 3. Diagram of padlock arm arrangement.

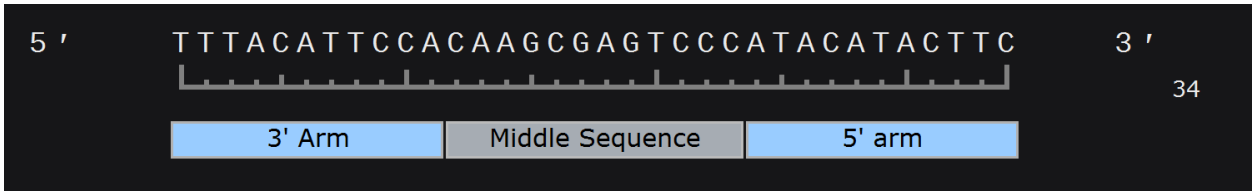


Figure 4. The entire padlock probe sequence that was used.

We then ordered the sequence from IDT so that Lambert iGEM could test with the padlock.

Test 1

Experimental Design

The result of a successful RCA reaction is a long ssDNA composed of repeats of the complement of the padlock probe arm and middle sequence. Since SYBR™ Safe was used as a gel stain that fluoresces if bound to the phosphate backbone of DNA strands, we inferred that directly adding SYBR™ Safe to RCA products would result in fluorescence that could be measured by the plate reader, if a significant amount of RCP was present. Therefore, we added SYBR™ Safe to the RCA products and read the fluorescence readout on the plate reader.

We developed our protocols under the guidance of Jonstrup et al.'s "A microRNA detection system based on padlock probes and rolling circle amplification" (2006) and Fang et al.'s "MiRNA Detection Using a rolling circle amplification and RNA-Cutting Allosteric Deoxyribozyme Dual Signal Amplification Strategy" (2021). Since SplintR is made better for ligation during the DNA-RNA hybridization, we made one change: we used the SplintR ligase instead of the T4 ligase used in the paper (Avantor Staff).

Protocols

1. miRNA - DNA hybridization and Ligation Reaction

1. Make sure all the PCR tubes are RNase free. Label the PCR tubes.
    - Label three PCR tubes A1. Label three PCR tubes A2. Label three PCR Tubes A3. Label three PCR Tubes A4. Label three PCR Tubes A5. Label three PCR Tubes A6. Label three PCR Tubes A7. Label three PCR Tubes A8. Label three PCR Tubes A9. Label three PCR Tubes A10. Pipette 1 µL of 10 x Ligation Buffer (from NEB) into all of the PCR tubes.

2. Add 0.5 µL of 100 nM padlock probe. Add .1 µL of 1000 pM of the miRNA-1. Add 6.9 µL of water.

3. Heat the solution at 65°C for 3 minutes, and slowly cool it to room temperature over 10 minutes using a thermocycler.

4. Then, add 20 U or 0.5 µL of RNase inhibitors.

5. Add 1 µL of Splint R Ligase.

6. Incubate the reaction for 2 hours at 37°C.

7. Bring reaction up to 65°C for 10 minutes to terminate. Additionally, to determine the ideal situations where the miRNA and the padlock do not detach, bring up the reaction to the gradient temperatures for 10 minutes as well as 20 minutes to determine the ideal temperature.

- Each of the A1- A5 tubes are on the gradient for 10 minutes. Each of the A6-A10 tubes are on the gradient for 20 minutes.

2. RCA Reaction (Reaction volume will be 25 µL so add 0.5 µL of water)

1. Pipette 2.5 µL of 10x phi29 buffer (from NEB) into previous ligation reaction mixture.

2. Add 12.5 units or 1.25 µL of phi29 DNA polymerase.

3. Add 6.75 ul of 10 mM dNTPs.

4. Incubate the reactions for 8 hours at 37°C.

5. Bring up the reactions to 65°C for 10 minutes to inactivate the DNA polymerase.

3. Readout

1. The readout reaction utilizes SYBR™ Safe: use a 5:1 ratio of SYBR™ Safe, the reaction volume.

2. Run all the reactions through the plate reader to obtain fluorescence values.

Additionally, we ran three triplicates of controls: a triplicate with just the padlock probes, a triplicate with just the miRNA, and a triplicate with the padlock and the miRNA. To analyze the results, we added SYBR™ Safe to all the samples, and read the results in a plate reader.

Results

We found two samples from two separate replicates that showed some improvement over our controls (see Fig. 5). The plate reader's readout from sample A6's readout was 9899.64 which showed results that showed a significant increase from our controls which averaged 103.39 for the control with both miR and padlock. However, due to inconsistency, these results showed that either our padlock likely did not produce RCP or the SYBR™ Safe detection mechanism did not succeed.

Sample	Trial 1	Trial 2	Trial 3
1	5864.642857	7073.642857	5119.642857
2	2861.642857	5927.642857	
3	6344.642857	5937.642857	
4	4972.642857	5348.642857	1696.642857
5	4142.642857	3313.642857	4198.642857
6	9899.642857	6683.642857	
7	3436.642857	5276.642857	
Control: pad	4177.642857	4332.642857	5840.642857
Control: miR	4944.642857	7241.642857	
Control: pad+miR	9636.642857	9261.642857	

Figure 5. The table above shows the plate reader's fluorescence reading after the addition of SYBR™ Safe to the RCA products. Samples 1-8 were all RCA reactions run on a temperature gradient to test the temperature at which RCA would work best. The controls consisted of the same concentration of miRNA and/or padlock as the RCA reactions in the same buffer solution, but without any other reagents. Due to sample evaporation, we could not read some trials.

Learn 1

Potential Problem	Solution
RCA reaction did not work.	To ensure that the RCA protocol was not the problem, we re-calculated and confirmed all enzyme concentrations and incubation temperatures and found that the RCA protocol was not problematic. Therefore,we considered the design of the padlock probe. We realized that the padlock probe did not contain a 5' phosphate group, which is necessary for DNA to be ligated by an enzyme (Liu et al., 2013). Therefore, we re-ordered the padlock probe with a 5' phosphate group.
SYBR™ Safe did not bind the RCP properly.	This is also a possible problem: the concentrations of the SYBR™ Safe and the binding of the SYBR™ Safe to the phosphate groups may have had issues. However, Lambert iGEM targeted one variable at a time to ensure that we have identified the problem correctly.

Round 2

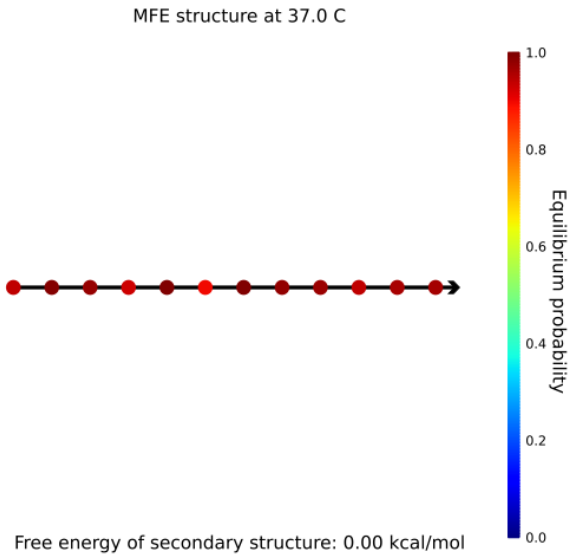
Design 2

The middle sequence was redesigned (BBa_K4245131) so that it could be used with potential future reporting mechanisms involving FAM (BBa_K4245130) and BHQ1

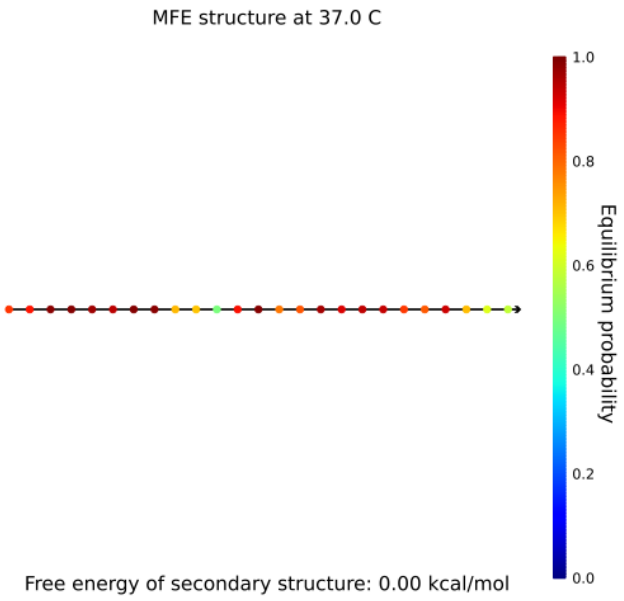
tagged DNA Linear Probe (BBa_K4245132) or the split lettuce DNA aptamer (left: BBa_K4245134/right: BBa_K4245135) (see Fig. 6).

Figure 6. Design of the middle sequence used in the second padlock probe design.

We initially sought to quantify our miRNA concentration, but unfortunately, SYBR™ Safe did not provide us a way to do that as it was a visualization mechanism. Therefore, we switched the reporting mechanism. The padlock's structure would not have changed much because both the previous middle sequence and the new one are both linear. As a result, this structure would not cause any additional folding (see Fig. 7). Additionally, we added a phosphate group to the 5' end of the new padlock probe since we found that SplintR ligase needs that phosphate to successfully ligate (Liu et al., 2013).



a)



b)

Figure 7. (a) Visualization of the Aid-DNA sequence through NuPack by CalTech (b) Visualization of the new version of the middle sequence through NuPack.

Build 2

We used a build process similar to the first build process to properly arrange the padlock probe arms and middle sequence. The miRNA-1 padlock probe (BBa_K4245200) was then ordered from Integrated DNA Technologies (IDT).

Test 2

Experimental Design

We used the same protocols from the last test step with only one change: the temperature gradient used in the last protocols was taken out, so the deactivation step was run at 65°C to reduce variance between samples.

Results

There was no significant increase in fluorescence when SYBR™ Safe was added to the RCA products as compared to the controls. However, the tubes of RCA reaction

appeared cloudy compared to the controls, suggesting that RCA could have been successful. Therefore, it was likely that the issue was using SYBR™ Safe as the mode of measurement.

Learn 2

Potential Problem	Solution
RCA did not work.	To ensure that the RCA protocol was not the problem, we re-calculated and confirmed all enzyme concentrations and incubation temperatures. We found that the RCA protocol was not problematic, so we then considered the design of the padlock probe. However, the design of the padlock probe should not have been a problem since the 5' phosphate end mistake was corrected. For the next round of experimentation, we decided to leave the RCA variable alone and isolate the SYBR™ Safe readout variable to make sure that we have identified the problem correctly.
SYBR™ Safe did not bind the RCP properly.	The concentrations of the SYBR™ Safe or the binding of the SYBR™ Safe to the phosphate groups may have had issues. Therefore, we double-checked the concentration which matched the given concentration by our supplier— New England Biolabs. Additionally, since the reaction appeared cloudy as compared to our controls, there is a likely possibility that RCA was successful. Therefore, to more clearly visualize our RCP (if one was, in fact, produced), we ran the reaction on a gel to visualize the DNA strand.

Round 3

Design 3

The use of SYBR™ Safe as a quantification method was not successful; therefore, we aimed to first find a way to visualize a long DNA product that formed as a result of RCA. We modified the mode of measurement from a plate reader fluorescence reading to a gel electrophoresis and adjusted our protocols to include the additional gel electrophoresis process..

Build 3

Our team aimed to test our reactions on different percent gels in order to view the short strands of the miRNA and padlock on our gel: we used the standard 1% agarose gel, but additionally included a 3% and 4% gel to better visualize the shorter strands of DNA and miRNA present in the controls. In addition, we adjusted our protocols to include the output mechanism of gel electrophoresis.

Test 3

Experimental Design

We ran RCA with the same protocols used in the previous round of DBTL, but changed the readout reaction. Since we saw some cloudiness in the previous sample, there was a possibility that RCA worked, so we then tested the reaction without quantification and changed the readout reaction to better reflect the procedures of running DNA on an agarose gel. Our team made 1%, 3%, and 4% agarose gels which were then loaded with either 10 µL of the control or 10 µL of the reaction with 2µL of loading dye.

Results

By analyzing the results on the gel, our team concluded that a very long strand of DNA, likely the RCP, was produced. A fluorescent band of DNA was present, very close to the well which indicates that a long strand of DNA (greater than 1 kB) was produced due to our reaction. Since the gel shows a bright fluorescent band near the well, we determined that the RCA reaction allowed the creation of a really long DNA stand—our RCP (see Fig. 8).

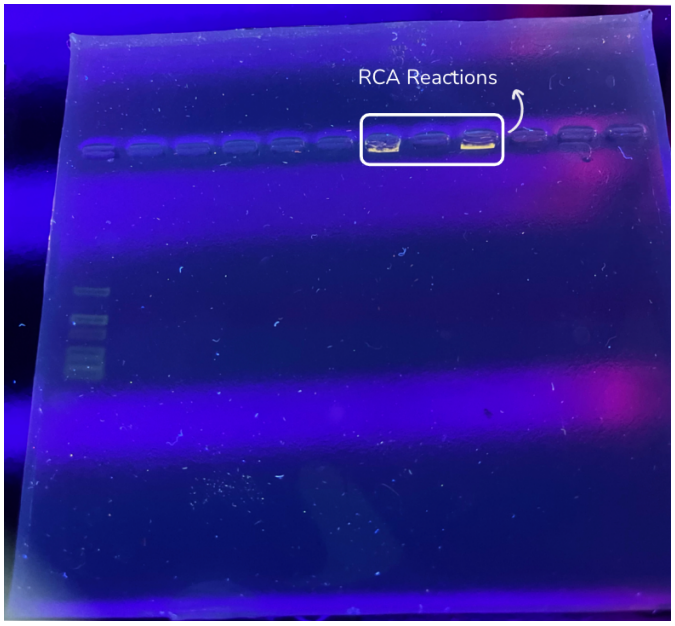


Figure 8. The gel above is a 1% gel showing a long strand present near the well of a gel showing that a very large strand of DNA was present.

However, we could not visualize the controls on any of the three gel types. Therefore, it was likely that the padlock sequences and the miRNA concentration were not high enough to be shown on a gel. In contrast, with RCA, it was possible for us to increase the concentration of the output, allowing the product to be visible on the gel.

Learn 3

The results of the gel showed a strong fluorescent band extremely close to the wells where we loaded the RCA reactions, indicating that an extremely long strand of DNA

was formed by RCA. Therefore, we concluded that RCP was successfully being produced. However, we needed to determine a method to quantify these results.

Lettuce

1) **Design 4**: To make Point of Care (POC) feasible, quantification of miRNA is required, which is not possible via gel electrophoresis. Therefore, our team decided to switch to using the split lettuce design in order to better quantify miRNA concentration.
2) **Build 4**: We derived the split Lettuce sequence by splitting the Lettuce DNA fluorogenic aptamer into left and right components. Using the same padlock sequence, our team designed the flanking sequences of the split lettuce, ordered as DNA oligos from IDT.
3) **Test 4**: We ran RCA with the miRNA-1 padlock with the reporter mechanism (the split lettuce and the DFHBI-1T dye) and measured the resulting fluorescence with the plate reader.
4) **Learn 4**: The increase in fluorescence after the split lettuce and dye were added was not significant. Our team determined that the binding of the left split lettuce sequence was inefficient.
5) **Design 5**: We decided to modify the lettuce left design to improve binding efficiency of the split lettuce. Additionally, we decided to test with a sequence simulant of the middle sequence of the RCP rather than the actual RCP to mitigate confounding variables.
6) **Build 5**: The lettuce right sequence remained unchanged. However, an extra guanine nucleotide was added at the left end of lettuce to increase binding efficiency. These sequences were ordered from IDT.
7) **Test 5**: We combined the new version of the split Lettuce aptamer, the “simulated RCP”, and DFHBI-1T and measured the resulting fluorescence output using the plate reader.
8) **Learn 5**: The increase in fluorescence after the split lettuce and dye were added was significant. We determined that the new Lettuce left design was capable of

binding efficiently to a “simulated RCP” and thus needed to be experimented with RCP from our RCA reactions.

9) **Design 6**: We wanted to confirm the ability of split Lettuce to bind to the RCP. Therefore, we adjusted our protocols to test the split lettuce reporter with RCP.
10) **Build 6**: We used the miRNA-1 padlock and split lettuce with the RCP and adjusted the protocols to test with RCP.
11) **Test 6**: We ran RCA with the same protocols as used in the previous rounds, tested the split Lettuce components, RCP, and DFHBI-1T, and measured the resulting fluorescence on the plate reader.
12) **Learn 6**: The increase in fluorescence after the split lettuce and dye were added was significant. We determined that the new Lettuce left design was capable of binding RCP efficiently, thus providing a mechanism through which the resulting fluorescence can be correlated to miRNA concentration.
13) **Future:** In the future, this biosensing and reporting mechanism will be implemented conjunctly to quantify miRNA extracted from blood serum to accurately screen for coronary artery disease.

Round 4a

Design 4a

While running an agarose gel proved the successful and significant production of RCP, we decided to switch to using FAM (BBa_K4245130) and BHQ1 tagged DNA Linear Probe (BBa_K4245132) or the split lettuce DNA aptamer (left: BBa_K4245136/right: BBa_K4245135) to quantify our results. This engineering success cycle focuses on the split lettuce aptamer. To make Lambert iGEM’s POC goal successful, we needed to quantify the miRNA, a method not possible using gel electrophoresis.

We used the same padlock probe design from the previous round, and designed the flanking sequences of the split lettuce probes to be complementary to the RCP (see Fig. 9).

When the two sides of the split lettuce come together, the DFHBI-1T dye binds into the secondary structures of the lettuce aptamer, allowing the dye to fluoresce (see Fig. 9) (VarnBuhler et al., 2022).

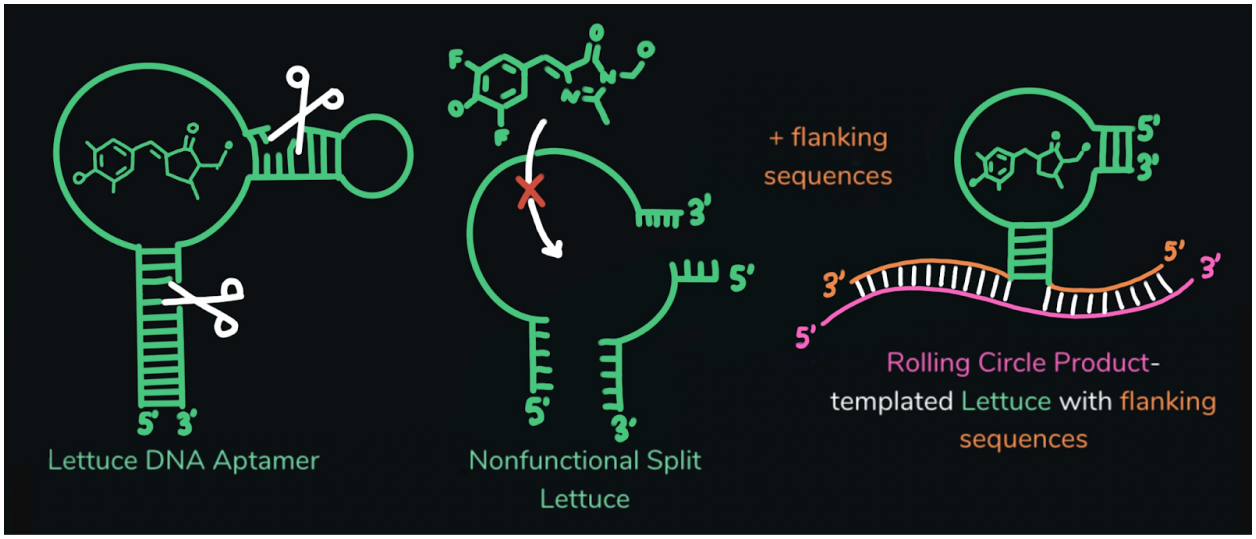


Figure 9. Diagram of the split lettuce mechanism (VarnBuhler et al., 2022).

Build 4a

We used the same miRNA-1 padlock probe (BBa_K4245200) and adopted the split lettuce sequences from the article, “Detection of SARS-CoV-2 RNA Using a DNA Aptamer Mimic of Green Fluorescent Protein” (VarnBuhler et al., 2022). The flanking sequences that were used were made to match the middle sequence of our previous padlock. The resulting probes were :

Lettuce Left: TAGTAGGGATGATGCGGCAGTGGGCTTCATCTAACCTCTTTCA
Lettuce Right: GCTTCCATCTGCAGATGAGGGGACTA

We then ordered these sequences with IDT so that they can be tested with RCA.

Test 4a

Experimental Design

We ran RCA with the same protocols used in the previous rounds of DBTL, but changed the readout reaction. Instead of using a gel readout, we added split lettuce. These are the following protocols:

1. In nuclease-free PCR tubes, add the following triplicates:

Dye Control (Background)	- 25 µL RNase-free water - 1 µL 10X Splint R Ligase Buffer - 2.5 µL 10X phi29 Reaction Buffer
Lettuce Left + Lettuce Right + Dye	- 19 µL RNase-free water - 1 µL 10X Splint R Ligase Buffer - 2.5 µL 10X phi29 Reaction Buffer - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock)
RCP + Lettuce Left + Lettuce Right + Dye	- 19.5 µL RNase-free water - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock) - 3 µL RCP
Padlock + miRNA + Lettuce Left + Lettuce Right + Dye	- 19.5 µL RNase-free water - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock) - 3 µL Control: Padlock + miRNA

2. Centrifuge the reagents.

3. On the sides of the centrifuged PCR tubes, carefully add 1.5 µL of DFHBI-1T (100 µM stock)

- For controls without the dye, we repeated steps 1-3 with 1.5 µL RNase-free water being added in place of the dye.

4. Vortex and centrifuge reagents.

5. Incubate the reaction at 70°C for 5 minutes, then cool to and incubate at 41°C for 1 hour.

6. Read fluorescence on the plate reader (the emission spectra is 528 nm, and the excitation spectra is 480 nm).

Results

If the split Lettuce sequences were able to successfully bind to the RCP, the change in fluorescence (from before the dye DFHBI-1T to after the dye was added) of the RCP-lettuce-dye reaction should have shown a greater increase than controls. However, the change in fluorescence for these three is lower from the change in fluorescence of the controls (see Fig. 10).

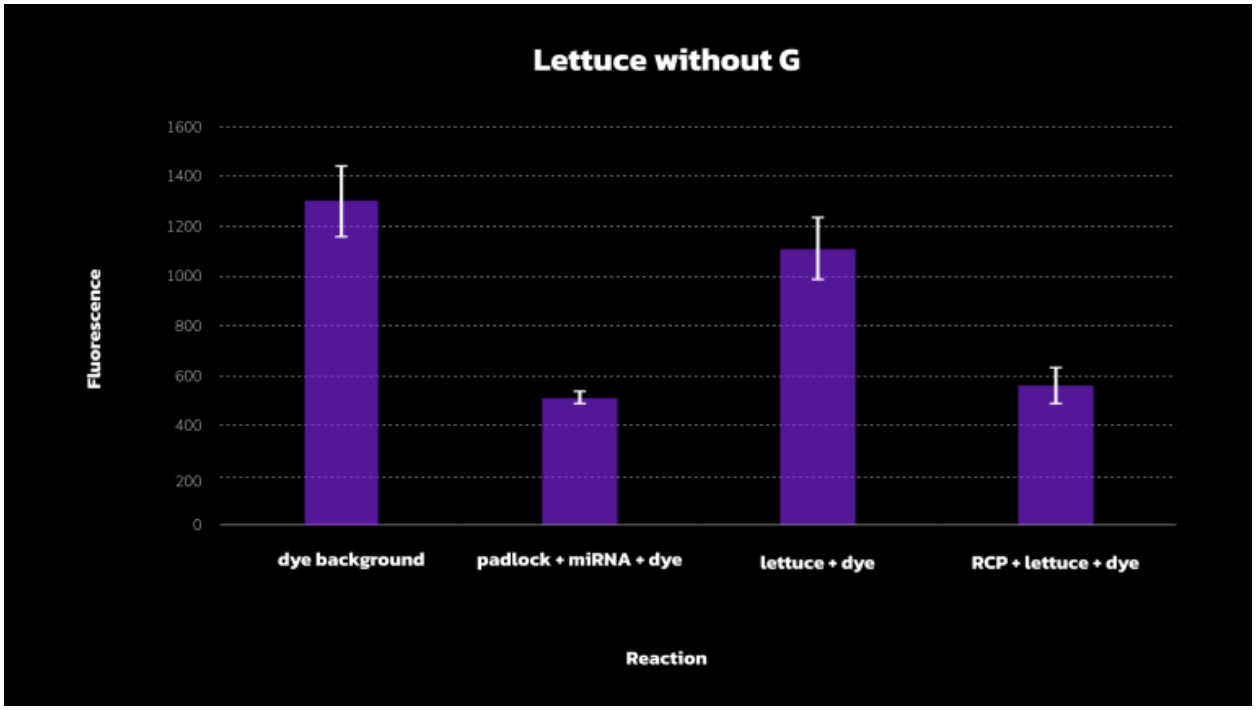


Figure 10. Graph of change in fluorescence after the dye was added to the controls and reaction. The fluorescence of the dye background reaction as well as the lettuce + dye reaction increased significantly greater than the reaction with RCP, lettuce, and dye.

Learn 4a

The increase in fluorescence after the split lettuce and dye were added was not significant.

Potential Problem	Solution
Binding efficiency of the lettuce aptamer is low.	One issue that could have inhibited the success of lettuce was the binding efficiency of the sequence. The lettuce sequence originally had an A-T bond at the end instead of a C-G bond. Therefore, the amount of lettuce aptamer that successfully bound could have ended up being lower. Therefore, our team ended up adding the guanine nucleotide in order to improve binding efficiency.
RCP has internal folding or damage.	The RCP could also have broken down. Since the RCP is ssDNA that is over 1 kB long, it is very unstable; therefore,

	external factors could have degraded or damaged it. As a result, we decided to use a simulated RCP by ordering just the middle sequences from IDT. By testing with these middle sequences we got rid of the RCP variable.

Round 5a

Design 5a

We decided to add a guanine-cytosine bond to the flanking sequence of the Lettuce left sequence to increase binding efficiency. Additionally, we used a simulated RCP sequence, so any damage done to the RCP did not affect the results of lettuce binding.

Build 5a

We did not modify the lettuce right sequence (BBa_K4245135), but added an extra guanine nucleotide to the flanking sequence of the Lettuce left sequence (BBa_K4245134), ordered as a DNA oligo from IDT. Additionally, we ordered the middle sequence of the RCP as a DNA oligo from IDT.

Modified Lettuce Left: TAGTAGGGATGATGCGGCAGTGGGCTTCATCTAACCTCTTTCAG
Lettuce Right: TGAAAGAGGTTGGCAGATGGAAGC

Test 5a

Experimental Design

We tested the new version of the split Lettuce aptamer, the middle sequence of the RCP, and DFHBI-1T and measured the fluorescence readout with the plate reader. These are the following protocols we used:

1. In nuclease-free PCR tubes add the following triplicates:
    1. In nuclease-free PCR tubes add the following triplicates:

Dye Control (Background)	- 25 µL water - 2.5 µL phi29 DNA polymerase buffer - 1 µL SplintR Ligase buffer
Lettuce Left + Lettuce Right + Dye	- 19 µL RNase-free water - 1 µL 10X Splint R Ligase Buffer - 2.5 µL 10X phi29 Reaction Buffer - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock)
Complement + Lettuce Left + Lettuce Right + Dye	- 17.5 µL RNase-free water - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock) - 1.5 µL complement - 1 µL 10X Splint R Ligase Buffer - 2.5 µL 10X phi29 Reaction Buffer

2. Centrifuge the reagents.

3. On the sides of the centrifuged PCR tubes, carefully add 1.5 µL of DFHBI-1T (100 µM stock)
    - For controls without the dye, we repeated steps 1-3 with 1.5 µL RNase-free water being added in place of the dye.

4. Vortex and centrifuge reagents.

5. Incubate the reaction at 70°C for 5 minutes, then cool to and incubate at 41°C for 1 hour.

6. Read fluorescence on the plate reader (the emission spectra is 528 nm, and the excitation spectra is 480 nm).

Results

From these results, we saw an increase in fluorescence with the presence of simulated RCP and lettuce in the reaction as compared to the controls (see Fig. 11). This shows that the lettuce aptamer hybridized to the RCP and the dye, causing fluorescence.

We observed a significant decrease in fluorescence in the lettuce reaction tube when compared to the dye background (see Fig. 11). This decrease is most likely due to the opaqueness of the lettuce DNA sequences. In contrast, the lettuce with complement reaction greatly increased in fluorescence when compared to the controls.

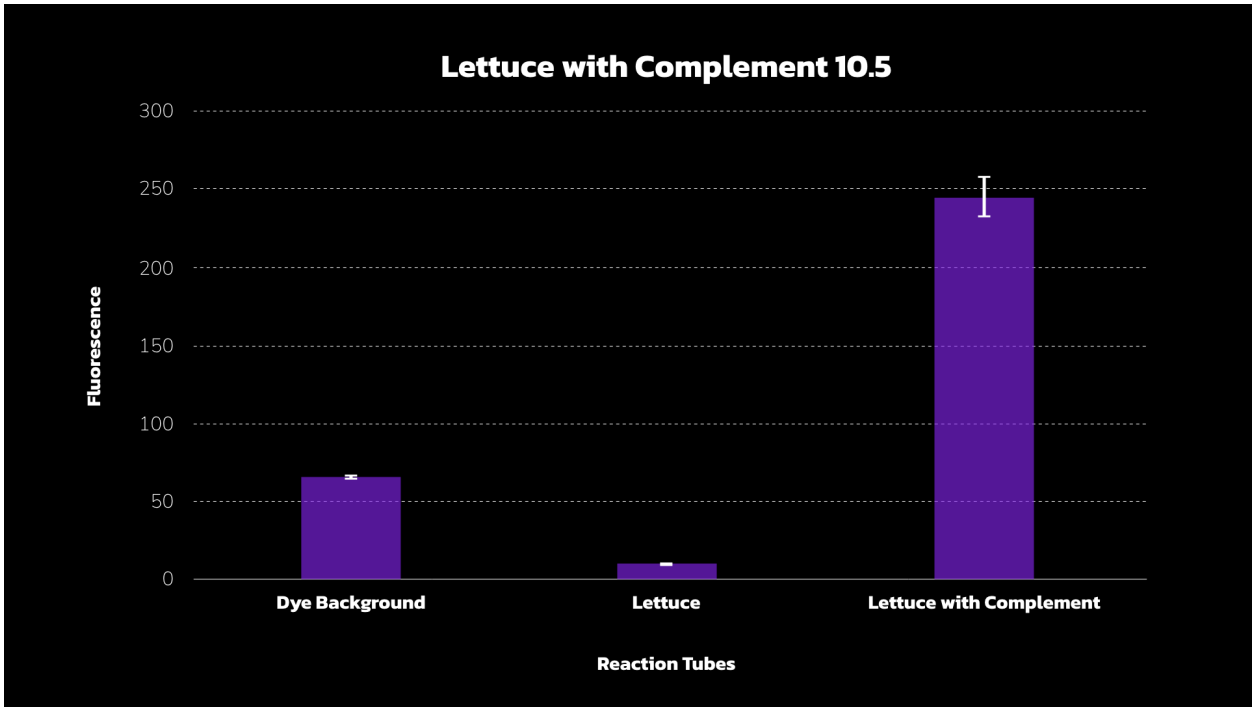


Figure 11. Lettuce with Complement Results change in fluorescence.

Learn 5a

The increase in fluorescence after the split lettuce and dye were added was significant. This suggests that the modified Lettuce left (BBa_K4245134) and Lettuce right (BBa_K4245135) sequences bounded efficiently to the middle sequence complement and trapped the DFHBI-1T dye. We determined that the new Lettuce left design needed to be experimented with RCP.

Round 6a

Design 6a

Now that we isolated and fixed the issue of the binding efficiency of the split Lettuce sequences (left: BBa_K4245134/right: BBa_K4245135), we wanted to confirm if the split Lettuce could effectively bind to the RCP. More specifically, we wanted to find out if the long length and potential folding of the RCP itself or any damage done to the RCP would affect our split Lettuce reporter mechanism.

Build 6a

We used the miRNA-1 padlock (BBa_K4245200) and the split Lettuce components from the previous round and used the same RCA protocols. Additionally, we used the same split Lettuce protocols used in previous rounds, but changed to reflect the use of RCP in place of the simulated RCP.

Test 6a

Experimental Design

We ran RCA with the same protocols as used in the previous rounds: we combined the split Lettuce components, RCP, and DFHBI-1T and measured the resulting fluorescence on the plate reader.

The protocols followed are below.

1. In nuclease-free PCR tubes add the following triplicates:

Dye Control (Background)	- 25 µL RNase-free water - 1 µL 10X Splint R Ligase Buffer - 2.5 µL 10X phi29 Reaction Buffer
Lettuce Left + Lettuce Right + Dye	- 19 µL RNase-free water - 1 µL 10X Splint R Ligase Buffer - 2.5 µL 10X phi29 Reaction Buffer - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock)
RCP + Lettuce Left + Lettuce Right + Dye	- 19.5 µL RNase-free water - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock) - 3 µL RCP
Padlock + miRNA + Lettuce Left + Lettuce Right + Dye	- 19.5 µL RNase-free water - 3 µL Split Lettuce Left Sequence (100 uM stock) - 3 µL Split Lettuce Right Sequence (100 uM stock) - 3 µL Control: Padlock + miRNA

2. Centrifuge the reagents.

3. On the sides of the centrifuged PCR tubes, carefully add 1.5 µL of DFHBI-1T (100 µM stock)

- For controls without the dye, we repeated steps 1-3 with 1.5 µL RNase-free water being added in place of the dye.

4. Vortex and centrifuge reagents.

5. Incubate the reaction at 70°C for 5 minutes, then cool to and incubate at 41°C for 1 hour.

6. Read fluorescence on the plate reader (the emission spectra is 528 nm, and the excitation spectra is 480 nm).

Results

The increase in fluorescence of the RCP + Lettuce + dye was significantly greater than the controls, which suggests that the split Lettuce was successfully bound to the RCP (see Fig. 12). In addition, the DFHBI-1T was also successfully bound within the split lettuce secondary folding. According to these results, RCA and the reaction between the split lettuce and RCP was successful.

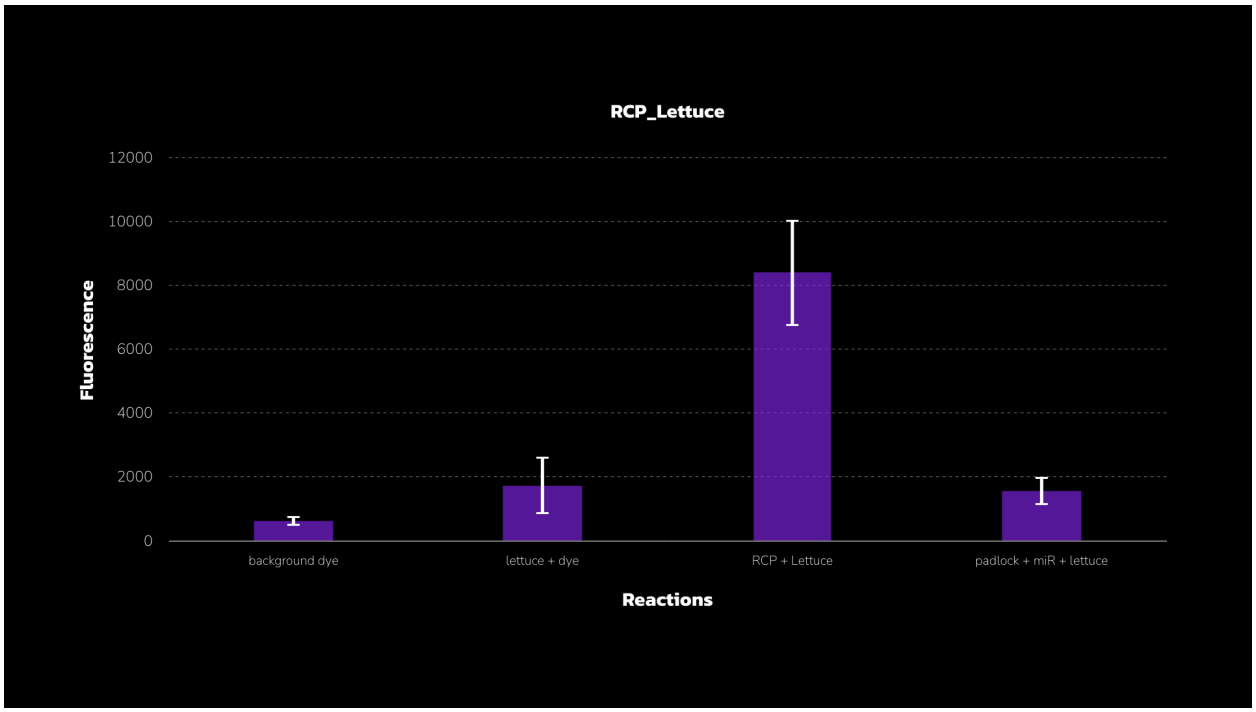


Figure 12. Graph of split lettuce with RCP results.

Learn 6a

We determined that the new Lettuce left design was capable of binding to RCP efficiently, providing a mechanism through which we could correlate the resulting fluorescence to the miRNA concentration.

Future

From the experimental results, We found that the split Lettuce aptamer works successfully as a reporter mechanism to measure rolling circle products. We plan to test and characterize the outputs of various miRNA concentrations through rolling circle amplification in conjunction with split Lettuce. Developing characterization data on rolling circle amplification will allow us to relate the output value of our reporting mechanisms to specific concentrations of miRNA, providing an additional point-of-care screening method for coronary artery disease.

Linear Probes Engineering Success

1. **Design 4b:** Lambert iGEM adopted FAM, and BHQ-1 Dye Tagged Linear DNA Probes in order to quantify RCP. We modified these probes to remove a guanine at the end to make this process relatively isothermal. We hand-built miRNA-1-3p padlock. These padlocks include the 5' and 3' arms and a linear middle sequence.
2. **Build 4b:** Our team designed these linear probes and padlock probes in SnapGene ordered the sequences from Integrated DNA Technologies (IDT). Protocols were taken from pre-existing research.
3. **Test 4b:** We ran RCA with the miRNA-1 padlock with the reporter mechanism (FAM/BHQ-1 Tagged Linear DNA Probes) and measured the resulting fluorescence with the plate reader.
4. **Learn 4b:** We expected to see a decrease in the fluorescence in our reaction compared to our FAM tagged linear probe control. We saw no such decrease. Our team troubleshooted the protocols and hypothesized that it is likely that our probes are not effectively binding.
5. **Design 5b:** We decided to modify the design of the FAM tagged linear DNA probe to improve binding efficiency to the RCP/middle sequence complement. Additionally, we decided to test with a sequence complement of the middle sequence of the RCP rather than the actual RCP to mitigate confounding variables.
6. **Build 5b:** The BHQ-1 tagged linear probe remained unchanged. However, an extra guanine nucleotide was added to FAM dye tagged linear DNA probe to increase binding efficiency. These sequences were ordered from IDT.
7. **Test 5b:** We combined the new version of the linear probes, the "simulated RCP", and measured the resulting fluorescence output using the plate reader.
8. **Learn 5b:** We observed a decrease in the fluorescence in our reaction compared to our FAM tagged probe control. To ensure that this principle could be applied to the product of our biosensor, we aimed to retest these probes on our (RCP).
9. **Design 6b:** We wanted to confirm the applicability of these linear DNA probes as a means of reporting our RCP.
10. **Build 6b:** We created RCP through the rolling circle amplification (RCA) process and reused the same probes as the previous step.

11. **Test 6b:** We combined the new version of the linear probes, the RCP and measured the resulting fluorescence output using the plate reader.
12. **Learn 6b:** The decrease in fluorescence after adding linear DNA probes was significant. We determined that the linear DNA probes were capable of binding RCP efficiently, thus providing a mechanism through which the resulting fluorescence can be correlated to miRNA concentration.

Round 4b

Design 4b

Even though gel electrophoresis showed the successful and significant production of RCP, we decided to switch to using FAM (BBa_K4245130) and BHQ1 tagged DNA Linear Probe (BBa_K4245132) and the split lettuce DNA aptamer (left: BBa_K4245134/ right: BBa_K4245135) to quantify our results. We focused on the quantification of the linear probes during this engineering success cycle.

We used the same padlock probe design from the previous round and adopted the linear probes sequences from previous research (Zhou et al., 2015).

To quantify the presence and concentration of target miRNA in the sample, Lambert iGEM decided to utilize fluorophore and quencher-tagged linear DNA probes. Each probe contains part of the complement to the middle sequence of the RCP. One probe is tagged with a fluorophore, and one probe is tagged with a quencher. Once the quencher- and fluorophore-tagged linear probes bind to the RCP, the quencher quenches the fluorophore, effectively shutting off the fluorescent signal (see Fig. 13).

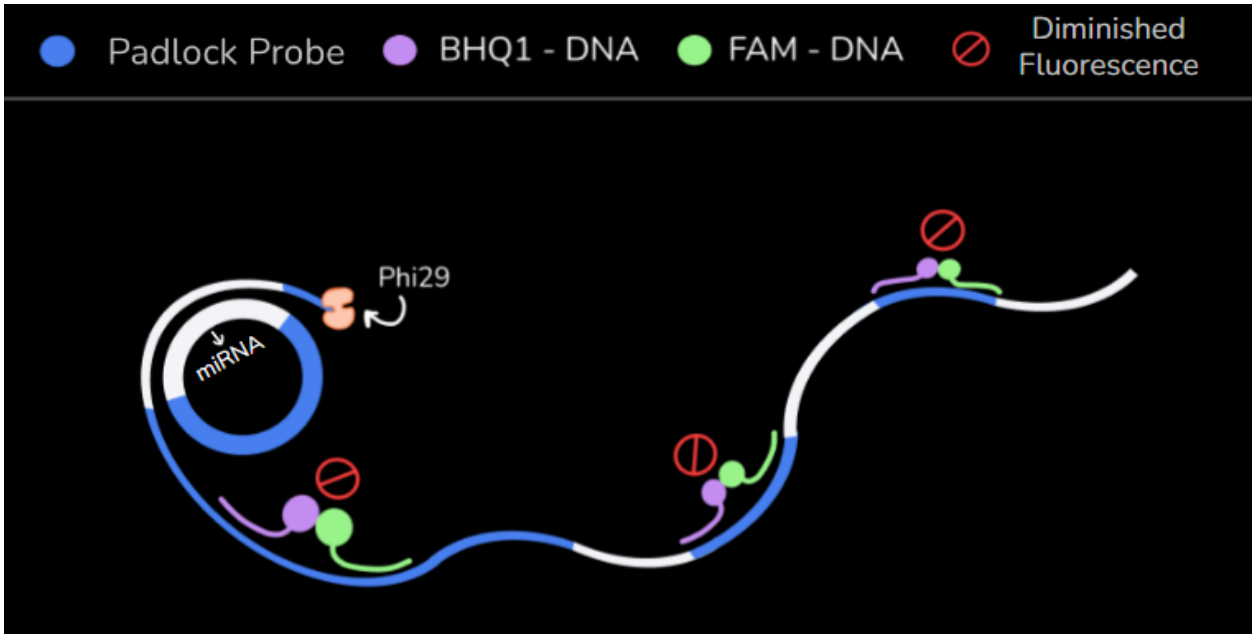


Figure 13. Diagram of the linear probe mechanism (Zhou et al., 2015)

We made modifications to the FAM dye tagged linear DNA probe created by labs at Shaanxi Normal University to create a more isothermal process. This modification entailed removing a guanine from the end of the FAM dye tagged linear DNA Probe.

Build 4b

We used the miRNA-1 padlock probe (BBa_K4245200) and adopted the linear probes from the Key Laboratory of Analytical Chemistry for Life Science of Shaanxi Normal University (Zhou et al., 2015).

We made modifications to the FAM dye tagged linear DNA probe to remove a guanine from the end of the FAM probe. This is reflected by the red strikeout below.

Modified FAM Dye Tagged Linear DNA Probe: 5'-FAM-CAA CCT CTT TCA ~~G~~-3'

BHQ-1 Tagged Linear DNA Probe: 5'-GCT TCC ATC TGC-BHQ1-3'

We then ordered these sequences from IDT so that they can be tested with the rolling circle product (RCP) produced by the miRNA-1 padlock probe (BBa_K4245200).

Test 4b

Experimental Design:

We ran RCA with the same protocols as used in the previous rounds: we combined the FAM/BHQ-1 Probes and linear probe complement and measured the resulting fluorescence on the plate reader.

The protocols followed are below.

1. Add the following to an amber microcentrifuge tube to make FAM-Probe Mastermix:

FAM Dye Tagged Linear DNA Probe Mastermix	- Volume of FAM Dye Tagged Linear DNA Probe: (# of reactions) x (1.6 µL FAM Dye Tagged Linear DNA Probe) x 1.1 - Volume of TE: (# of reactions) x (29 µL TE) x 1.1

2. Add the following to a PCR tube:

Tris-EDTA Buffer (Background)	- 34 µL TE Buffer
FAM Dye Tagged Linear DNA Probe + TE	- 29.4 µL Mastermix - 4.6 µL TE Buffer
BHQ-1 Quencher Tagged Linear DNA Probe + TE	- 1.6 µL BHQ-1 Quencher Tagged Probe - 32.4 µL TE Buffer
FAM Dye Tagged Linear DNA Probe + BHQ-1 Quencher Tagged Linear DNA Probe + RCP + TE	- 29.4 µL Mastermix - 1.6 µL BHQ-1 Quencher Tagged Probe - 3 µL RCP

3. Vortex several seconds and spin down tubes.

4. Place tubes in the thermocycler at 41°C for 1 minute.
5. Place tubes in thermocycler 37°C for 1 minute.
6. Pipette all 32 µL of solution into a 384 well plate to measure fluorescence at excitation wavelength of 480 nm and emission intensity at 518 nm using a plate reader.

Results:

The results display no significant decrease in the fluorescence intensity of a triplicate with FAM Probe, BHQ Probe, and Linear Probe Complement as compared to a triplicate of just FAM tagged Probes (see Fig. 14). We are expecting to see a significant decrease between these two trials, but saw no decrease.

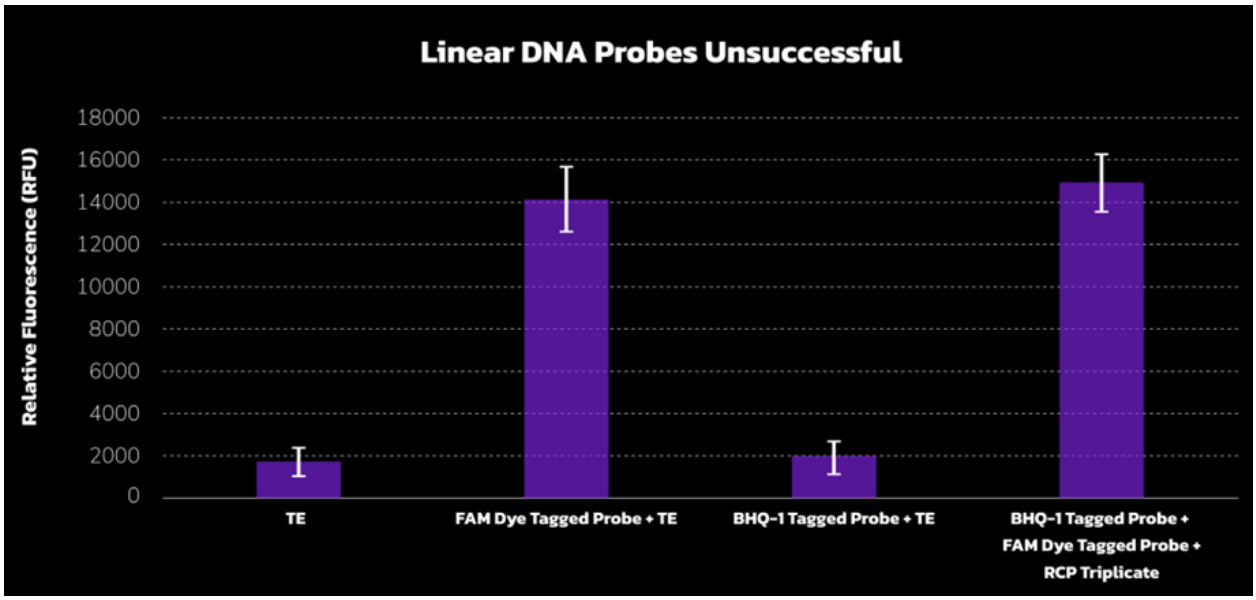


Figure 14. Initial linear DNA Probe failure with removed guanine

Learn 4b

The increase in fluorescence after the split lettuce and dye were added was not significant.

Potential Problem	Solution
Binding efficiency of the lettuce aptamer is low.	One issue that could have inhibited the success of lettuce was the binding efficiency of the sequence. The linear probes sequence originally had an A-T bond at the end instead of a C-G bond. Therefore, the amount of linear probes that successfully bound could have ended up being lower. Therefore, our team ended up adding the guanine nucleotide in order to improve binding efficiency.
RCP has internal folding or damage.	The RCP could also have broken down. Since the RCP is ssDNA that is over 1 kB long, it is very unstable; therefore, external factors could have degraded or damaged it. As a result, we decided to use a simulated RCP by ordering just the middle sequences from IDT. By testing with these middle sequences we got rid of the RCP variable.

Round 5b

Design 5b

We decided to read a guanine-cytosine bond to the 3’ end of the 6-carboxyl-fluorescein (FAM) Labeled DNA Probe sequence to increase binding efficiency with the RCP. Additionally, we used the complement to the linear DNA probe sequence to effectively isolate this section of the experimentation process.

Build 5b

We did not modify the Black Hole Quencher 1 (BHQ1) DNA Probe sequence (BBa_K4245132), but we added an extra guanine nucleotide to the 3' end of the 6-carboxyl-fluorescein (FAM) Labeled DNA Probe sequence, ordered as a DNA oligo from IDT. Additionally, we ordered the middle sequence of the RCP as a DNA oligo from IDT.

FAM Tagged Linear Probe (BBa_K4245130): 5'-FAM-CAA CCT CTT TCA G-3'

BHQ-1 Tagged Linear Probe (BBa_K4245132) : 5'-GCT TCC ATC TGC-BHQ1-3'

Test 5b

Experimental Design:

We ran RCA with the same protocols as used in the previous rounds: we combined the FAM/BHQ-1 Probes and RCP and measured the resulting fluorescence on the plate reader.

The protocols followed are below.

1. Add the following to an amber microcentrifuge tube to make FAM-Probe Mastermix:

FAM Dye Tagged Linear DNA Probe Mastermix	Volume of FAM Dye Tagged Linear DNA Probe: (# of reactions) x (1.6 µL FAM Dye Tagged Linear DNA Probe) x 1.1 Volume of TE: (# of reactions) x (29 µL TE) x 1.1

2. Add the following to a PCR tube:

Tris-EDTA Buffer (Background)	34 µL TE Buffer
FAM Dye Tagged Linear DNA Probe + TE	29.4 µL Mastermix 4.6 µL TE Buffer
BHQ-1 Quencher Tagged Linear DNA Probe + TE	1.6 µL BHQ-1 Quencher Tagged Probe 32.4 µL TE Buffer
FAM Dye Tagged Linear DNA Probe + BHQ-1 Quencher Tagged Linear DNA Probe + Linear Probe Complement + TE	29.4 µL Mastermix 1.6 µL BHQ-1 Quencher Tagged Probe 3 µL Linear Probe Complement

3. Vortex several seconds and spin down tubes.
4. Place tubes in the thermocycler at 41°C for 1 minute.
5. Place tubes in thermocycler 37°C for 1 minute.
6. Pipette all 32 µL of solution into a 384 well plate to measure fluorescence at excitation wavelength of 480 nm and emission intensity at 518 nm using a plate reader.

Results:

The results display a significant decrease in the fluorescence intensity of a triplicate with FAM Probe, BHQ Probe, and Linear Probe Complement as compared to a triplicate of just FAM tagged Probes, indicating that the probes had efficient binding (see Fig. 15).

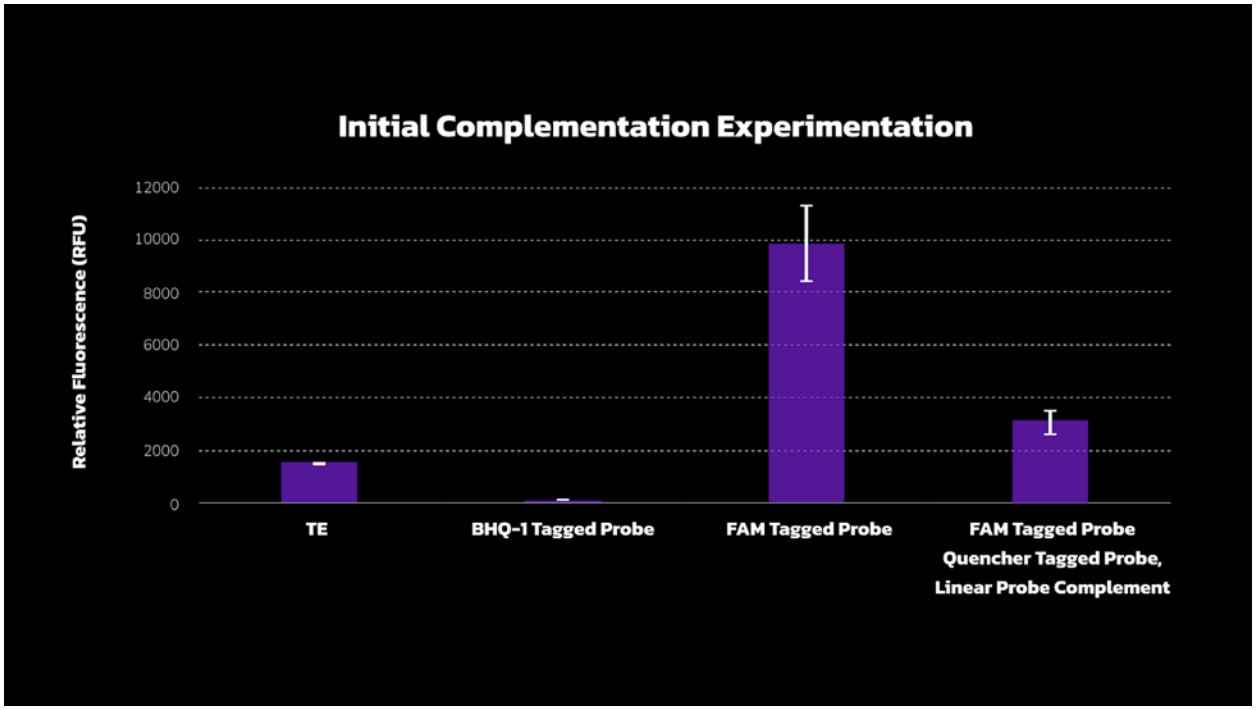


Figure 15. Linear Probe Complement Fluorescent Readout

Learn 5b

We determined that the new FAM/BHQ-1 Tagged Probe designs were capable of binding to linear probe complement efficiently, providing evidence that these probes are an effective means of sensing. This process could be applied to the RCP through which we could correlate the resulting fluorescence to the miRNA concentration.

Round 6b

Design 6b

Now that we isolated and fixed the issue of the binding efficiency of the linear probes to the RCP, we wanted to confirm if the linear probes could effectively bind to the RCP. More specifically, we wanted to find out if the long length and potential folding of the

RCP itself or any damage done to the RCP would affect our linear probes reporter mechanism.

Build 6b

We used the miRNA-1 padlock (BBa_K4245200) and the linear probes from the previous round, and we used the same RCA protocols. Additionally, we used the same linear probes protocols used in the previous round but changed to reflect the use of RCP in place of the linear probe complement.

FAM Dye Tagged Linear Probe (BBa_K4245130): 5'-FAM-CAA CCT CTT TCA G-3'

BHQ-1 Tagged Linear Probe (BBa_K4245132): 5'-GCT TCC ATC TGC-BHQ1-3’

Test 6b

Experimental Design:

Add the following to an amber microcentrifuge tube to make FAM-Probe Mastermix:

FAM Dye Tagged Linear DNA Probe Mastermix	Volume of FAM Dye Tagged Linear DNA Probe: (# of reactions) x (0.4 µL FAM Dye Tagged Linear DNA Probe) x 1.1 Volume of TE: (# of reactions) x (29 µL TE) x 1.1

1. Add the following to a PCR tube:

Tris-EDTA Buffer (Background)	34 µL TE Buffer

FAM Dye Tagged Linear DNA Probe + TE	29.4 µL Mastermix 4.6 µL TE Buffer
BHQ-1 Quencher Tagged Linear DNA Probe + TE	1.6 µL BHQ-1 Quencher Tagged Probe 32.4 µL TE Buffer
FAM Dye Tagged Linear DNA Probe + BHQ-1 Quencher Tagged Linear DNA Probe + rolling circle product + TE	29.4 µL Mastermix 1.6 µL BHQ-1 Quencher Tagged Probe 3 µL Linear Probe Rolling Circle Product

1. Vortex several seconds and spin down tubes.
2. Place tubes in the thermocycler at 41°C for 1 minute.
3. Place tubes in thermocycler 37°C for 1 minute.
4. Pipette all 32 µL of solution into a 384 well plate to measure fluorescence at excitation wavelength of 480 nm and emission intensity at 518 nm using a plate reader.

Results:

There is a statistically significant decrease in the fluorescent output of a triplicate with FAM Probe, BHQ Probe, and RCP as compared to a triplicate of just FAM tagged Probes (see Fig. 16). This confirms that we did produce our desired RCP in the RCA reaction for our miRNA-1-3p and miRNA-133a-3p sensors and that this mechanism was an effective reporting method for our sensor.

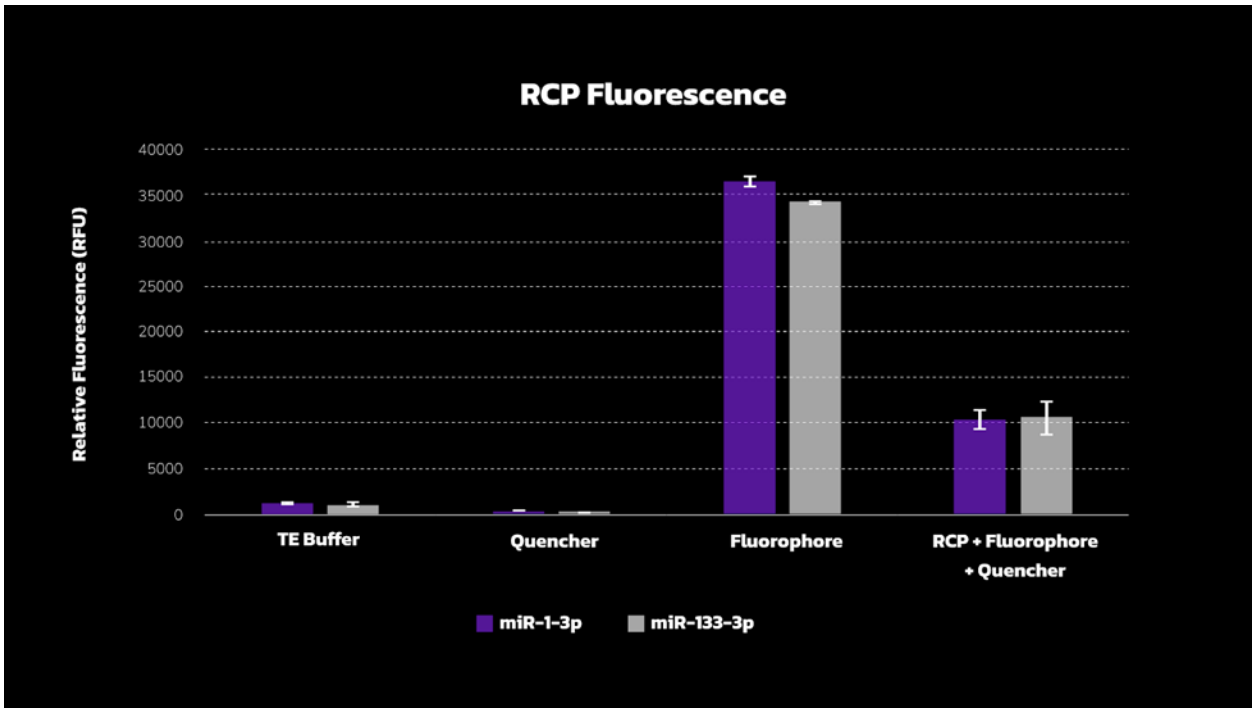


Figure 16. Fluorescent Read of Rolling Circle Product for miRNA-133a-3p and miRNA-1-3p

Learn 6b

We determined that the new FAM/BHQ-1 Tagged Probe designs were capable of binding to the rolling circle product (RCP) efficiently, providing evidence that these probes are an effective means of reporting the RCP, which is correlative with our miRNA. This experimentation validates the use of our sensor to detect the presence of miR-1-3p, a biomarker for coronary artery disease (CAD). Understanding the

relationship between the RCP (and by corollary the miRNA), and the decrease in fluorescence is the next step in the experimentation process.

Future

From the experimental results, We found that the FAM/BHQ-1 tagged linear DNA probes worked successfully as reporter mechanisms to measure rolling circle products. We have already tested and characterized the outputs of various miRNA concentrations through rolling circle amplification in conjunction with FAM/BHQ-1 tagged linear DNA probes.

Our next steps are to characterize these linear DNA probes in serum to get a curve correlating miRNA concentrations to fluorescence. Developing characterization data on rolling circle amplification from serum allows us to relate the output value of our reporting mechanisms to specific concentrations of miRNA, providing an additional point-of-care screening method for coronary artery disease.

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