Molecular Beacon Documentation

Purdue iGEM - 2020

cArgo: A COVID-19 Diagnostic Device

August 5th, 2020

GOALS: List the seven potential molecular beacon sequences previously obtained and determine any constraints on molecular beacon design and ion concentration in the molecular beacon solution.

Seven Potential MB Sequences:

1. MB: TGCGCACTTGCTTTACATAGAGCGCA
2. MB: AGCGCACTTGCTTTACATAGAGCGCT
3. MB: ACCGCACTTGCTTTACATAGAGCGGT
4. MB: ACGCCACTTGCTTTACATAGAGGCGT
5. MB: ACGGCACTTGCTTTACATAGAGCCGT
6. MB: ACGCGACTTGCTTTACATAGACGCGT
7. MB: TGCCCACTTGCTTTACATAGAGGGCA

Constraints on molecular beacon design:

In order to work properly, free beacons must remain in the hairpin structure to keep fluorophore quenched as they will produce fluorescence without binding to the target if they do not. This is extremely important in order to minimize background noise in the results. Therefore, factors such as the probe length, melting temperature, stem length, stem composition, and the lowest free energy structure are extremely important to designing a functional beacon for a specific usage.

Probe Length: A molecular beacon must have a probe length of 13 to 19 base pairs to be considered functional. This ensures that the probe will accurately recognize the target sequence and be able to bind to it, producing fluorescence.

Melting Temperature: A molecular beacon's melting temperature must be suitable for the temperature range in which it will be used to avoid both a lack of fluorescence and incorrectly produced fluorescence. To ensure a molecular beacon will work accurately, they should only be used in instances where the temperature is below the beacon's Tm. The fluoropore must remain quenched when a structure is not formed with the target sequence or the beacon will produce incorrect results. If a molecular beacon is used at or above its determined Tm, undesired instances such as the unbound hairpin structure developing a random coil configuration and incorrectly producing fluorescence or the bound hairpin probe dissociating with target and returning to the closed hairpin state and incorrectly not producing fluorescence may occur. If necessary, the Tm for unbound fluorophore and bound fluorophore target duplex can be

adjusted independently. Tm for the molecular beacon largely depends on its stem length and composition. It is proposed to use both argonautes and isothermal amplification to produce target sequence DNA in the same reaction chamber. The temperature of operation for argonautes is typically around 70 C. The temperature of operation for RPA is around 37 C. Therefore, it is recommended that our ideal beacon should have a Tm that is 7 to 10 C above the range of these values.***

Stem Length: The stem length is an important factor to consider in molecular beacon design. Longer stems, defined as 4 to 7 bases long, are generally used more often than shorter stems, defined as less than 4 bases. Longer stems result in a more stable structure with more specificity, a higher ability to discern between targets in a larger range of temperatures, decreased MB-target duplex stability, and a decreased rate of hybridization. On the other hand, shorter stems are associated with faster hybridization kinetics.

Stem Composition: In addition to stem length, stem content also influences a molecular beacon's melting temperature. Tm largely depends on the stem's GC-pair content as stems with a higher percentage of GC pairings will have a higher melting temperature due to the additional hydrogen bond a GC pair has when compared to an AT pair.

Free Energy: To ensure the molecular beacon forms at all, it is extremely important that a negative ΔG is used. In addition, we generally want a molecular beacon with a ΔG between -1.5 kcal/mol and -2.0 kcal/mol, but this specific range is less important than the probe length, melting temperature, stem length, and stem composition of the beacon. It is most important that free energy is below 0.

Ion Concentration Constraints

Two ions, Mg^{+2} and Na^{+}, are identified as important to proper beacon hybridization. Therefore, these must be changed according to literature and the QuickFold structures formed at each.

For this situation, it is important to first review the general ion composition of RPA and argonaute kits. The RPA kit used as reference is the TwistDx RPA kit. It was found that [Mg^{+2}] in RPA is irrelevant. RPA kit contents make up a total of 50 microliters. Argonaute contents make up a total of 20 microliters. There is 300 nM of Na^{+} in 1 microliter of argonaute, and there is 2 mM of Mg^{+2} in 2 microliters of thermopol buffer. These concentrations are very low, so it was determined that the ion concentration within the hybridization buffer for the molecular beacons was more important.

To see what ion concentrations are typically used in the hybridization buffer, a literature search was performed. It was found that 10 mM of Na^{+} and 5 mM Mg^{+2} are suggested, with at least 1 mM of Mg^{+2} in the assay medium to ensure stem hybridization (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC150230/).

Final Molecular Beacon Constraints (Listed in Order of Importance):

- Negative ΔG value*
- 4-7 base pair stem length
- 13-17 base pair probe length
- Ion concentrations at or near [Na+] = 10 mM and [Mg++] = 5 mM**
- Tm around 7 to 10 C above temperature of operation***

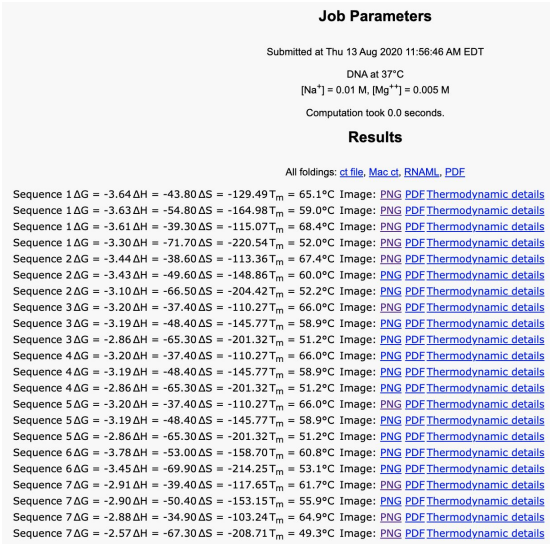
**Preferred ΔG is within a range of -1 to -2 kJ/mol. This is not a hard constraint, however.*
***At the very least, [Mg^{+2}] must meet the constraint of being greater or equal to 1 mM.*
****NOTE: We were unable to find a molecular beacon that met this constraint. Therefore, we will need to work around the argonaute process as it was not possible to find a beacon that met all other constraints with a melting temperature of over 77 ℃.*

August 7th, 2020

GOAL: Find a molecular beacon sequence and structure that meets the previous constraints.

To begin an iterative process that was used to find the best molecular beacon candidate, an online software developed by SUNY named QuickFold was used to predict the structure of each molecular beacon sequence at different temperatures and ion concentrations. The QuickFold software also provided information regarding the characteristics of each molecular beacon structure, such as ΔG, ΔH, ΔS, Tm, at specific values of [Na^{+}] and [Mg^{+2}]. Thus, multiple concentration levels within a prespecified range of acceptable values were tested to find the most ideal molecular beacon candidates. Additionally, a temperature of 37°C was initially used as a baseline condition as it was determined to be the optimal temperature of the RPA reaction.

The first run completed assumed ***[Na^{+}] = 10 mM*** and ***[Mg^{+2}] = 5 mM***. The results for this initial run are below:



Job Parameters

Submitted at Thu 13 Aug 2020 11:56:46 AM EDT

DNA at 37°C
[Na^{+}] = 0.01 M, [Mg^{++}] = 0.005 M

Computation took 0.0 seconds.

Results

All foldings: ct file, Mac ct, RNAML, PDF

Sequence 1	ΔG = -3.64	ΔH = -43.80	ΔS = -129.49	T_m = 65.1°C	Image:	PNG	PDF	Thermodynamic details
Sequence 1	ΔG = -3.63	ΔH = -54.80	ΔS = -164.98	T_m = 59.0°C	Image:	PNG	PDF	Thermodynamic details
Sequence 1	ΔG = -3.61	ΔH = -39.30	ΔS = -115.07	T_m = 68.4°C	Image:	PNG	PDF	Thermodynamic details
Sequence 1	ΔG = -3.30	ΔH = -71.70	ΔS = -220.54	T_m = 52.0°C	Image:	PNG	PDF	Thermodynamic details
Sequence 2	ΔG = -3.44	ΔH = -38.60	ΔS = -113.36	T_m = 67.4°C	Image:	PNG	PDF	Thermodynamic details
Sequence 2	ΔG = -3.43	ΔH = -49.60	ΔS = -148.86	T_m = 60.0°C	Image:	PNG	PDF	Thermodynamic details
Sequence 2	ΔG = -3.10	ΔH = -66.50	ΔS = -204.42	T_m = 52.2°C	Image:	PNG	PDF	Thermodynamic details
Sequence 3	ΔG = -3.20	ΔH = -37.40	ΔS = -110.27	T_m = 66.0°C	Image:	PNG	PDF	Thermodynamic details
Sequence 3	ΔG = -3.19	ΔH = -48.40	ΔS = -145.77	T_m = 58.9°C	Image:	PNG	PDF	Thermodynamic details
Sequence 3	ΔG = -2.86	ΔH = -65.30	ΔS = -201.32	T_m = 51.2°C	Image:	PNG	PDF	Thermodynamic details
Sequence 4	ΔG = -3.20	ΔH = -37.40	ΔS = -110.27	T_m = 66.0°C	Image:	PNG	PDF	Thermodynamic details
Sequence 4	ΔG = -3.19	ΔH = -48.40	ΔS = -145.77	T_m = 58.9°C	Image:	PNG	PDF	Thermodynamic details
Sequence 4	ΔG = -2.86	ΔH = -65.30	ΔS = -201.32	T_m = 51.2°C	Image:	PNG	PDF	Thermodynamic details
Sequence 5	ΔG = -3.20	ΔH = -37.40	ΔS = -110.27	T_m = 66.0°C	Image:	PNG	PDF	Thermodynamic details
Sequence 5	ΔG = -3.19	ΔH = -48.40	ΔS = -145.77	T_m = 58.9°C	Image:	PNG	PDF	Thermodynamic details
Sequence 5	ΔG = -2.86	ΔH = -65.30	ΔS = -201.32	T_m = 51.2°C	Image:	PNG	PDF	Thermodynamic details
Sequence 6	ΔG = -3.78	ΔH = -53.00	ΔS = -158.70	T_m = 60.8°C	Image:	PNG	PDF	Thermodynamic details
Sequence 6	ΔG = -3.45	ΔH = -69.90	ΔS = -214.25	T_m = 53.1°C	Image:	PNG	PDF	Thermodynamic details
Sequence 7	ΔG = -2.91	ΔH = -39.40	ΔS = -117.65	T_m = 61.7°C	Image:	PNG	PDF	Thermodynamic details
Sequence 7	ΔG = -2.90	ΔH = -50.40	ΔS = -153.15	T_m = 55.9°C	Image:	PNG	PDF	Thermodynamic details
Sequence 7	ΔG = -2.88	ΔH = -34.90	ΔS = -103.24	T_m = 64.9°C	Image:	PNG	PDF	Thermodynamic details
Sequence 7	ΔG = -2.57	ΔH = -67.30	ΔS = -208.71	T_m = 49.3°C	Image:	PNG	PDF	Thermodynamic details

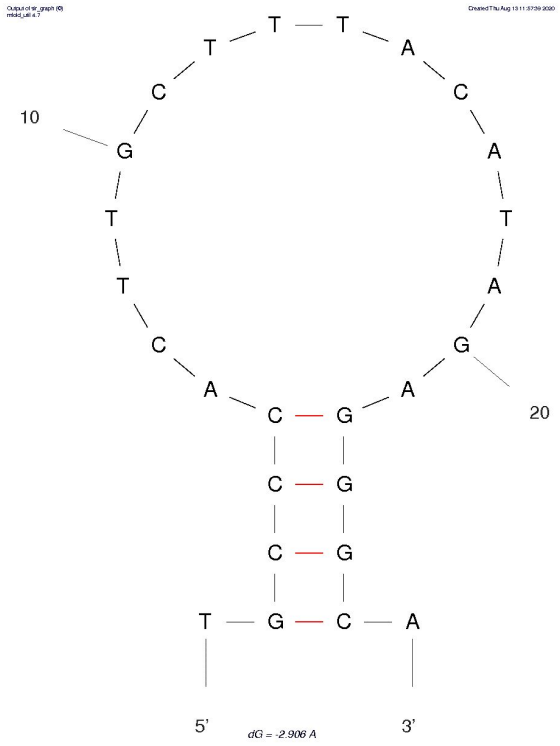
Figure 1: *QuickFold run results using concentrations of [Na^{+}] = 10 mM and [Mg^{+2}] = 5 mM.*

As all these results met the requirements set regarding [Na^{+}] and [Mg^{+2}], these potential structures were checked to see if they met all remaining constraints, notably having a negative ΔG value and adequate stem and probe lengths. Qualified beacons were included in the following figures. Each sequence number corresponds to the number designated

Sequence 7, Structure 1:

ΔG = -2.91 ΔH = -39.40 ΔS = -117.65 Tm = 61.7°C

Probe Length: 16 bp Stem Length: 4 bp



Sequence 7, structure 1

ΔG = -2.91 kcal/mol ΔH = -39.40 kcal/mol ΔS = -117.65 e.u. T_m = 61.7°C

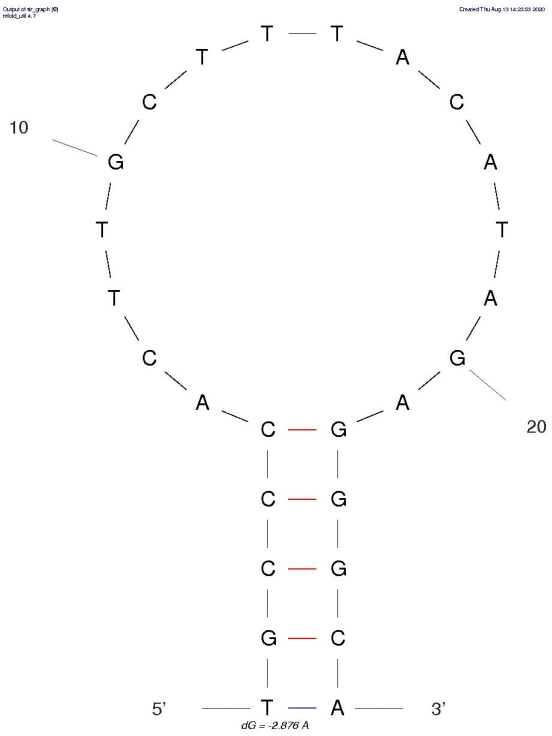
Structural element	δδG	Information
External loop	-1.27	2 ss bases & 1 closing helices
Stack	-2.08	External closing pair is G^{2}-C^{25}
Stack	-1.68	External closing pair is C^{3}-G^{24}
Stack	-1.68	External closing pair is C^{4}-G^{23}
Helix	-5.44	4 base pairs
Hairpin loop	+3.80	Closing pair is C^{5}-G^{22}

Figure 2: *Sequence 7, Structure 1 at [Na^{+}] = 10 mM and [Mg^{+2}] = 5 mM*

Sequence 7, Structure 3:

ΔG = -2.88 ΔH = -34.90 ΔS = -103.24 Tm = 64.9°C

Probe Length: 16 bp Stem Length: 5 bp



Sequence 7, structure 3

ΔG = -2.88 kcal/mol ΔH = -34.90 kcal/mol ΔS = -103.24 e.u. T_m = 64.9°C

Structural element	δδG	Information
External loop	+0.05	0 ss bases & 1 closing helices
Stack	-1.29	External closing pair is T^1-A^{26}
Stack	-2.08	External closing pair is G^2-C^{25}
Stack	-1.68	External closing pair is C^3-G^{24}
Stack	-1.68	External closing pair is C^4-G^{23}
Helix	-6.73	5 base pairs
Hairpin loop	+3.80	Closing pair is C^5-G^{22}

Figure 3: *Sequence 7, Structure 3 at [Na^+] = 10 mM and [Mg^{+2}] = 5 mM*

Sequence 7, Structure 1 and Sequence 7, Structure 3 meet all designated requirements other than melting temperature. Instead of having a melting temperature 7 to 10 °C above the argonaute operating temperature, both structures have a Tm below 65 °C. Both sequences have the same probe length of 16 base pairs and stem lengths that differ by 1 base pair. Sequence 7, Structure 3 is slightly more ideal as it has a higher Tm, but Sequence 7, Structure 1 is slightly more energetically favorable and thus more likely to form under these conditions.

August 8th, 2020

GOAL: Continue to search for a molecular beacon sequence and structure that meets the previous constraints.

To see if a molecular beacon that met all requirements by having a higher melting temperature, different concentrations of ions were tested. Specifically, the lower bound of [Mg^{+2}] was tested to see the impact of [Mg^{+}] on Tm. QuickFold results corresponding to ***[Na^{+}] = 10 mM*** and ***[Mg^{+2}] = 1 mM*** are included on the following pages.

DNA at 37°C
[Na^{+}] = 0.01 M, [Mg^{++}] = 0.001 M

Computation took 0.0 seconds.

Results

All foldings: ct file, Mac ct, RNAML, PDF

Sequence 1 ΔG = -3.29 ΔH = -43.80 ΔS = -130.61 T_m = 62.2°C Image: PNG PDF Thermodynamic details
Sequence 1 ΔG = -3.26 ΔH = -39.30 ΔS = -116.20 T_m = 65.1°C Image: PNG PDF Thermodynamic details
Sequence 1 ΔG = -3.03 ΔH = -54.80 ΔS = -166.92 T_m = 55.2°C Image: PNG PDF Thermodynamic details
Sequence 1 ΔG = -2.44 ΔH = -71.70 ΔS = -223.31 T_m = 47.9°C Image: PNG PDF Thermodynamic details
Sequence 2 ΔG = -3.09 ΔH = -38.60 ΔS = -114.49 T_m = 64.0°C Image: PNG PDF Thermodynamic details
Sequence 2 ΔG = -2.83 ΔH = -49.60 ΔS = -150.80 T_m = 55.8°C Image: PNG PDF Thermodynamic details
Sequence 2 ΔG = -2.24 ΔH = -66.50 ΔS = -207.19 T_m = 47.8°C Image: PNG PDF Thermodynamic details
Sequence 3 ΔG = -2.85 ΔH = -37.40 ΔS = -111.40 T_m = 62.6°C Image: PNG PDF Thermodynamic details
Sequence 3 ΔG = -2.59 ΔH = -48.40 ΔS = -147.70 T_m = 54.5°C Image: PNG PDF Thermodynamic details
Sequence 3 ΔG = -2.00 ΔH = -65.30 ΔS = -204.09 T_m = 46.8°C Image: PNG PDF Thermodynamic details
Sequence 4 ΔG = -2.85 ΔH = -37.40 ΔS = -111.40 T_m = 62.6°C Image: PNG PDF Thermodynamic details
Sequence 4 ΔG = -2.59 ΔH = -48.40 ΔS = -147.70 T_m = 54.5°C Image: PNG PDF Thermodynamic details
Sequence 4 ΔG = -2.00 ΔH = -65.30 ΔS = -204.09 T_m = 46.8°C Image: PNG PDF Thermodynamic details
Sequence 5 ΔG = -2.85 ΔH = -37.40 ΔS = -111.40 T_m = 62.6°C Image: PNG PDF Thermodynamic details
Sequence 5 ΔG = -2.59 ΔH = -48.40 ΔS = -147.70 T_m = 54.5°C Image: PNG PDF Thermodynamic details
Sequence 5 ΔG = -2.00 ΔH = -65.30 ΔS = -204.09 T_m = 46.8°C Image: PNG PDF Thermodynamic details
Sequence 6 ΔG = -3.18 ΔH = -43.20 ΔS = -129.03 T_m = 61.7°C Image: PNG PDF Thermodynamic details
Sequence 6 ΔG = -3.18 ΔH = -53.00 ΔS = -160.63 T_m = 56.8°C Image: PNG PDF Thermodynamic details
Sequence 6 ΔG = -2.59 ΔH = -69.90 ΔS = -217.02 T_m = 48.9°C Image: PNG PDF Thermodynamic details
Sequence 7 ΔG = -2.56 ΔH = -39.40 ΔS = -118.78 T_m = 58.6°C Image: PNG PDF Thermodynamic details
Sequence 7 ΔG = -2.53 ΔH = -34.90 ΔS = -104.37 T_m = 61.2°C Image: PNG PDF Thermodynamic details
Sequence 7 ΔG = -2.30 ΔH = -50.40 ΔS = -155.09 T_m = 51.8°C Image: PNG PDF Thermodynamic details
Sequence 7 ΔG = -1.71 ΔH = -67.30 ΔS = -211.48 T_m = 45.1°C Image: PNG PDF Thermodynamic details

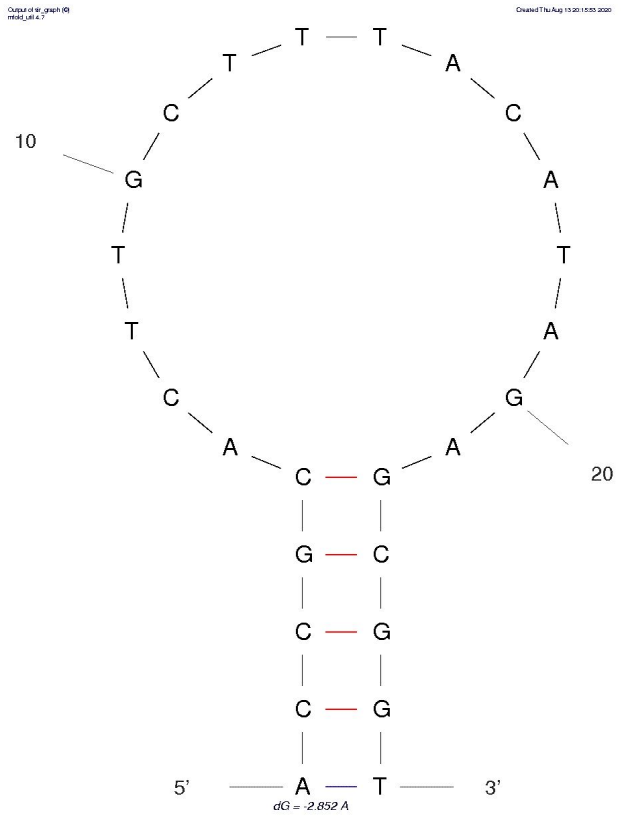
Figure 4: *QuickFold run results using concentrations of [Na^{+}] = 10 mM and [Mg^{+2}] = 1 mM.*

Similar to what was done for the previous run, molecular beacon structures formed at these conditions which met requirements were further considered.

Sequence 3, Structure 1

ΔG = -2.85 ΔH = -37.40 ΔS = -111.40 Tm = 62.6°

Probe Length: 16 bp Stem Length: 5 bp



Sequence 3, structure 1

ΔG = -2.85 kcal/mol ΔH = -37.40 kcal/mol ΔS = -111.40 e.u. T_m = 62.6°C

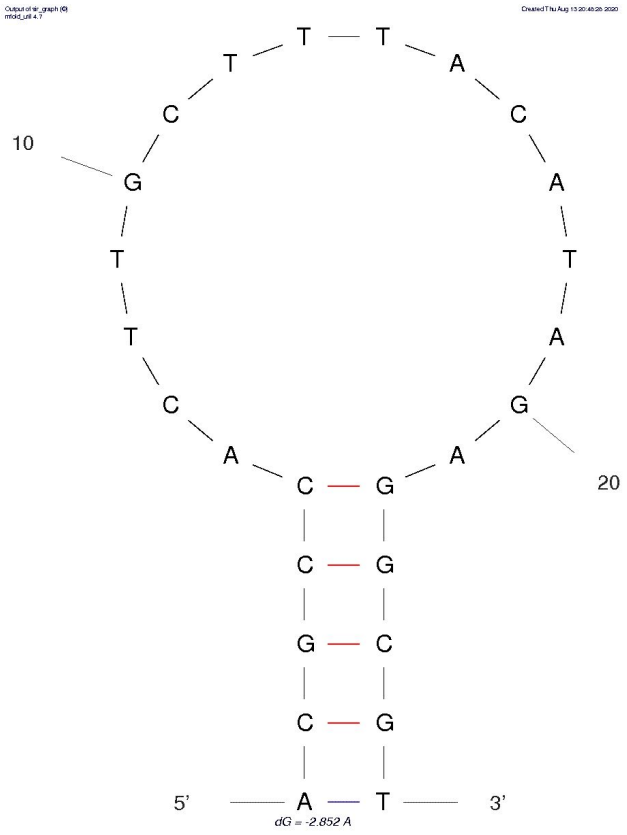
Structural element	δδG	Information
External loop	+0.05	0 ss bases & 1 closing helices
Stack	-1.19	External closing pair is A^1-T^{26}
Stack	-1.59	External closing pair is C^2-G^{25}
Stack	-1.92	External closing pair is C^3-G^{24}
Stack	-1.99	External closing pair is G^4-C^{23}
Helix	-6.70	5 base pairs
Hairpin loop	+3.80	Closing pair is C^5-G^{22}

Figure 5: *Sequence 3, Structure 1 at [Na^+] = 10 mM and [Mg^{+2}] = 1 mM*

Sequence 4, Structure 1

ΔG = -2.85 ΔH = -37.40 ΔS = -111.40 Tm = 62.6°C

Probe Length: 16 Stem Length: 5



Sequence 4, structure 1

ΔG = -2.85 kcal/mol ΔH = -37.40 kcal/mol ΔS = -111.40 e.u. T_m = 62.6°C

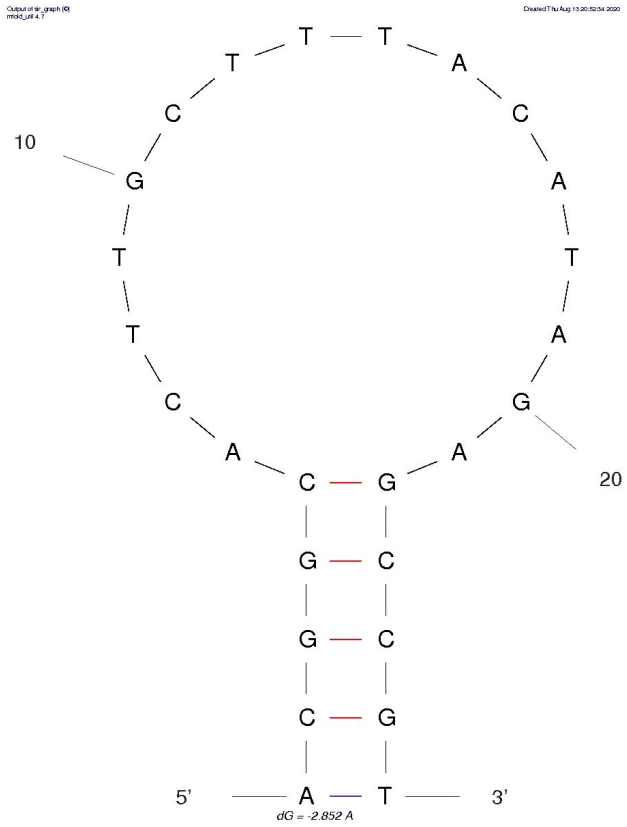
Structural element	δδG	Information
External loop	+0.05	0 ss bases & 1 closing helices
Stack	-1.19	External closing pair is A^1-T^{26}
Stack	-1.92	External closing pair is C^2-G^{25}
Stack	-1.99	External closing pair is G^3-C^{24}
Stack	-1.59	External closing pair is C^4-G^{23}
Helix	-6.70	5 base pairs
Hairpin loop	+3.80	Closing pair is C^5-G^{22}

Figure 6: *Sequence 4, Structure 1 at [Na^+] = 10 mM and [Mg^{+2}] = 1 mM*

Sequence 5, Structure 1

ΔG = -2.85 ΔH = -37.40 ΔS = -111.40 Tm = 62.6°C

Probe Length: 16 bp Stem Length: 5 bp



Sequence 5, structure 1

ΔG = -2.85 kcal/mol ΔH = -37.40 kcal/mol ΔS = -111.40 e.u. T_m = 62.6°C

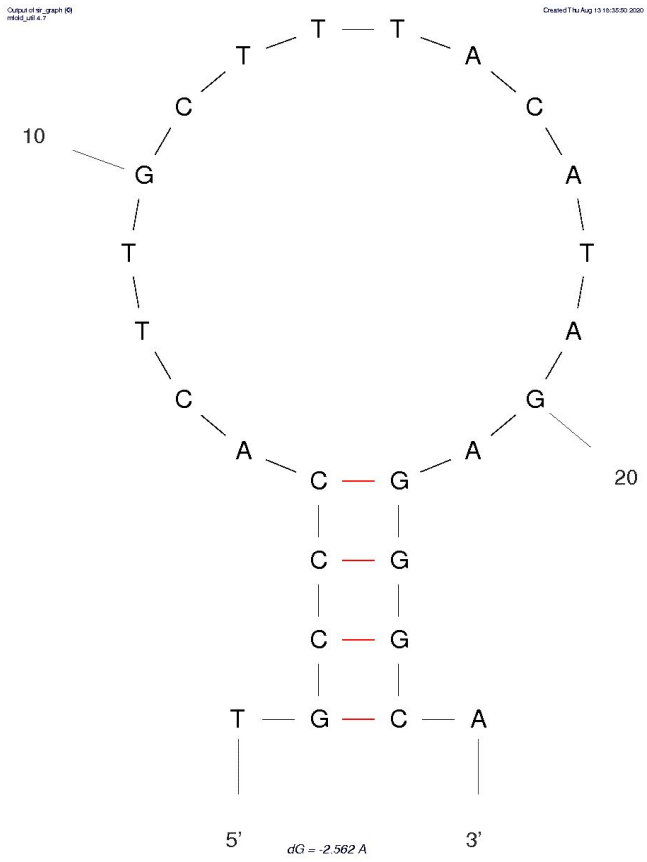
Structural element	δδG	Information
External loop	+0.05	0 ss bases & 1 closing helices
Stack	-1.19	External closing pair is A^1-T^{26}
Stack	-1.92	External closing pair is C^2-G^{25}
Stack	-1.59	External closing pair is G^3-C^{24}
Stack	-1.99	External closing pair is G^4-C^{23}
Helix	-6.70	5 base pairs
Hairpin loop	+3.80	Closing pair is C^5-G^{22}

Figure 7: *Sequence 5, Structure 1 at [Na^+] = 10 mM and [Mg^{+2}] = 1 mM*

Sequence 7, Structure 1

ΔG = -2.56 ΔH = -39.40 ΔS = -118.78 Tm = 58.6°C

Probe Length: 16 bp Stem Length: 4 bp



Sequence 7, structure 1

ΔG = -2.56 kcal/mol ΔH = -39.40 kcal/mol ΔS = -118.78 e.u. T_m = 58.6°C

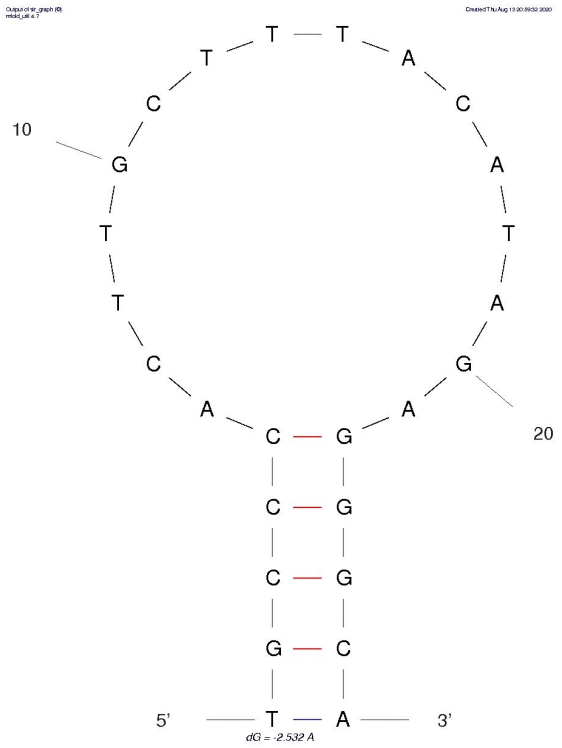
Structural element	δδG	Information
External loop	-1.18	2 ss bases & 1 closing helices
Stack	-1.99	External closing pair is G^{2}-C^{25}
Stack	-1.59	External closing pair is C^{3}-G^{24}
Stack	-1.59	External closing pair is C^{4}-G^{23}
Helix	-5.18	4 base pairs
Hairpin loop	+3.80	Closing pair is C^{5}-G^{22}

Figure 8: *Sequence 7, Structure 1 at [Na^{+}] = 10 mM and [Mg^{+2}] = 1 mM*

Sequence 7, Structure 2

ΔG = -2.53 ΔH = -34.90 ΔS = -104.37 Tm = 61.2°C

Probe Length: 16 bp Stem Length: 5 bp



Sequence 7, structure 2

ΔG = -2.53 kcal/mol ΔH = -34.90 kcal/mol ΔS = -104.37 e.u. T_m = 61.2°C

Structural element	δδG	Information
External loop	+0.05	0 ss bases & 1 closing helices
Stack	-1.20	External closing pair is T^{1}-A^{26}
Stack	-1.99	External closing pair is G^{2}-C^{25}
Stack	-1.59	External closing pair is C^{3}-G^{24}
Stack	-1.59	External closing pair is C^{4}-G^{23}
Helix	-6.38	5 base pairs
Hairpin loop	+3.80	Closing pair is C^{5}-G^{22}

Figure 9: *Sequence 7, Structure 2 at [Na^{+}] = 10 mM and [Mg^{+2}] = 1 mM*

While these structures meet most of the set constraints and have desirable probe and stem lengths, no structures experienced at this concentration level were found to have a sufficient melting temperature. The highest Tm of these proposed structures is 62.6°C, which is lower than melting temperatures of the structures observed at [Na^{+}] = 10 mM and [Mg^{+2}] = 5 mM. Therefore, these structures were determined to not be significantly more ideal than those at the previous concentration level tested.

August 10th, 2020

GOAL: Continue to search for a molecular beacon sequence and structure that meets the previous constraints, notably melting temperature.

Changing the concentration of the magnesium ion proved to not significantly impact the melting temperature of previous molecular beacon structures. As a result, it was determined that other variables should be altered, such as the temperature of operation at which the structure would be observed at. Moreover, the program written to generate molecular beacon sequences was edited and produced new potential molecular beacon sequences as a result. Therefore, these were tested in addition to the seven sequences included in the first entry. In addition to the generation of these new sequences, it was also realized that each molecular beacon structure considered must be the most energetically-favorable structure associated with that specific sequence as this was the most likely conformation the beacon sequence would form. Furthermore, it was also decided that each considered structure should not have more than one loop or buckle to ensure that target DNA matching with the probe sequence would be the only cause of fluorescence. These additional constraints were emphasized in all QuickFold runs made following this decision. The updated list of constraints, arranged by priority, is included below:

UPDATED (First Time) Molecular Beacon Constraints (Listed in Order of Importance):

- Most energetically favorable structure (lowest ΔG value)
- No additional loops or buckles other than the primary probe sequence
- Negative ΔG value*
- 4-7 base pair stem length
- 13-17 base pair probe length
- Ion concentrations at or near $[Na^{+}]$ = 10 mM and $[Mg^{+2}]$ = 5 mM**
- Tm around 7 to 10 C above temperature of operation***

**Preferred ΔG is within a range of -1 to -2 kJ/mol. This is not a hard constraint, however.*
***At the very least, $[Mg^{+2}]$ must meet the constraint of being greater or equal to 1 mM.*
****NOTE: We were unable to find a molecular beacon that met this constraint. Therefore, we will need to work around the argonaute process as it was not possible to find a beacon that met all other constraints with a melting temperature of over 77 °C.*

Because the RPA reaction works within a large range of temperatures, it was decided that a change in the temperature at which the molecular beacon should be considered. The optimal temperature at which RPA works is 37°C, but the reaction has been observed at similar efficiency within a temperature range of 37°C to 42°C. Therefore, QuickFold was used to generate molecular beacon structures at a temperature of 39°C and ion concentrations of ***$[Na^{+}]$ = 10 mM*** and ***$[Mg^{+2}]$ = 5 mM.***

Unfortunately, these results did not yield a melting temperature above 70°C. In addition, all molecular beacons observed were found to have more energetically-favorable structures with short probes or with a secondary loop, which was determined to be highly undesirable and be a source of potential error. Therefore, these structures could not be used. Examples of such structures generated were included in Figures 10 and 11.

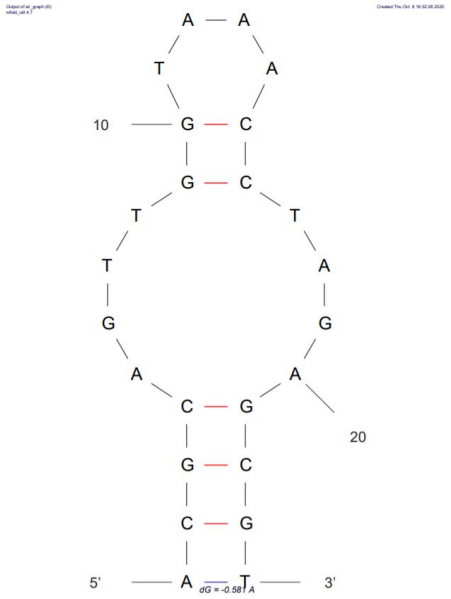


Figure 10: *An example of an undesirable molecular beacon structure. In addition to its short probe sequence, the loop in the middle of the stem disqualifies this molecular beacon conformation.*

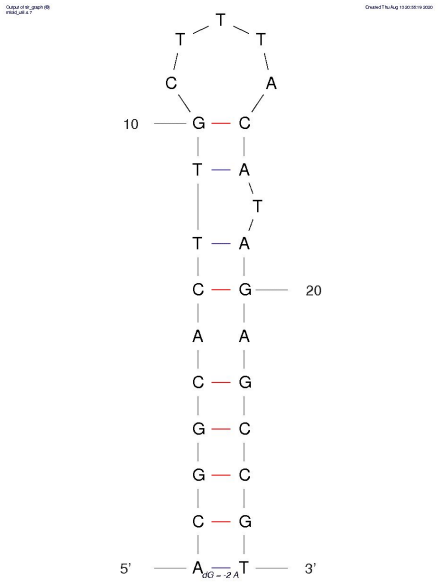


Figure 11: *Another example of an undesirable molecular beacon structure. In addition to its short probe sequence and long stem, the buckle near the top of the stem disqualifies this molecular beacon conformation.*

Following this run, it was decided that the biological processes using this molecular beacon must be conducted below the melting temperature or designed to occur at different times as obtaining a melting temperature over 70°C was deemed unreasonable. Therefore, this requirement was removed from the previously-defined list of constraints, as shown below:

UPDATED (Second Time) Molecular Beacon Constraints (Listed in Order of Importance):

- Most energetically favorable structure (lowest ΔG value)
- No additional loops or buckles other than the primary probe sequence
- Negative ΔG value*
- 4-7 base pair stem length
- 13-17 base pair probe length
- Ion concentrations at or near $[Na^{+}]$ = 10 mM and $[Mg^{+2}]$ = 5 mM**
- ~~Tm around 7 to 10 C above temperature of operation***~~

**Preferred ΔG is within a range of -1 to -2 kJ/mol. This is not a hard constraint, however.*
***At the very least, $[Mg^{+2}]$ must meet the constraint of being greater or equal to 1 mM.*

Due to the presence of less secondary structures than at 37°C, it was decided subsequent runs were completed at 39°C. Another QuickFold run with the same molecular sequences at 39°C and ion concentrations of ***$[Na^{+}]$ = 10 mM*** and ***$[Mg^{+2}]$ = 1 mM*** was completed to check molecular beacon structures associated with the lower bound of $[Mg^{+2}]$. Once again, there were no sequences found without an undesirable secondary structure. For one of the sequences, however, the structure associated with the ΔG met all requirements, but an undesirable secondary sequence was observed. These structures and their associated sequence are included in Figure 12.

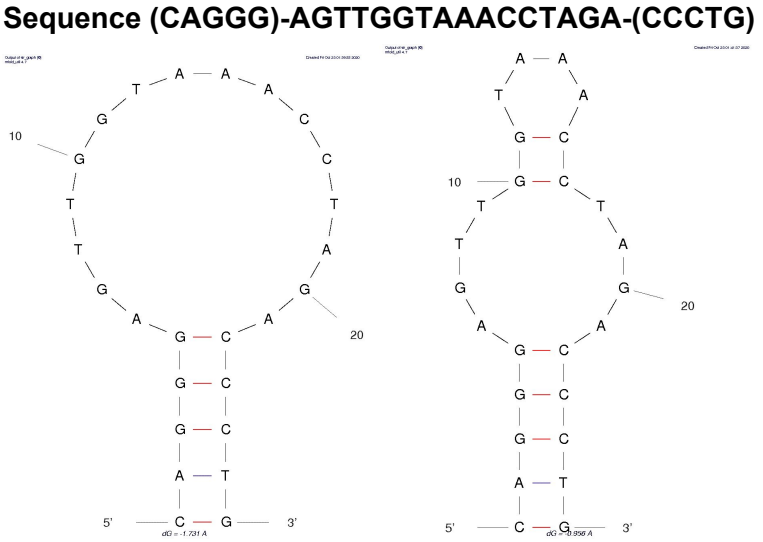


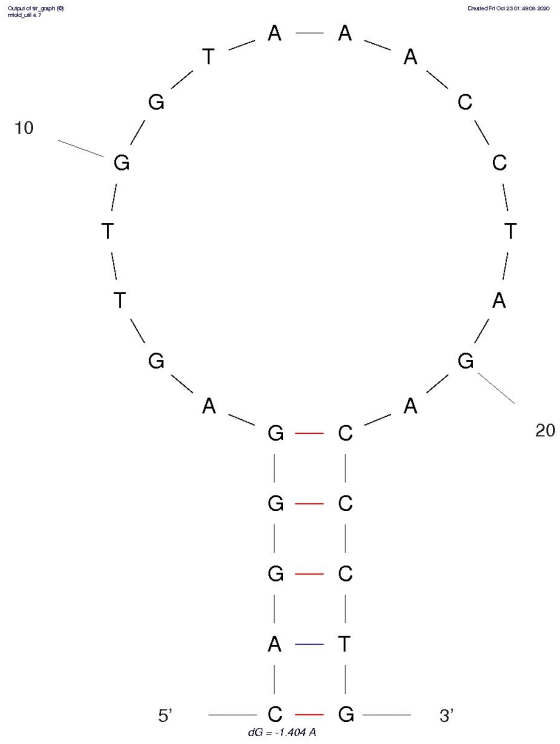
Figure 12: *A desirable structure (left) which meets all of the set constraints at 39°C and $[Na^{+}]$ = 10 mM and $[Mg^{+2}]$ = 1 mM except for having an undesirable second structure (right)*

To observe the effects of changing [Na^+], a run at ***[Na^+] = 15 mM*** and ***[Mg^{+2}] = 1 mM*** was completed. This was also set to 39°C. These results included one structure that met all set constraints, featured in Figure 13. This was chosen as the final molecular beacon.

Sequence (CAGGG)-AGTTGGTAAACCTAGA-(CCCTG), Structure 1

ΔG = -1.40 ΔH = -38.30 ΔS = -118.21 Tm = 50.8°C

Probe Length: 16 bp Stem Length: 5 bp



ΔG = -1.40 kcal/mol ΔH = -38.30 kcal/mol ΔS = -118.21 e.u. T_m = 50.8°C

Structural element	δδG	Information
External loop	+0.00	0 ss bases & 1 closing helices
Stack	-1.16	External closing pair is C^1-G^{26}
Stack	-0.99	External closing pair is A^2-T^{25}
Stack	-1.56	External closing pair is G^3-C^{24}
Stack	-1.56	External closing pair is G^4-C^{23}
Helix	-5.27	5 base pairs
Hairpin loop	+3.86	Closing pair is G^5-C^{22}

Figure 13: *Sequence (CAGGG)-AGTTGGTAAACCTAGA-(CCCTG), Structure 1 at [Na^+] = 15 mM and [Mg^{+2}] = 1 mM*

CONCLUSION: SEQUENCE (CAGG)-GAGTTGGTAAACCTAGA-(CCCTG)

Structure 1 of the molecular beacon sequence **(CAGG)-GAGTTGGTAAACCTAGA-(CCCTG)** at ***T = 39°C***, ***[Na^+] = 15 mM***, and ***[Mg^{+2}] = 1 mM*** was chosen as the final beacon as it sufficiently met all constraints previously set with exception of the constraint that Tm should be 7 to 10°C above argonaute functioning temperature, defined as 70°C. However, it was decided to disregard this constraint as no functional molecular beacons observed had a melting temperature above 70°C. Consequently, the order in which reactions will take place within the reaction chamber must be altered to accommodate the melting temperature of the molecular beacon. This will especially affect how and when the argonaute reaction is completed as this reaction takes place above the molecular beacon's melting temperature of 50.8°C. The RPA reaction will not be directly impacted by this as it runs at 37°C, over 10°C lower than the beacon's melting temperature.

This final list of constraints used to determine whether or not this structure was acceptable are listed below. All of these were sufficiently met by the chosen molecular beacon.

Final Molecular Beacon Constraints (Listed in Order of Importance):

- Most energetically favorable structure (lowest ΔG value)
- No additional loops or buckles other than the primary probe sequence
- Negative ΔG value*
- 4-7 base pair stem length
- 13-17 base pair probe length
- Ion concentrations at or near [Na^+] = 10 mM and [Mg^{+2}] = 5 mM**

**Preferred ΔG is within a range of -1 to -2 kcal/mol. This is not a hard constraint, however.*
***At the very least, [Mg^{+2}] must meet the constraint of being greater or equal to 1 mM.*

This sequence was chosen to be the final molecular beacon as it met all of these final constraints within the most ideal range specified (if applicable). First, the final molecular beacon structure was the only structure present for the specific sequence used, so it was definitely the most energetically favorable and could be assumed to form spontaneously. Moreover, as the structure formed by the sequence is associated with a ΔG value of -1.40 kcal/mol, it falls within the desired -1 kcal/mol to -2 kcal/mol interval, which is viewed as extremely desirable. Additionally, the structure did not have any loops or buckles other than the probe, which signifies a lower chance of error due to incorrect hybridization from DNA sequences other than the target sequence. Furthermore, the beacon's structure met the required stem and probe length requirements with a 16 base pair long probe and a 5 base pair long stem. Last, the ion concentrations in the buffer used to produce this molecular beacon structure did not deviate far from the recommended [Na^+] = 10 mM and [Mg^{+2}] = 5 mM without going under [Mg^{+2}] = 1 mM: the final concentration of magnesium ions was determined to be [Mg^{+2}] = 1 mM, and the final concentration of sodium ion was determined to be [Na^+] = 15 mM.

Overall, this molecular beacon successfully met all structural requirements and is assumed to be functional. For these reasons, it is determined that using sequence (CAGG)-GAGTTGGTAAACCTAGA-(CCCTG) under conditions of 39°C produces an ideal molecular beacon that is predicted to work well in the final cArgo device.

It should be noted that the portion of the molecular beacon in parentheses make up the stem of the beacon while the bases in between form the probe. This is seen in the final beacon image, shown in Figure 13.

References:

QuickFold software:
http://unafold.rna.albany.edu/?q=DINAMelt/Quickfold

General info about MBs and basic thermodynamic properties:
https://www.biosyn.com/tew/Design-rules-for-Molecular-Beacons.aspx
http://www.molecular-beacons.org/MB_SC_design.html
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC150230/