Confirmation of mScarlet Megaprimer Generation

Participants: Brianna Branson, Rori Hoover, Patrick Jiang

Date: Wednesday, May 10, 2023

Protocol:

1. Prepared a 1% agarose running gel:
    a. Added 0.25 g agarose to 25 mL 1X TBE in an Erlenmeyer flask
    b. Heated the flask in a microwave in 10 second increments until fully dissolved
    c. Cooled the flask until it was manageable to touch
    d. Added 2.5 µL SYBR Safe and swirled the flask to mix
    e. Poured the solution into a gel mold with a comb and left it to solidify for 20 minutes
    f. Removed the comb and covered gel cast completely with 1X TBE
2. Loaded gel:
    a. Loaded 5 µL 1 kb DNA ladder into the first well
    b. Added 3 µL gel loading dye to the PCR product
    c. Loaded 5 µL of PCR product into the second well
3. Placed the gel cast in a blueGel electrophoresis system and left the sample to run for 30 minutes

Results: The gel was foggy and unclear, so we were unable to see our samples after running the gel.

Conclusion: We should reprepare the 1% agarose running gel and rerun our sample for clearer results.

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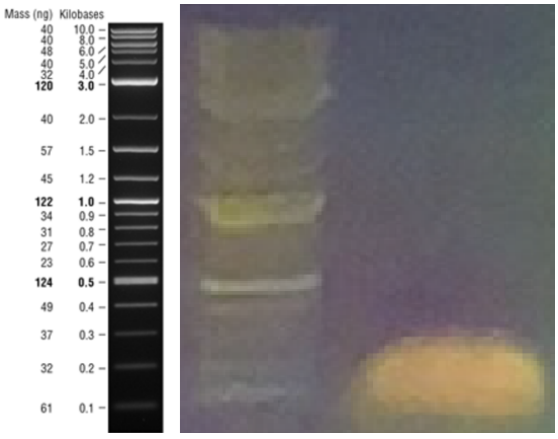
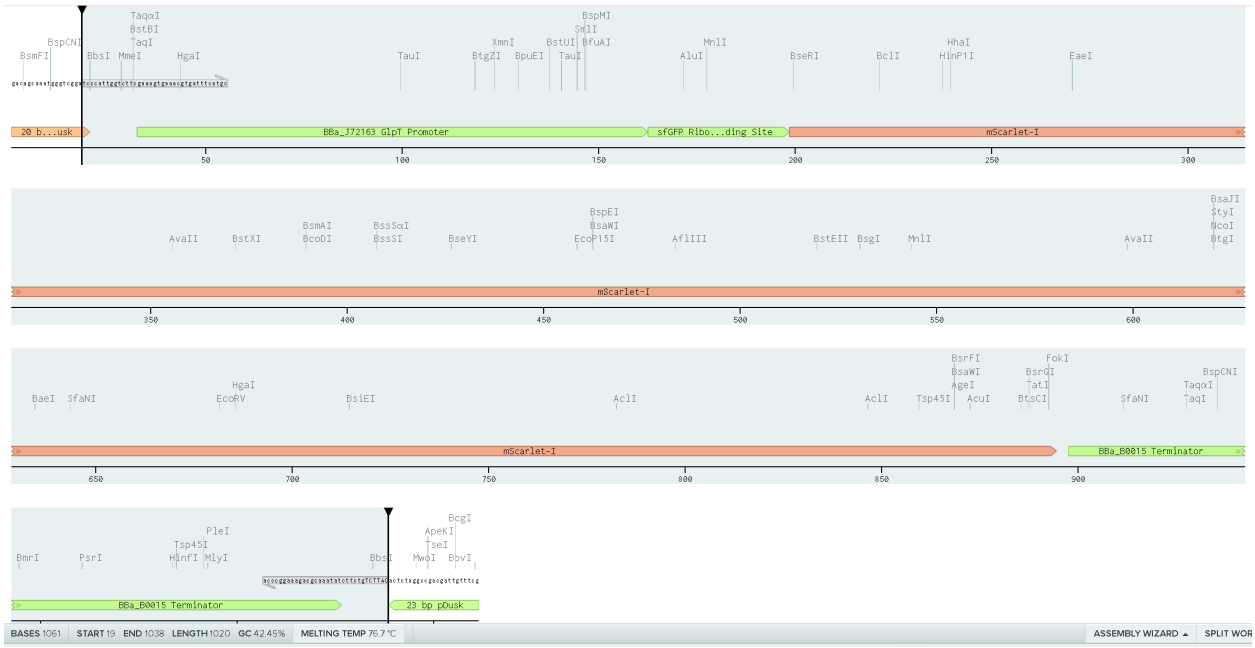
Protocol: see protocol on page 1

Results:



Conclusion:

According to our plasmid map in Benchling, the mScarlet megaprimer should be **994 bp long (shouldn't the sample be 1020 bp? Doesn't the bp count include the primers at both ends?)**



However, after performing gel electrophoresis and comparing the migration distance of our sample to the 1kb DNA ladder, we determined that our sample was only approximately 300 bp. Thus, we were unsuccessful in extracting the mScarlet megaprimer, and must redo the extraction.

Since our sample had too few base pairs, it is possible that the annealing temperature was wrong. Using the NEB temperature calculator, we recalculated the annealing temperature to be 59 °C. However, to ensure that there were no further issues with the annealing temperature, we decided to perform PCR at 8 different annealing temperatures: 50.9 °C, 52 °C, 53.4 °C, 54.8 °C, 56.2 °C, 57.6 °C, 59 °C, and 60.1 °C to determine which one would yield a band closest to **994 bp** following gel electrophoresis.

mScarlet Megaprimer Generation with 8 Different Annealing Temperatures

Participants: Brianna Branson, Rori Hoover, Patrick Jiang

Date: Wednesday, May 10, 2023

Protocol:

1. Prepared a 25 µL reaction in a PCR tube with the following components:

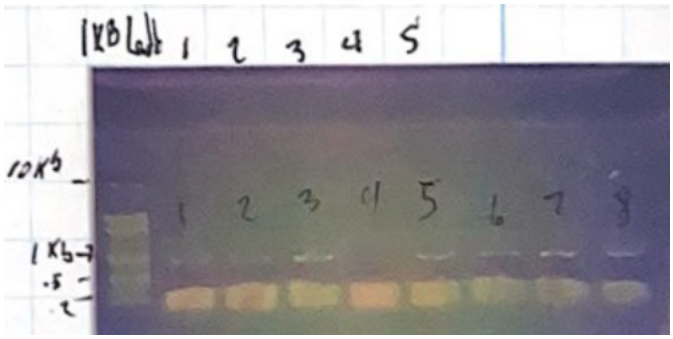
Component	Volume
10 µM forward primer	1.25 µL
10 µM reverse primer	1.25 µL
10 ng template DNA (R8)	2.1 µL
Q5 High-Fidelity 2X Master Mix	12.5 µL
Nuclease-free water	7.9 µL
Total	**25 µL**

2. Amplified the DNA in the 25 µL reaction with a thermocycler, using the following conditions:

Step	Temperature	Time	Number of cycles
Initial denaturation	98 °C	30 sec	1
Denaturation	98 °C	10 sec	30
Annealing	50.9-60.1 °C (see conclusion on page 3)	30 sec	30
Extension	72 °C	30 sec	30
Final extension	72 °C	2 min	1
Hold	10 °C	Forever	-

3. Prepared a 1% agarose running gel:
    a. Added 0.25 g agarose to 25 mL 1X TBE in an Erlenmeyer flask
    b. Heated the flask in a microwave in 10 second increments until fully dissolved
    c. Cooled the flask until it was manageable to touch
    d. Added 2.5 µL SYBR Safe and swirled the flask to mix
    e. Poured the solution into a gel mold with a comb and left it to solidify for 20 minutes
    f. Removed the comb and covered gel cast completely with 1X TBE
4. Loaded gel:
    a. Loaded 3 µL 1 kb DNA ladder into the first well
    b. Added 3 µL gel loading dye to the PCR product
    c. Loaded 3 µL of each sample into wells 2-9; the samples were loaded from left to right from lowest temperature to highest temperature
5. Placed the gel cast in a blueGel electrophoresis system and left the sample to run for 30 minutes

Results:



Conclusion:

We were successful in generating the mScarlet megaprimer because a thin band appeared at 1 kb. We belatedly realized that we had forgotten to dilute the forward and reverse primer from 100 µM to 10 µM, so the larger bands appearing at approximately 200 bp are likely the result of self-primerization.

We can now move on to performing gel extraction to harvest mScarlet megaprimer.

Gel Extraction of mScarlet Megaprimer

Participants: Brianna Branson, Rori Hoover, Patrick Jiang

Date: Wednesday, May 10, 2023

Protocol:

Note: these steps follow those outlined in the Monarch® DNA Gel Extraction Kit Protocol (NEB #T1020) by New England Biolabs.

1. Excised mScarlet megaprimer DNA from the agarose gel and placed them in a microfuge tube. The weights of each agarose gel piece are listed in the table below.

Test tube #	1	2	3	4
Mass	224.9 mg	177.4 mg	215 mg	214.7 mg
Volume of ethanol	899.6 µL	709.6 µL	860 µL	858.8 µL

2. Ethanol was added to each agarose gel piece in the volumes listed in the table above. The volume of ethanol was calculated to be four times the mass of the agarose piece.
3. Incubated the agarose pieces at 50 °C while shaking at 400 rpm until the gel completely dissolved
4. Inserted a spin column into a 4 collection tubes and loaded each sample into their respective column
5. Centrifuged all 4 tubes at 13,000 rpm for 1 minute and discarded the flow-through
6. Added 200 µL DNA Wash Buffer 1 to each column and centrifuged all of the columns at 13,000 rpm for 1 minute
7. Added another 200 µL DNA Wash Buffer 1 to each column and centrifuged all of the columns at 13,000 rpm for 1 minute
8. Transferred each spin column to a clean microfuge tube
9. Added 30 µL DNA Elution Buffer
10. Waited 1 minute before centrifuging all of the tubes at 13,000 rpm for 1 minute

Results: N/A

Conclusion: Next time we are in the lab, we will perform PCR to insert mScarlet megaprimer into G1.