Tear-Inducing Bacteria

Holly Milbach, Jenna Fox, Neha Yadav, Heather Goodrich

Goals

- The goal of our project was to isolate the Alliinase gene from an onion, which is the gene responsible for producing tears when onions are cut.
- In our project, we worked with the gene sequence that codes for this particular enzyme, clone it, and check its expression in E. coli.
- Our main goal was to achieve the expression of the gene behind the alliinase enzyme, ALL 1, in E. coli.

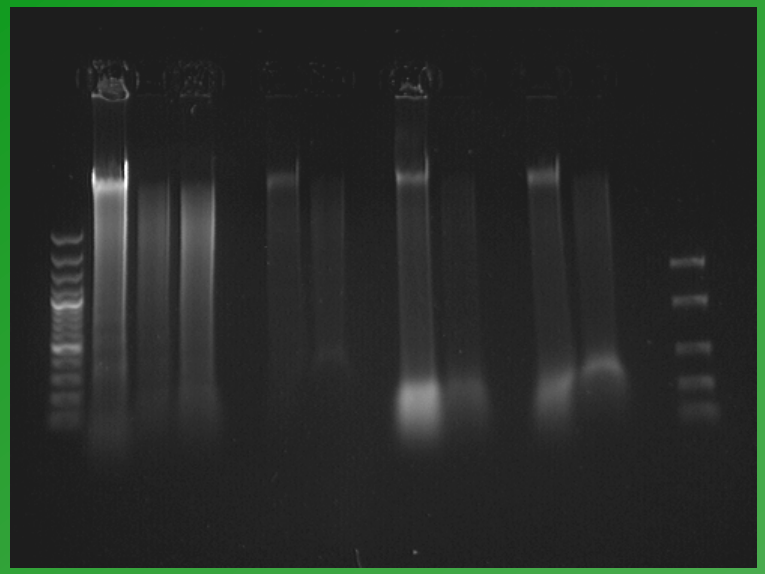
Project Description

- We started our experiment with the extraction of DNA from the roots and leaves of an onion using the protocol Extraction of Total Cellular DNA.

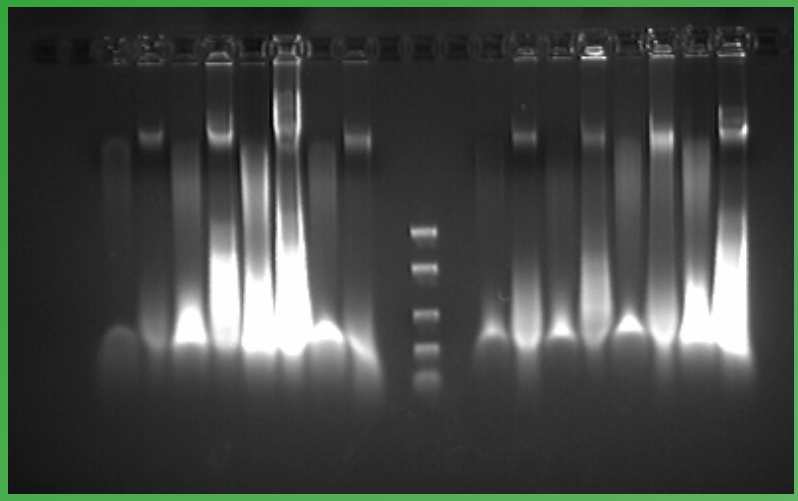
- We used the extraction method based on the CTAB nucleic acid extraction procedures of Murray and Thompson (1980), Taylor and Powell (1982), Rogers and Bendich (1985, 1988, 1994), Rogers, et al. (1989), and Shivji et al. (1992) that makes it possible to extract high molecular weight DNA without the use of expensive equipment and time-consuming procedures.

DNA Extraction

- Our results from using this protocol yielded DNA from both the onion leaves and roots. Throughout the semester, we repeated this protocol three times and received the following results.

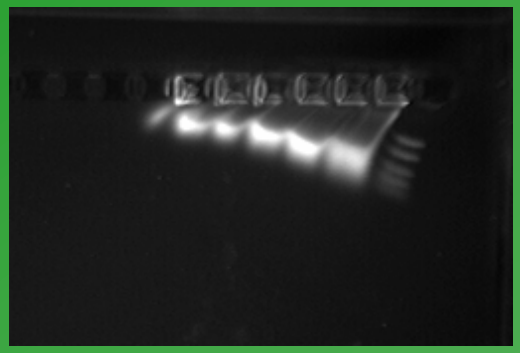


DNA Extraction 1



DNA Extraction 2

- From our gel electrophoresis results, we were able to conclude that each extraction yielded a usable amount of DNA for further experimentation.



DNA Extraction 3

- Originally, we chose four plasmids from the 2008 Parts Registry:

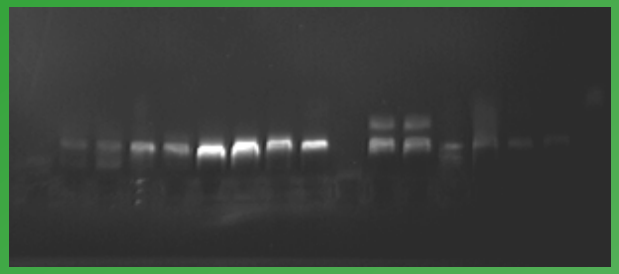
  (BBa_I719005 T7 Promoter Library: Spring 2008 Well: 3G Plasmid: pSB1A2; BBa_I712074 T7 promoter (strong promoter from T7 bacteriophage) Library: Spring 2008 Plasmid: pSB1AK8; BBa_I715038 pLac-RBS-T7 RNA Polymerase Library: Spring 2008 Well: 6H Plasmid: pSB1AK3; BBa_J13002 TetR repressed POPS/RIPS generator Library: Spring 2008 Plasmid: pSB1A2).

- Restriction enzymes were examined on the Fermentas Life Science website and we found that our gene contains an EcoR1 site. To make the gene biobrick compatible, we would have performed site directed mutagenesis to remove the EcoR1 site.

Registry Parts

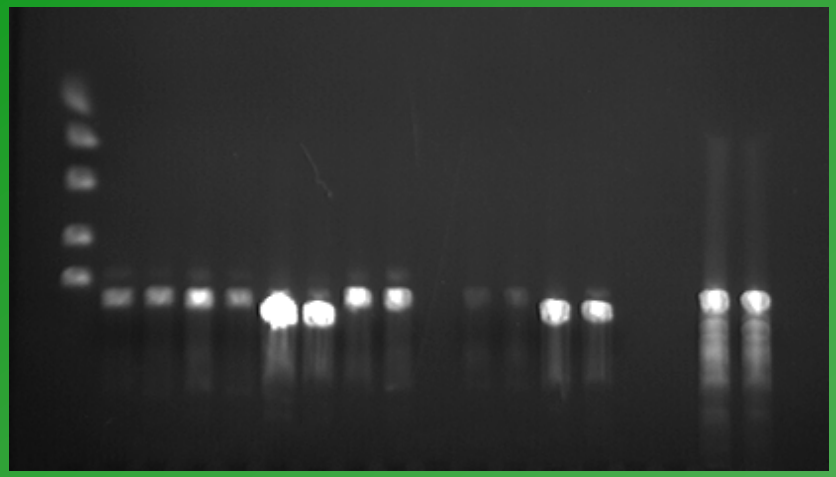
- From our original selection of plasmids from the 2008 parts registry, we were unable to transform our plasmids into competent to obtain clones to use as our vector for the onion DNA.
- We then selected new plasmids from the 2007 registry, but again we were unsuccessful.

- We were then provided with other plasmids to use as vectors, which yielded the results we needed.
- With these plasmids, we made glycerol stocks to keep cells viable for later use.
- Next we made mini-preps, using the Fermentas Life Sciences protocol (kit) to isolate the plasmids and verify the identity of the plasmids.



Plasmid DNA 1

Second trial of gel electrophoresis of the plasmid DNA.

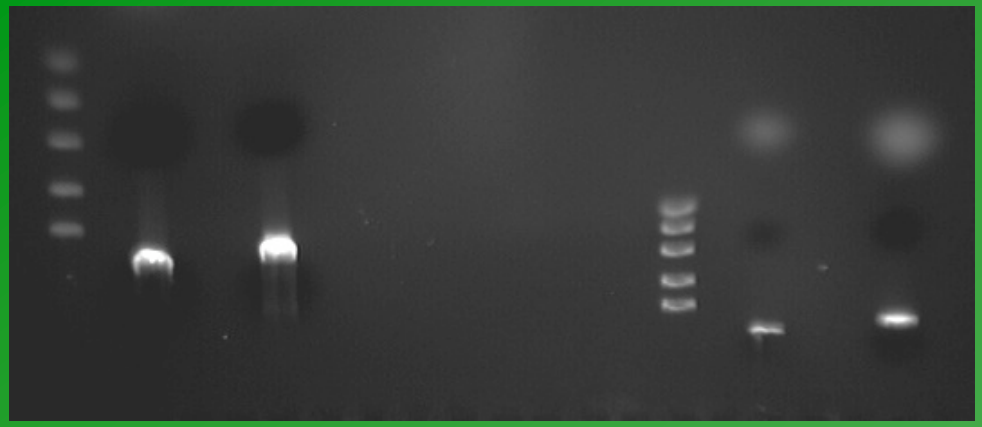


Plasmid DNA 2

- We then chose two plasmids (R0080 and pSB1A3) to purify to prepare them for insertion ALL1 gene.
- We cut the plasmids using Pst 1 and Spe 1.
- The protocol we used was from the QIAquick Purification kit.

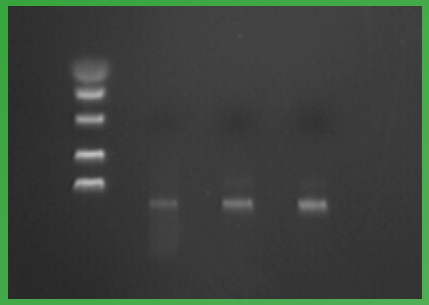
Results

Trial 1 Trial 2



Plasmid DNA (R0080 & pSB1A3)

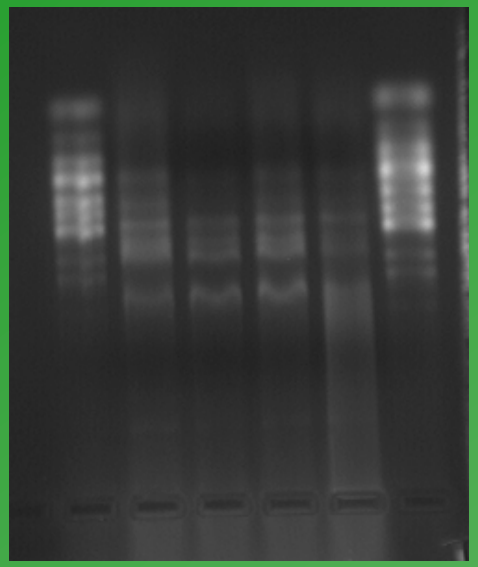
We then cut and purified our remaining plasmids (R0010, R0040, and R0051).



Plasmid DNA (R0010, R0040, R0051)

RNA Purification

- Our next step was to extract and purify the RNA from the onion tissue using the QIAquick RNA purification protocol and kit.



Onion RNA

Goals

- In order to clone our All1 gene we needed to first check our primers. We performed PCR using our DNA to make sure our primers worked. If the primers worked we would be able to extract RNA and use the primers to amplify RNA since they don't contain introns.

Checking Primers with Onion DNA

Attempt #1

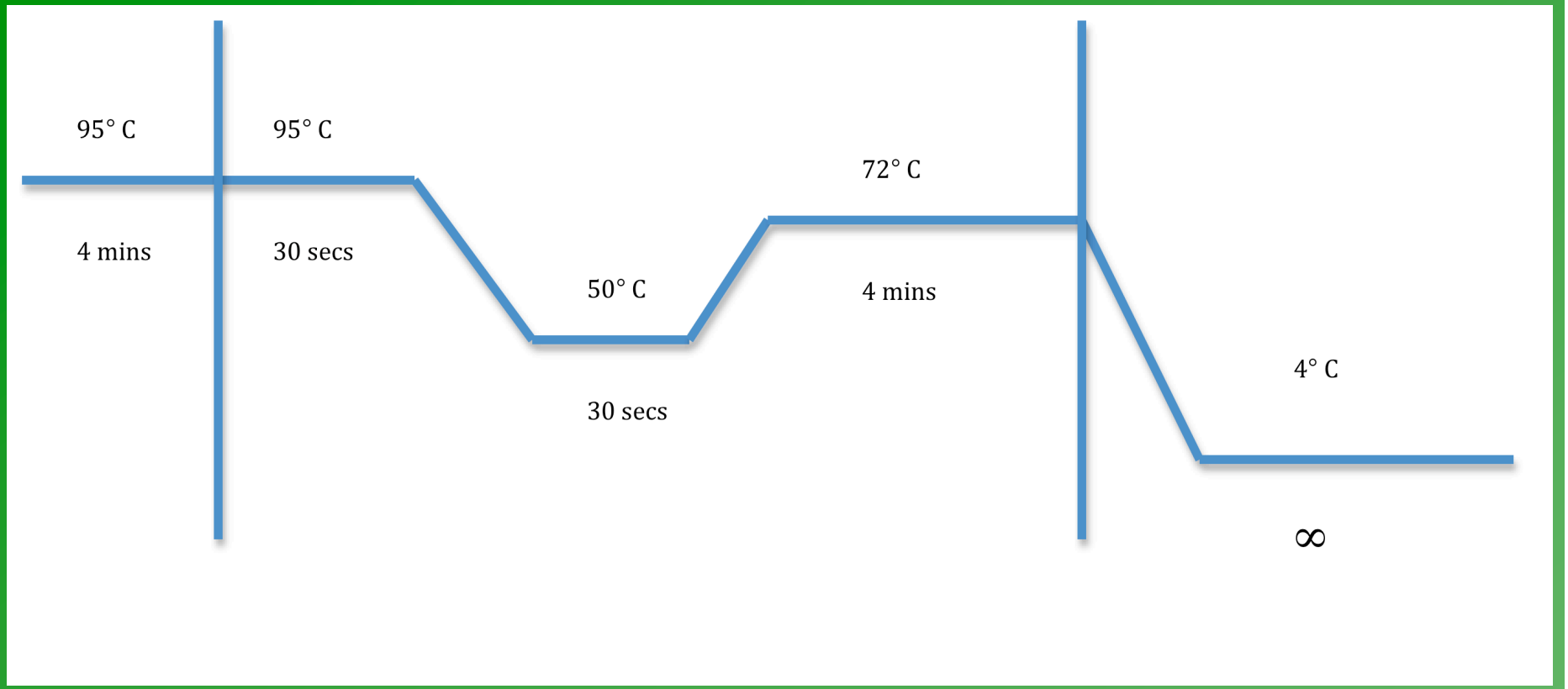
- DNA was used from leaves and roots.
- We ran PCR to check if our primers would work with our DNA.
- The protocol that we used was found on openwetware.com

Checking Primers with Onion DNA

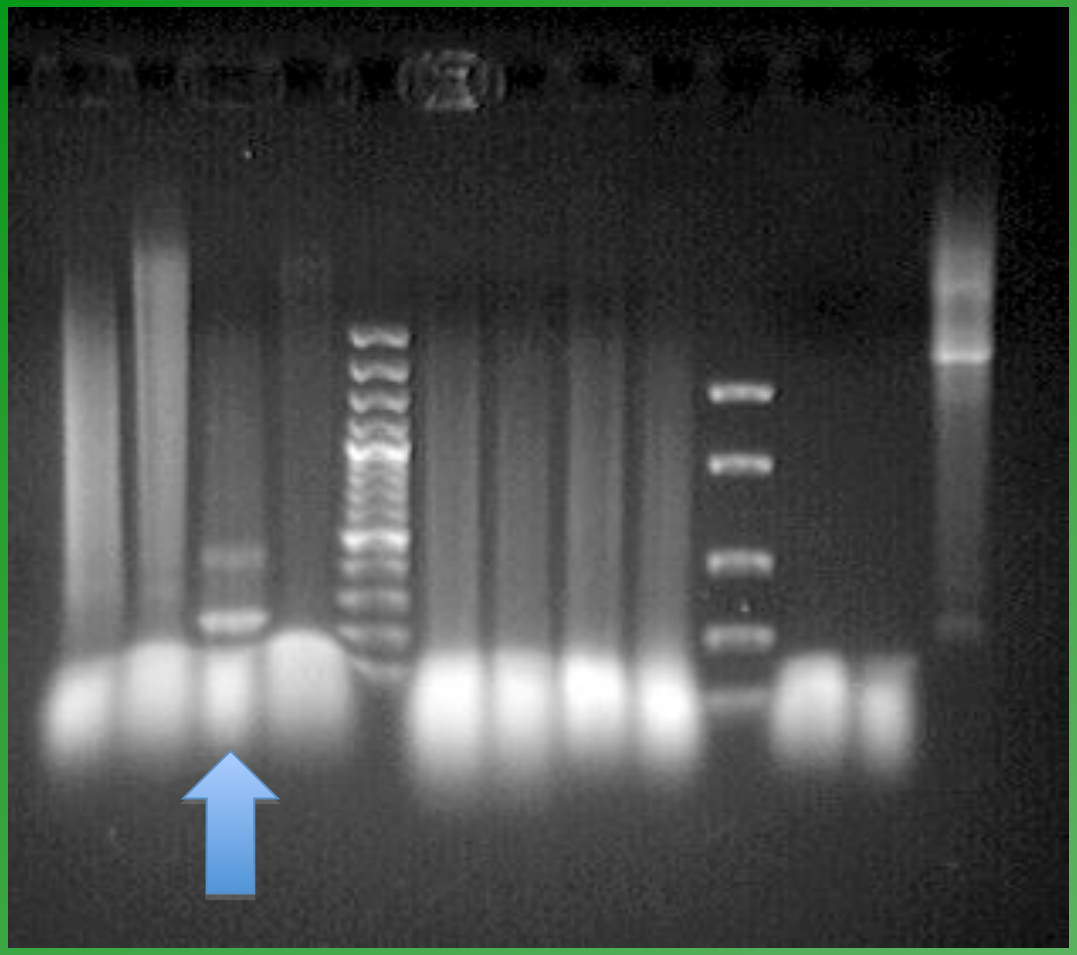
Attempt #1

- We are using two forward primers (F1, F2) and one reverse (R).
- We are using two forward primers since there are two signal peptides in our gene of interest.
- Expectations:
  - F1 & R: 1817 basepairs
  - F2 & R: 1714 basepairs

PCR Cycle Attempt 1



Results

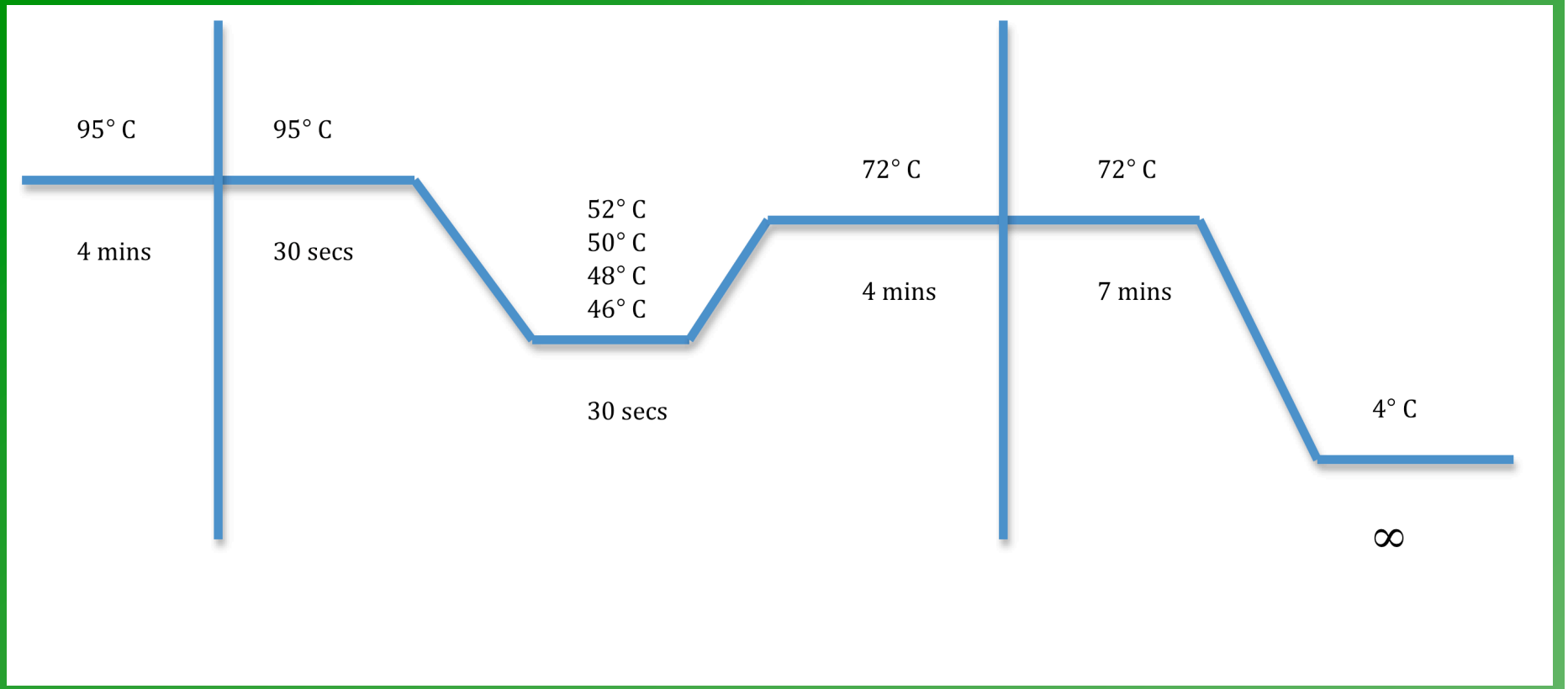


Checking Primers with Onion DNA

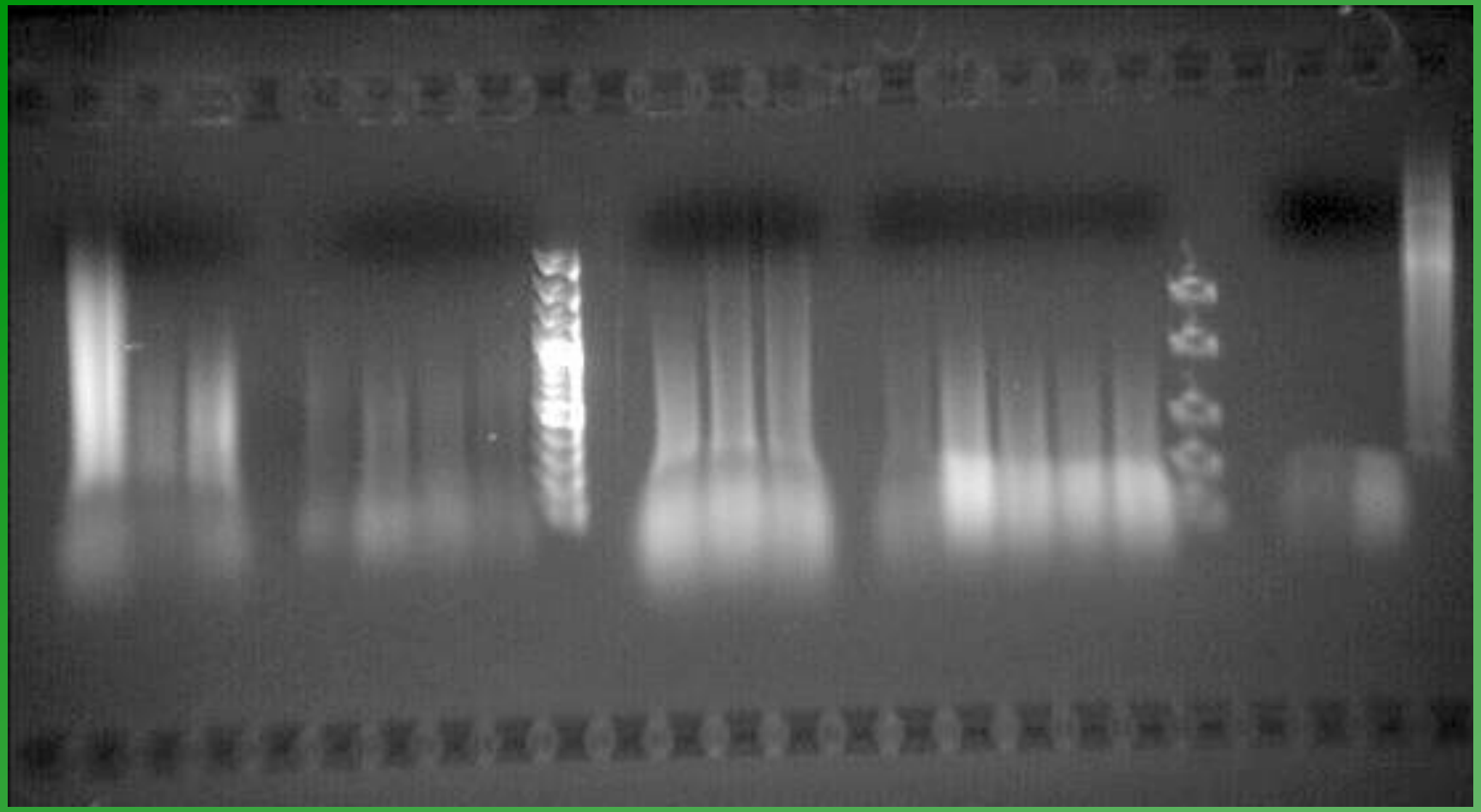
Attempt #2

- Same primers were used along with the same protocol with the exception of the temperature gradient.
- We also added in a step after the PCR cycle at 72° for 7 minutes.
- Expectations:
  - F1 & R: 1817 basepairs
  - F2 & R: 1714 basepairs

PCR Cycle Attempt #2



Results

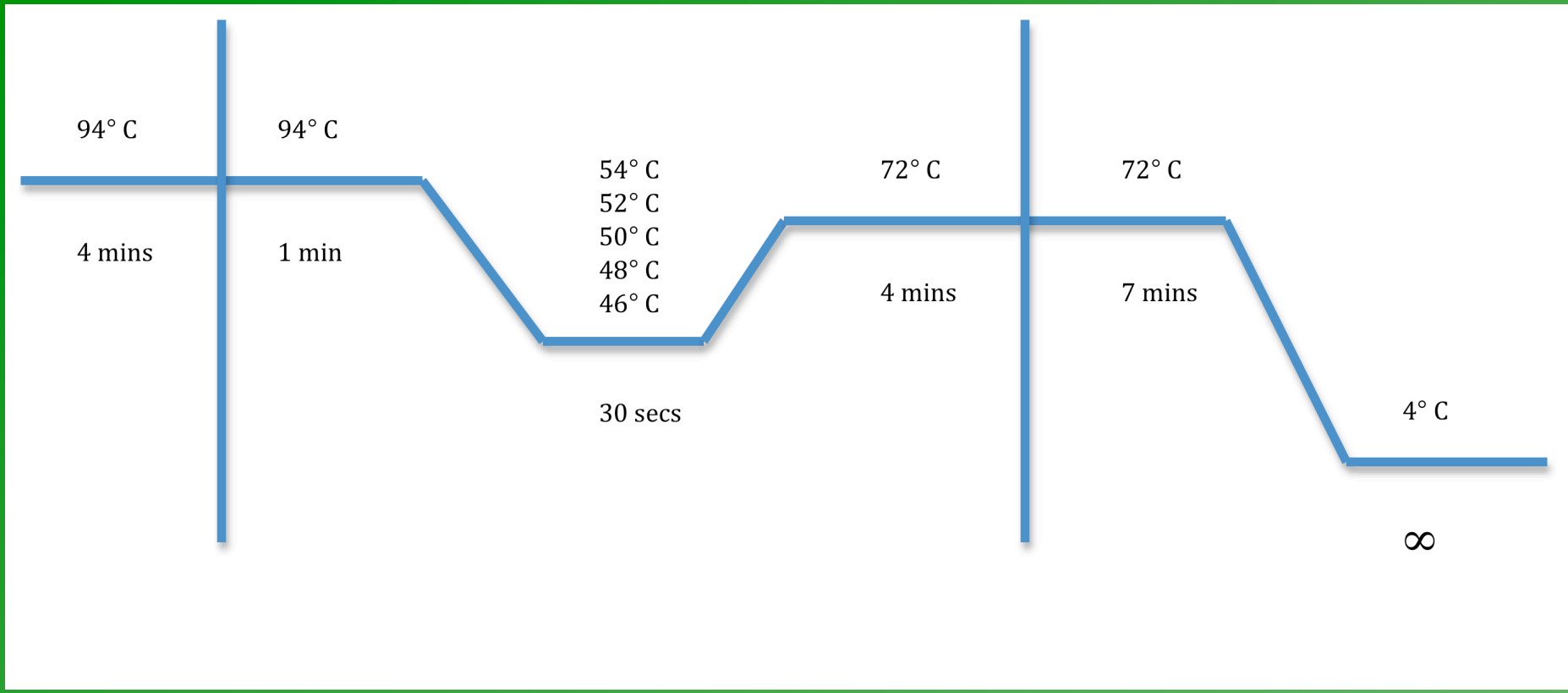


Checking Primers with Onion DNA

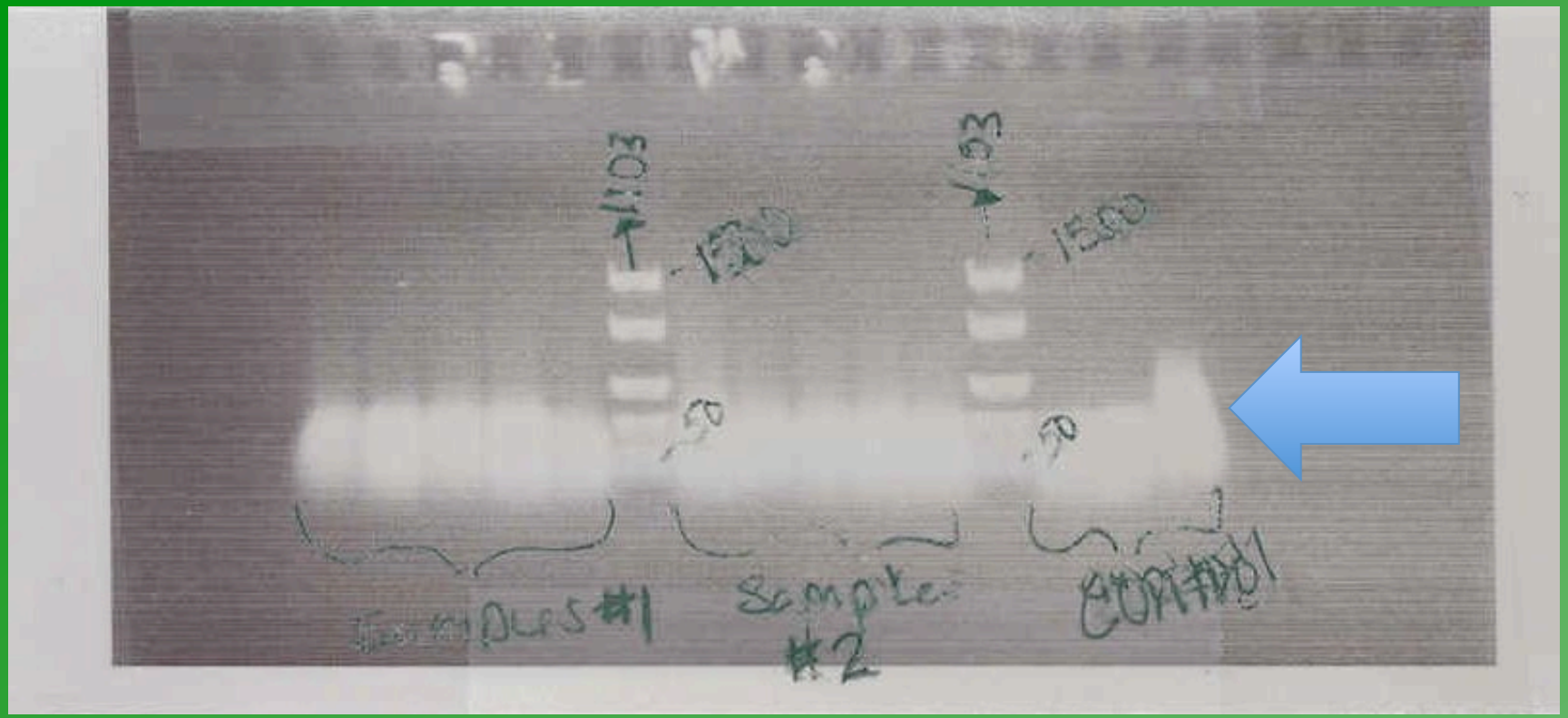
Attempt #3

- This PCR was performed after extracting DNA for a second time. Extraction of DNA was purely from our leaves since previously we did have some results.
- We also extended our denaturation time from 30 seconds to one minute.
- Expectations:
  - F1 & R: 1817 basepairs
  - F2 & R: 1714 basepairs

PCR Cycle Attempt #3



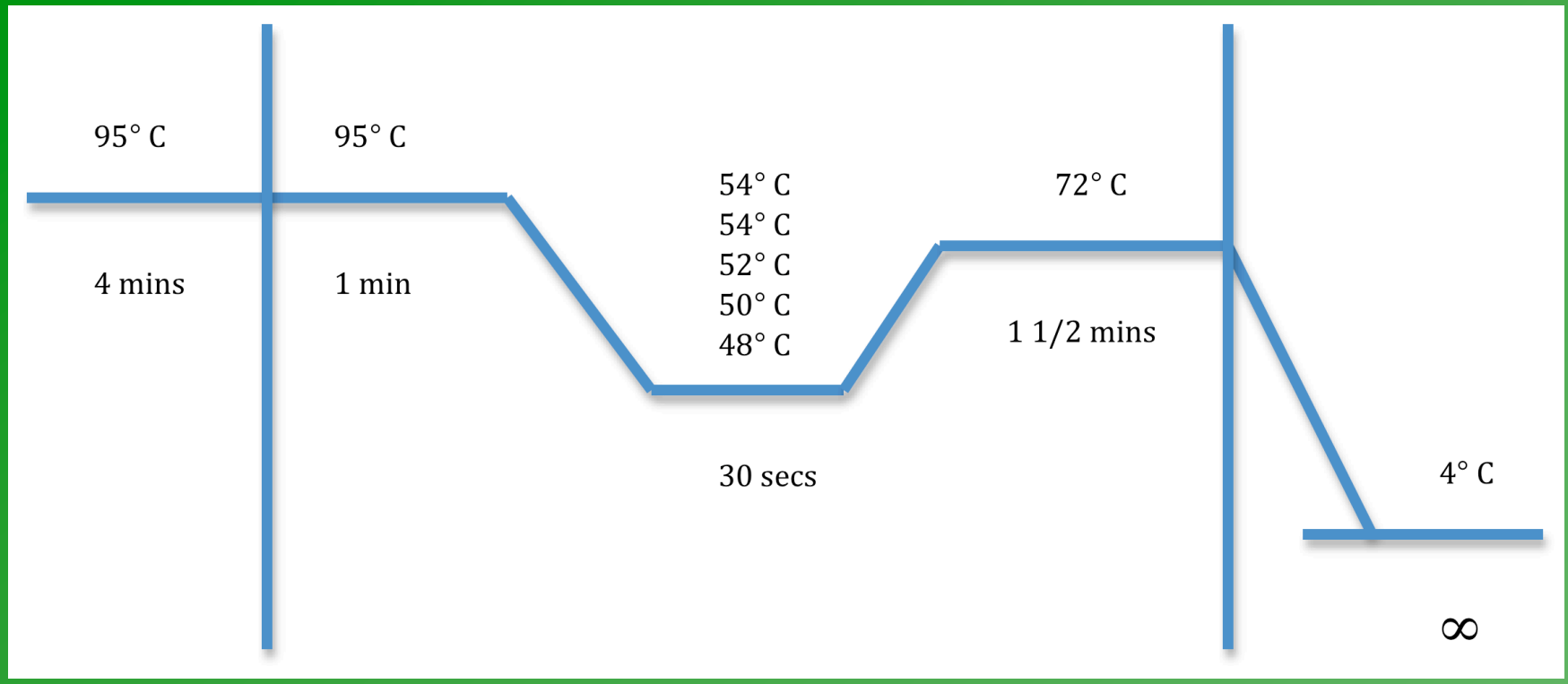
Results



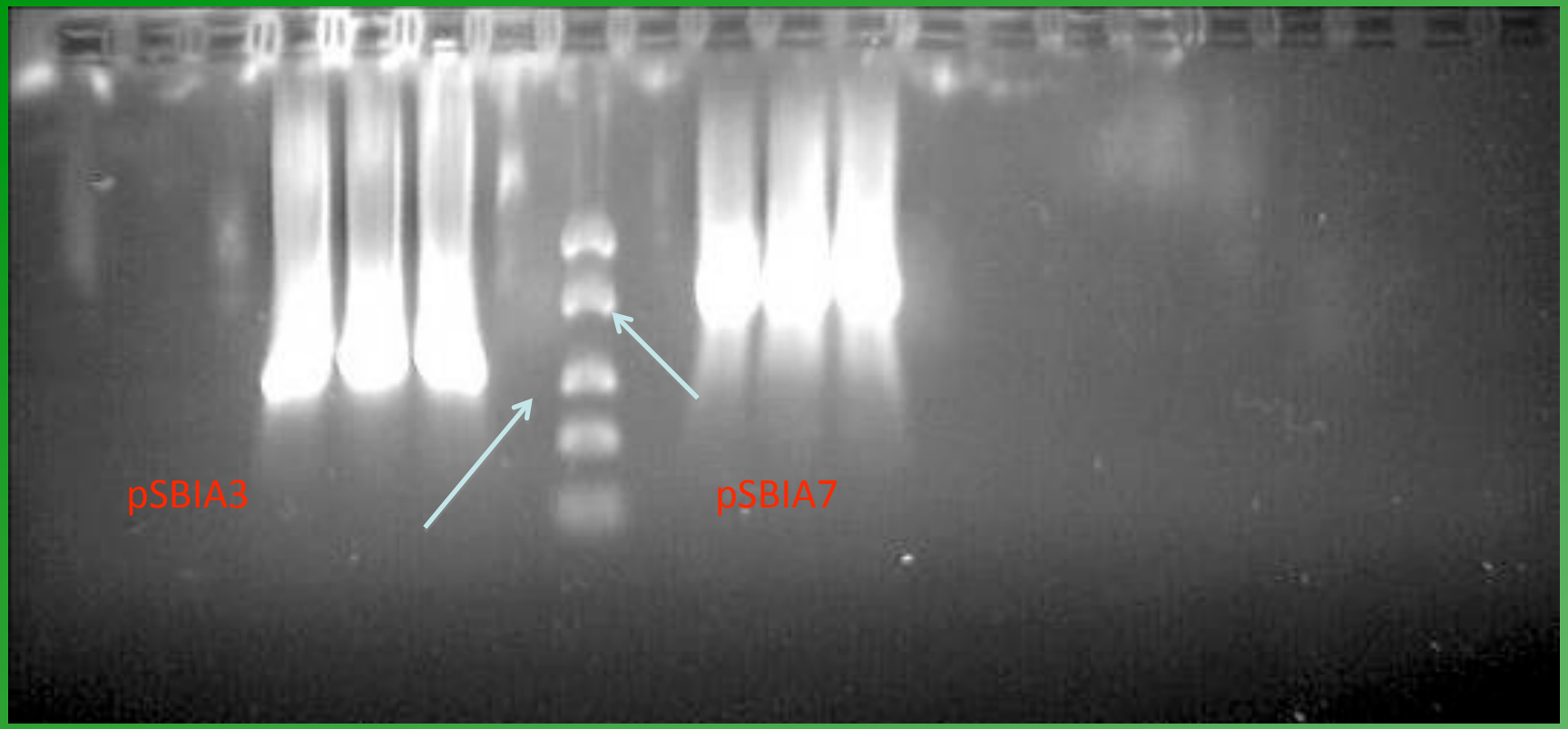
Trouble Shooting PCR

- Since our positive control did not work in the last experiment we decided to check and ensure that our mastermix was working.
- To do this we are running positive controls with two plastmids: pSBIA3 & pSBIA7
- Expectations:
  - pSBIA3: 362 basepairs
  - pSBIA7: 667 basepairs

Trouble Shooting PCR



Results

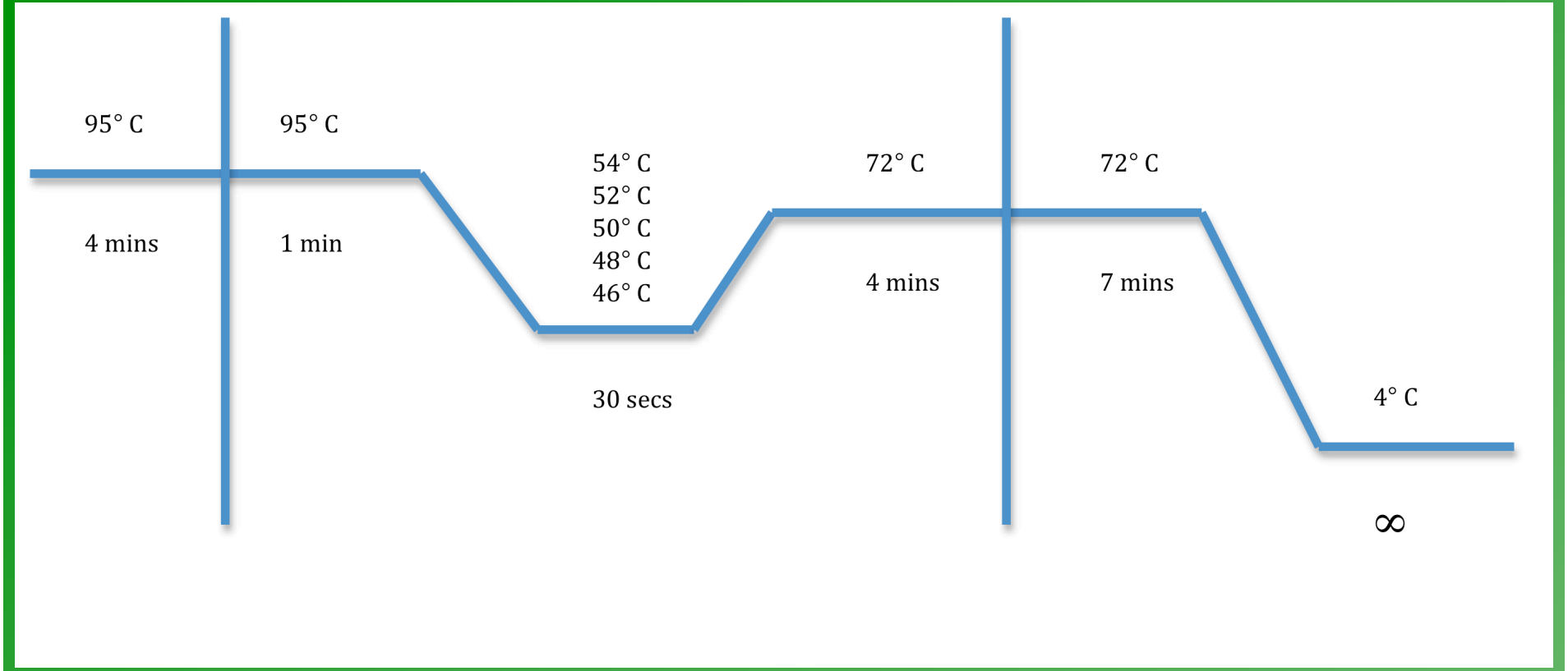


Checking Primers with Onion DNA

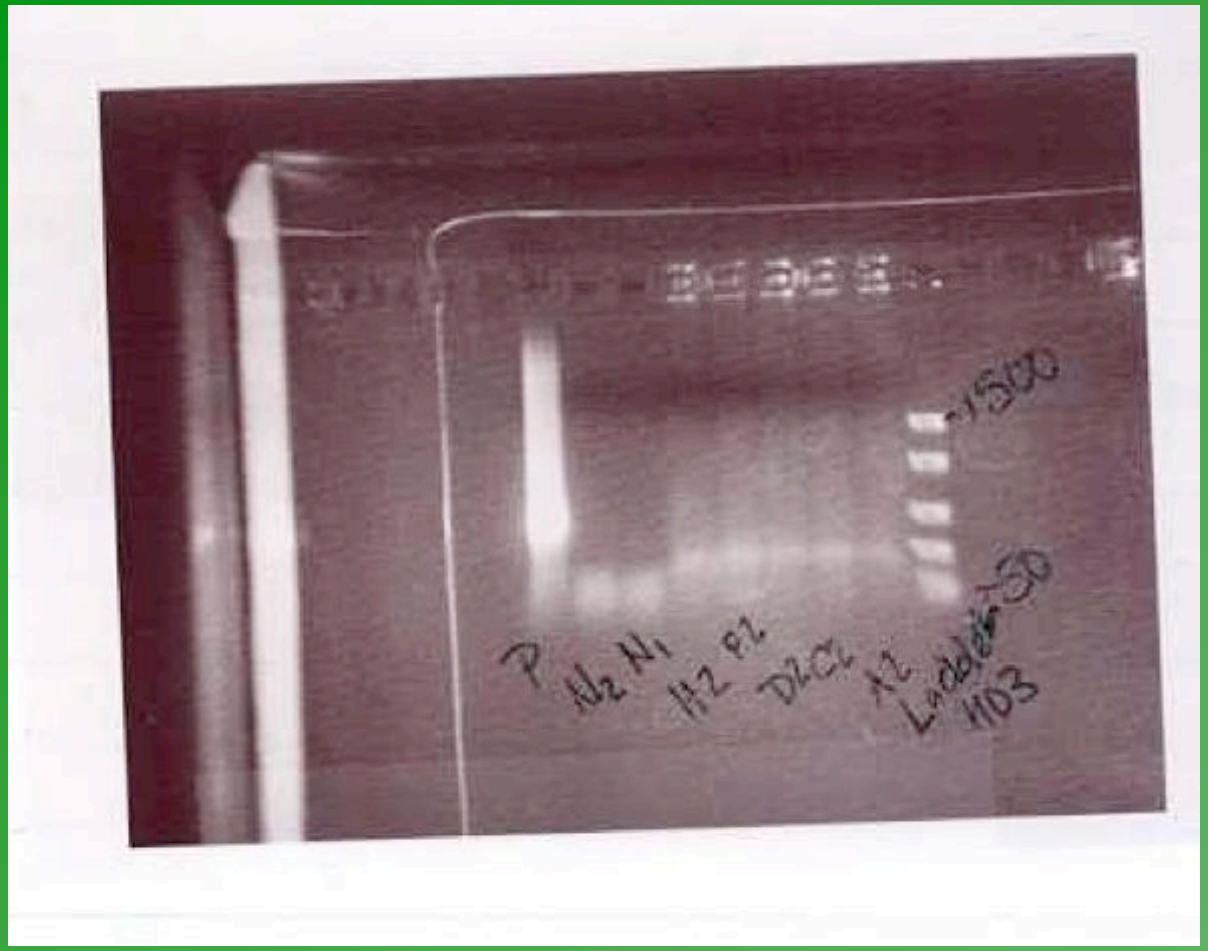
Attempt #4

- We know our mastermix is working so we will continue using the same mastermix with our primers.
- One modification, we are using pSBIA7 as our positive control since we already know it works.
- Expectations:
  - pSBIA3: 362 basepairs
  - pSBIA7: 667 basepairs

PCR Cycle Attempt #4



Results



Designing New Primers

- Since there has been little luck with our primers we designed 5 new primers.
  - P1: F1 primer without extension
  - P2: F2 primer without extension
  - R1: R primer without extension
  - P3: From IDT's website
  - R2: From IDT's website
- P3 & R2 include our gene of interest plus a little extra.

Checking New Primers with Onion DNA

Attempt #5

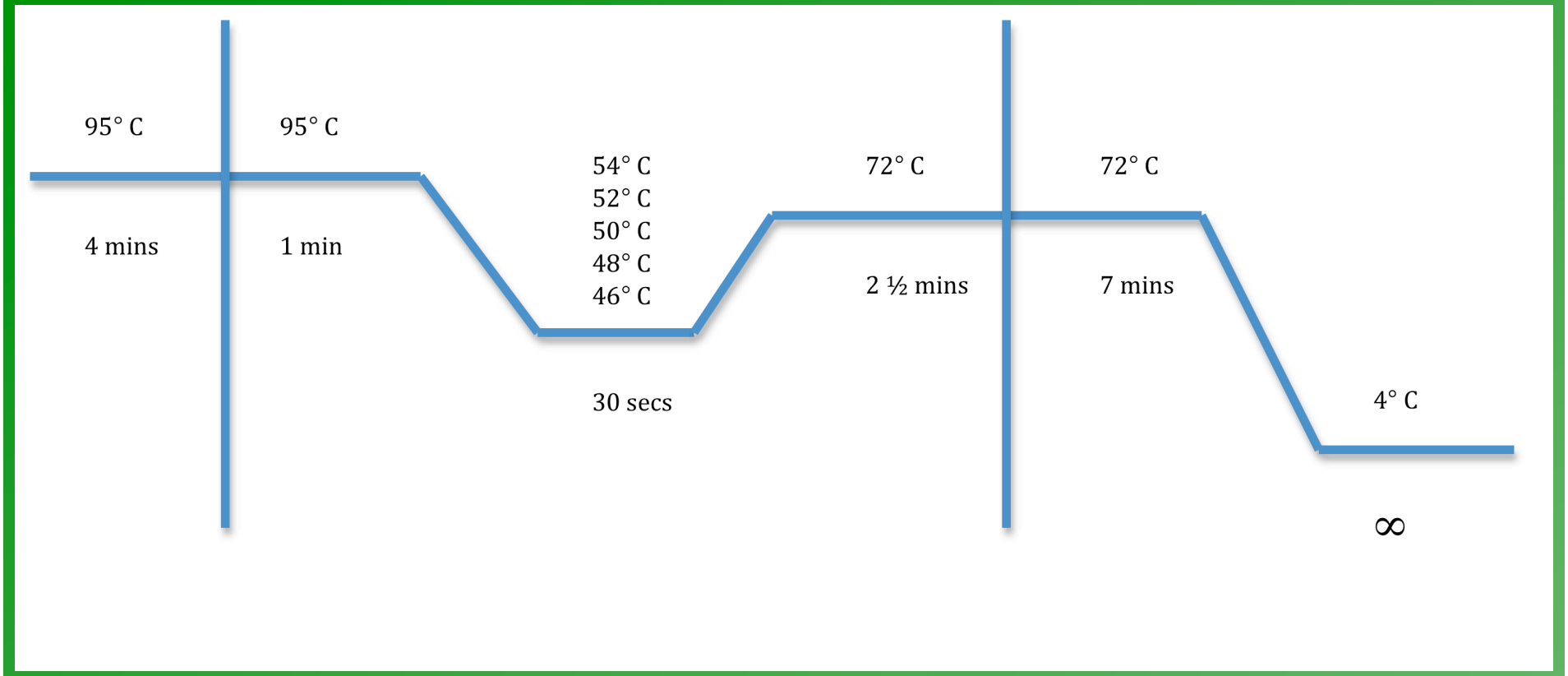
- PCR will be the same except our extension time changed from 4 minutes to 2 ½ minutes.
- We used 6 samples and 3 different temperatures for each sample and 2 DNA concentrations: 1) Our original DNA concentration 2) 1:10 dilution DNA
  - 36 samples plus 2 controls

Checking New Primers with Onion DNA

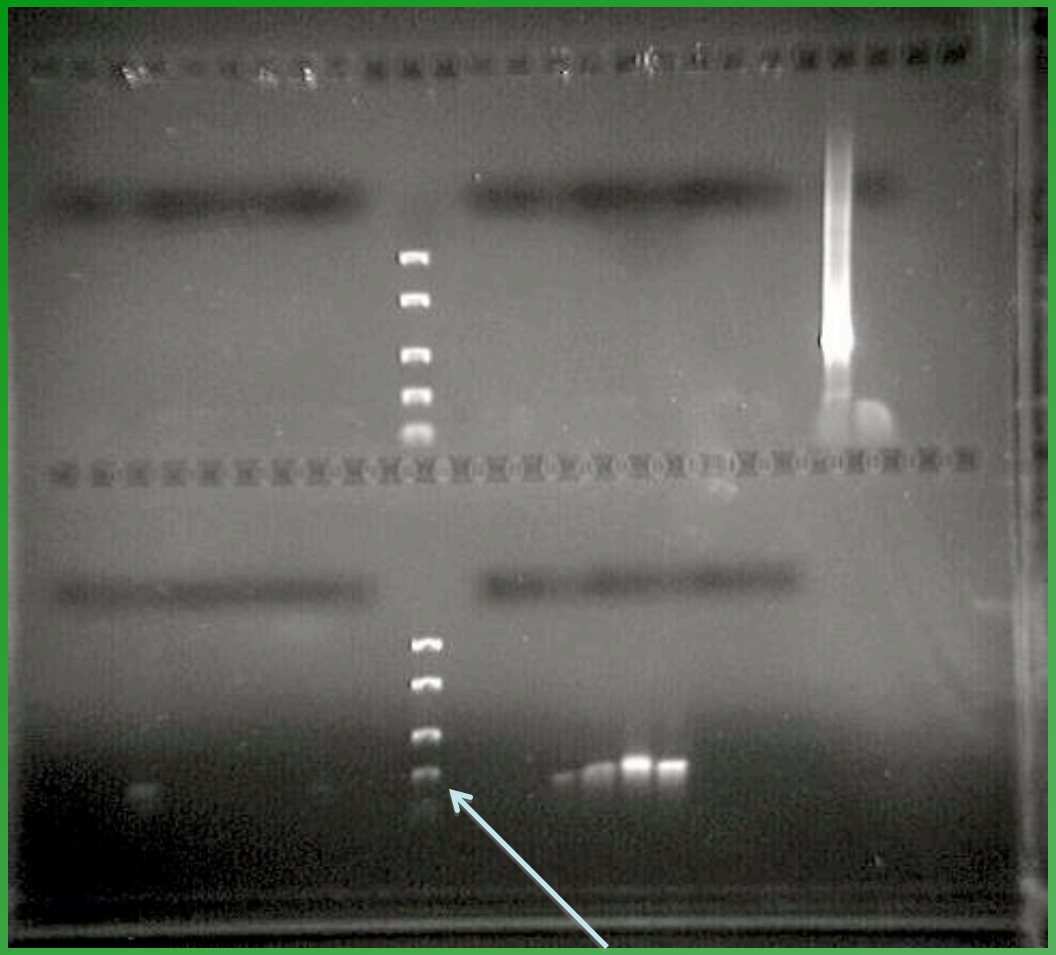
Attempt #5

- Expectations:
  - P1 & R1: 1816 basepairs
  - P1 & R2: 1714 basepairs
  - P2 & R1: 1942 basepairs
  - P2 & R2: 2107 basepairs
  - P3 & R1: 2005 basepairs
  - P3 & R2: 2233 basepairs

PCR Cycle Attempt #5



Results



Normal DNA Concentration

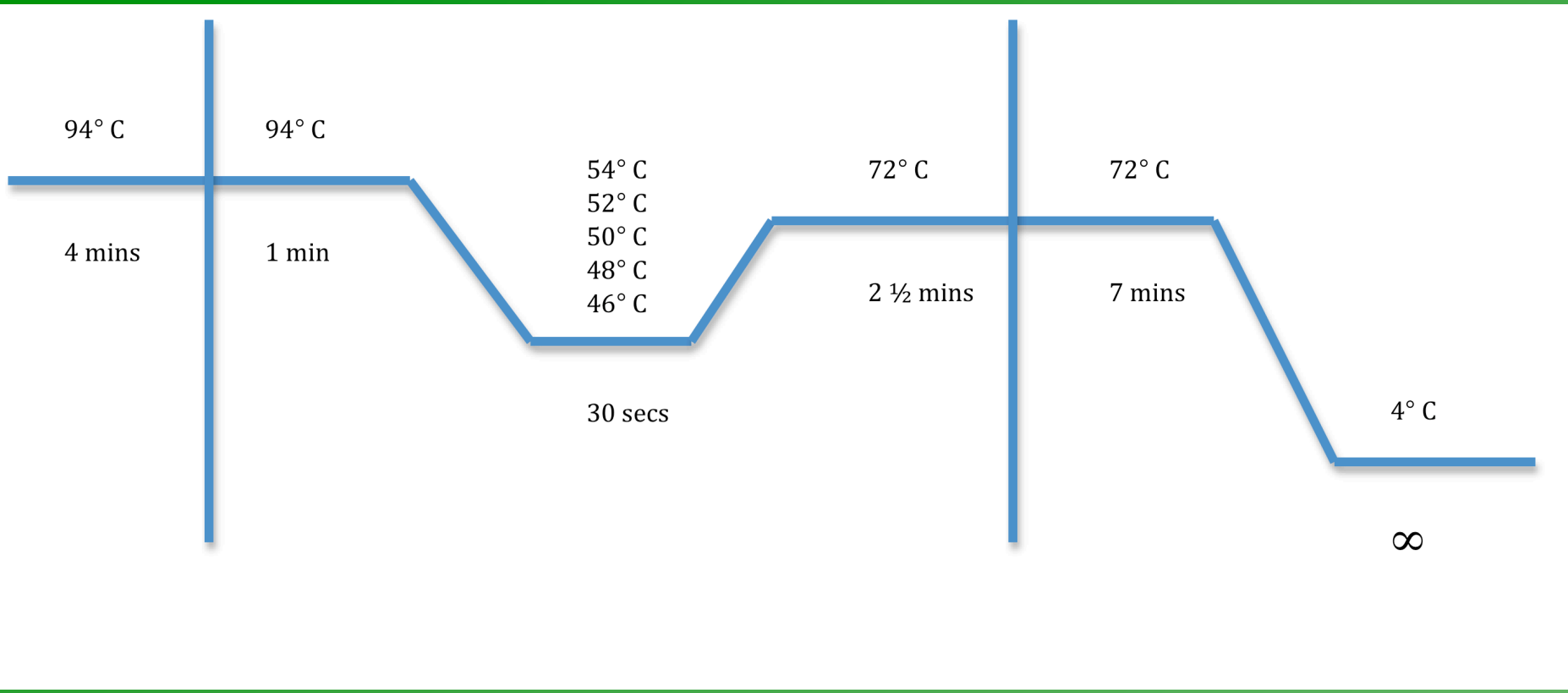
1:10 Diluted DNA concentration

Checking Primers with Onion DNA

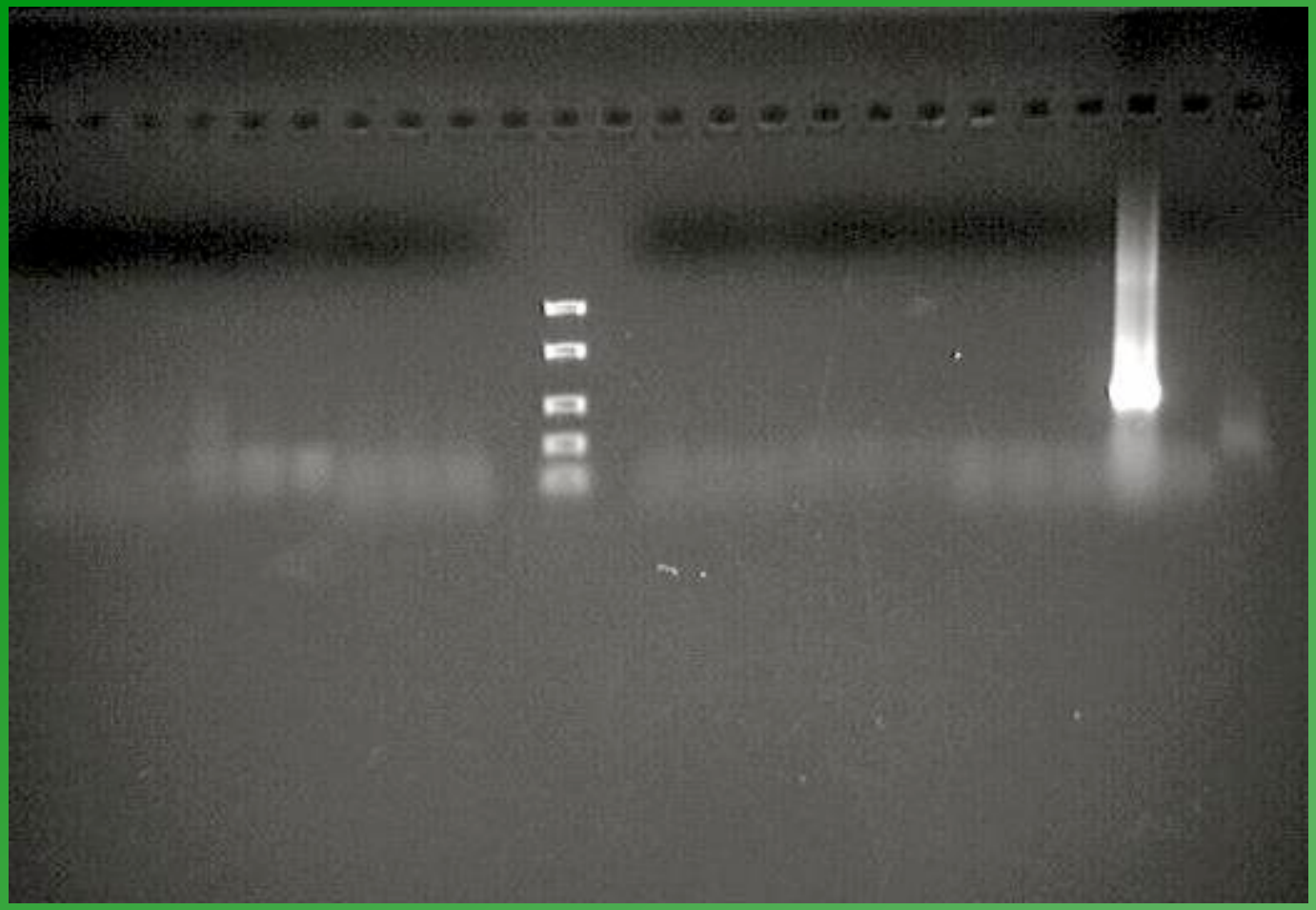
Attempt #6 with Purification

- Since some results were seen with the diluted DNA, we concluded that our DNA has some impurities that is interfering with our primers. So we purified our DNA with PEG.
- We are using the same PCR cycle as previously.
- Expectations:
  - P1 & R2: 2107 basepairs
  - P2 & R2: 2005 basepairs
  - P3 & R2: 2233 basepairs
  - HOPS DNA: 320 basepairs
  - HOPS RNA: 250 basepairs

PCR Cycle #6


94° C
4 mins
94° C
1 min
54° C
52° C
50° C
48° C
46° C
30 secs
72° C
2 ½ mins
72° C
7 mins
4° C
∞

Results



Checking New Primers with Onion RNA

Attempt #1

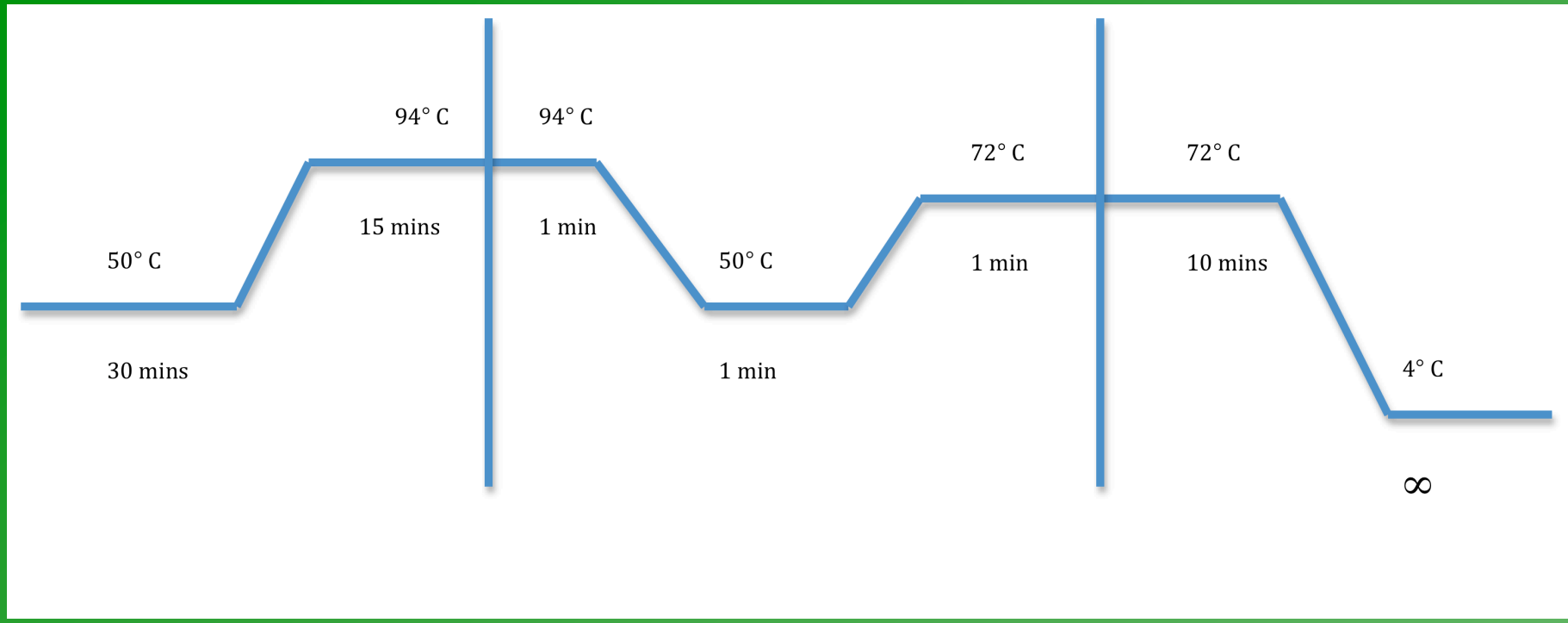
- Since we no longer had any DNA left we decided to run a RT-PCR with our extracted RNA.
- We used HOPS as an internal control along with pSBIA7 as a positive control.
- The only difference in RT-PCR and PCR is the added RT cycle. Also one sample is left in the ice.
- We used the protocol QIAGEN OneStep RT-PCR Kit

Checking New Primers with Onion RNA

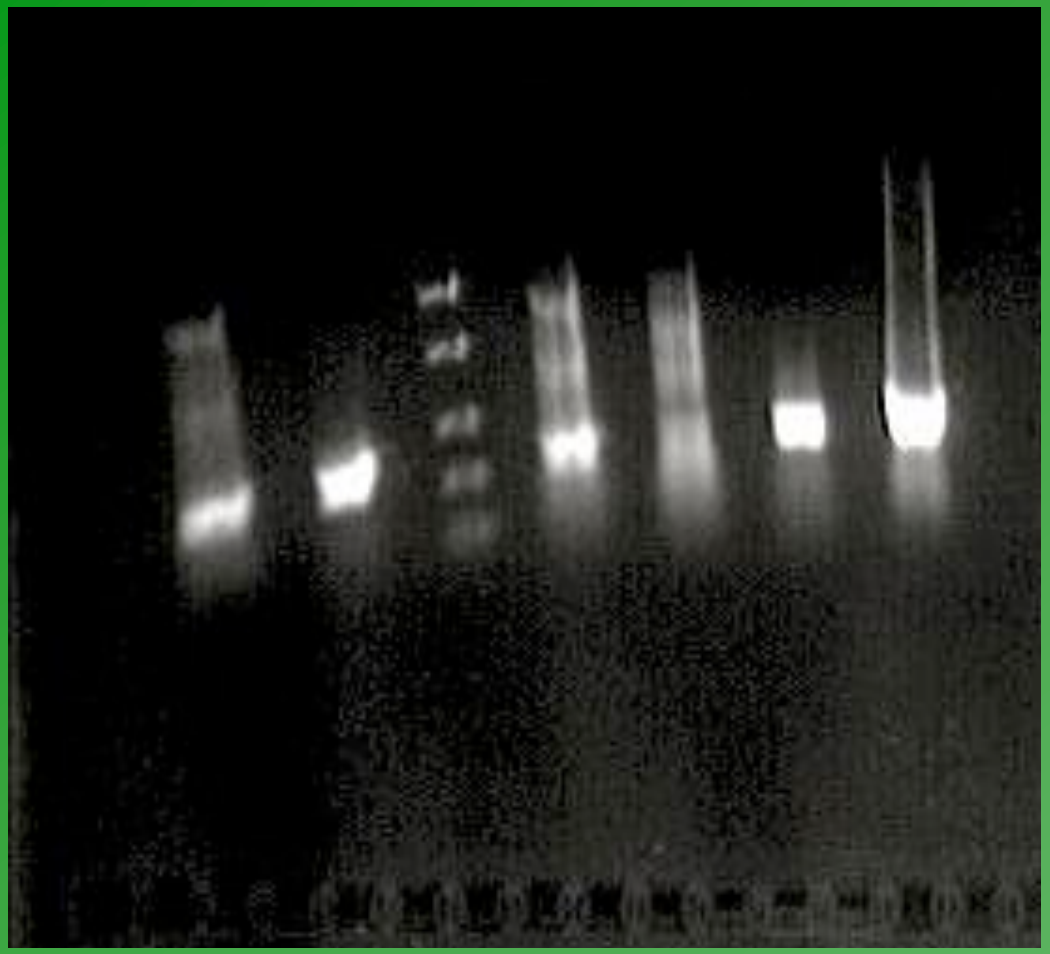
Attempt #1

- Expectations:
  - P1 & R2: 1734 basepairs
  - P2 & R2: 1632 basepairs
  - HOPS DNA: 320 basepairs
  - HOPS RNA: 250 basepairs

RT-PCR Cycle Attempt #1



Results

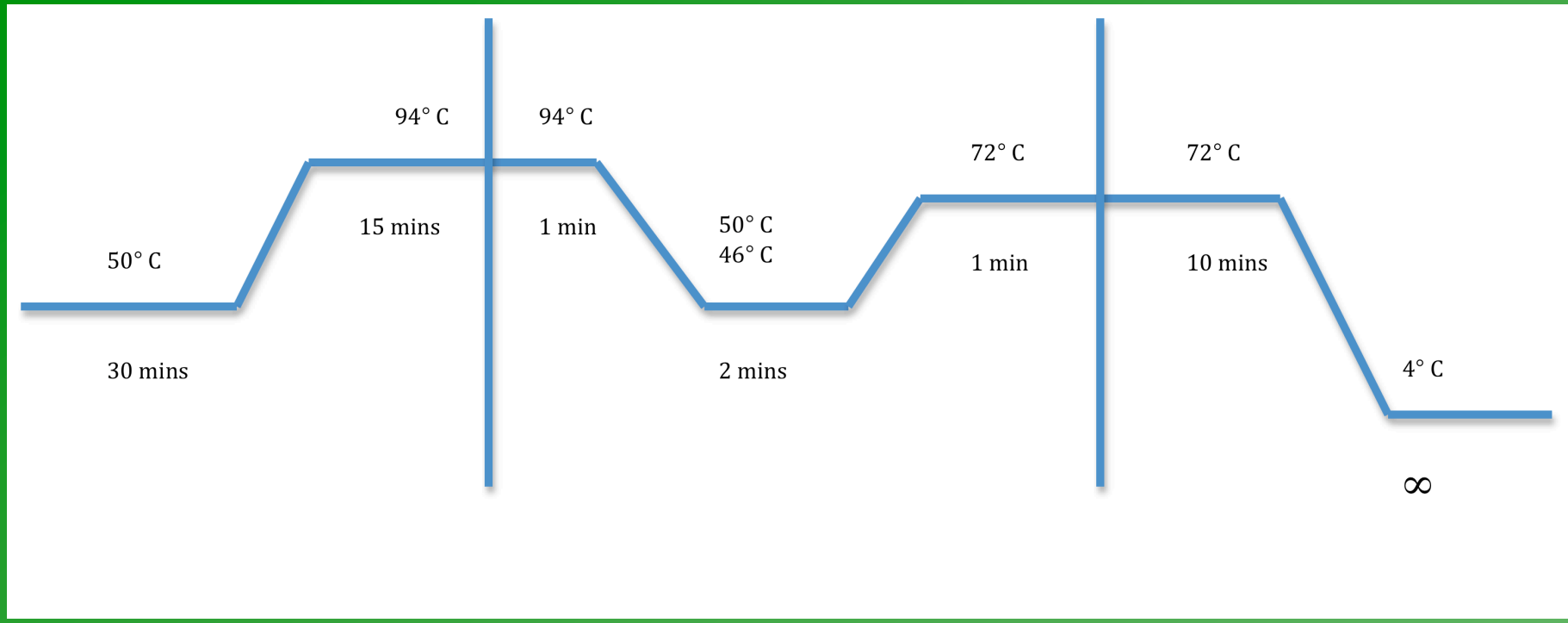


Checking Primers with Onion RNA

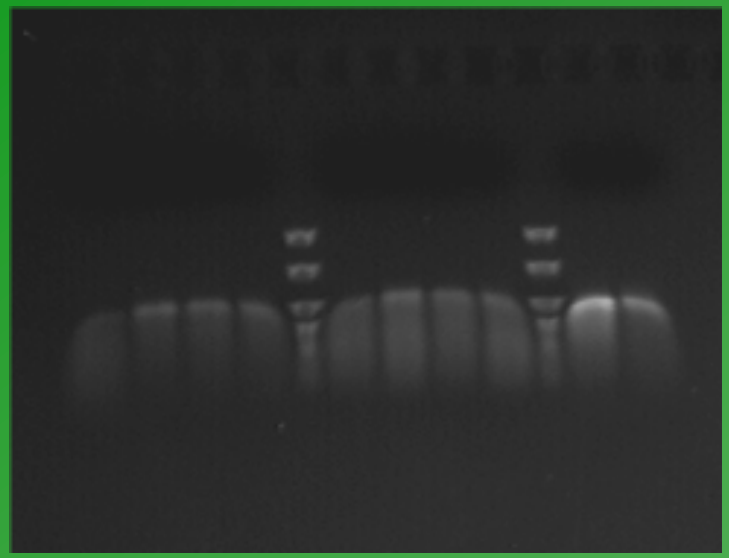
Attempt #2

- This is the same protocol as pervious with the exception of a temperature gradient. We are now using two different temperatures.
- We also changed our annealing time from 1 minute to 2 minutes.

RT-PCR Cycle Attempt #2



Results



RT-PCR of RNA

Conclusions

- We successfully extracted good-quality DNA and RNA from the onion tissue.
- We also successfully purified our plasmids to use as vectors.
- We performed PCR to amplify the DNA, but did not receive significant results.
- We also performed RT-PCR with new primers to amplify the RNA, but we did not receive significant results from this either.