Optimizing RT-LAMP Primers Targeting *Mycobacterium tuberculosis* for DRonA Universal Susceptibility Test (DUST)

Jin Ah Bellefuil (Ballard High School) and Sophie Zhang (Skyline High School)

July 5th, 2022 - August 19th, 2022

Mentors: Dr. Eliza Peterson and Julie Do

Baliga Lab

Introduction/Background/Aim:

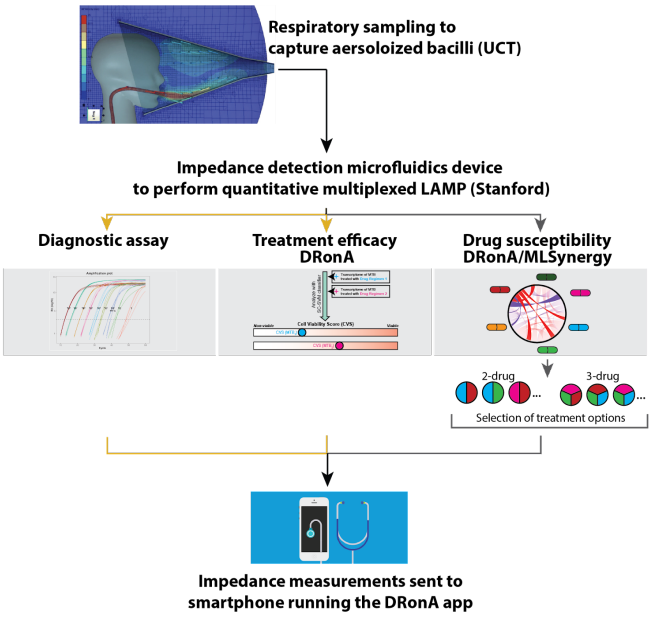
Mycobacterium tuberculosis (Mtb) is a species of bacteria that causes tuberculosis, a disease that mainly attacks the lungs, but can also attack other parts of the body such as the spine, brain, and kidney. Antibiotics have been developed to treat the disease, but multidrug resistant (MDR) strains of Mtb are on the rise, and are projected to cause around one quarter of antimicrobial resistance deaths in the world. Mtb is a slow growing pathogen that is able to grow into phenotypically diverse subpopulations, allowing it to survive various drug treatments. The World Health Organization (WHO) stated a need for a fast and affordable drug susceptibility test (DST) that tests if drug-resistant Mtb is present and which drugs will be able to inhibit the growth of the bacterium, allowing for early selection of treatments for patients with Mtb.

Current methods to observe Mtb response to drug treatments have many limitations. The lack of knowledge of resistance-causing genes and mutations of Mtb leads to limitations of a genotypic DST. There are also phenotypic DST methods. For example, by growing Mtb on a solid agar plate, the amount of colonies formed can be counted, which are known as colony forming units (CFU). Additionally, the time it takes for a liquid culture sample to become positive for Mtb can be measured, providing the term time to positivity. Both these phenotypic DST methods must be grown over long periods of time, which can lead to contamination. Although these culture-based phenotypic DST can directly determine Mtb response to antibiotics, they are time-consuming as results may not be known for weeks. Furthermore, these DST are unable to distinguish dead versus non-cultivable (dormant) Mtb cells, leading to potential false negatives. These limitations lead to a demand for a more efficient method, which is answered by a DST utilizing DRonA (DRoNA universal susceptibility test or DUST).

Drug Response Assayer (DRonA) is a machine learning algorithm that calculates the cell viability score (CVS) of Mtb to a panel of drugs, quantifying the degree of susceptibility Mtb DNA is to combinations of antibiotics. The DRonA algorithm uses a multigene transcriptional signature of cell viability to accurately detect Mtb susceptibility to diverse antibiotics, regardless of their mechanism of killing and treatment context. The algorithm was calculated through publicly available transcriptomes of Mtb grown in diverse conditions to detect gene signatures

that connect to loss of viability. The degree of deviation of the transcriptomes fed into DRonA from the viable Mtb transcriptomes in the DRonA database calculates the CVS. This CVS provides how susceptible Mtb is to the combination of drugs. Antibiotic exposure associated with loss of survivability triggers characteristic transcriptional responses in susceptible, but not resistant Mtb, providing specificity for detection of bacteriostatic and bactericidal effects within a few minutes. DRonA can be leveraged in this manner to create a culture-free phenotypic DST that meets the WHO's target product proposal profiles (TPP) for TB DST. DUST can deliver drug susceptibility results within hours with high sensitivity and specificity for any strain of Mtb (including multi-strain infections).

DRonA initially used whole transcriptome profiling with RNA-seq, an expensive, technically demanding, and often time-consuming process to discover and analyze the transcriptome. This diminished the practicality of DUST in low-resource, developing regions that have the majority of TB cases. However, DRonA is capable of effectively detecting Mtb drug susceptibility with as few as 12 transcripts. This allows loop-mediated isothermal amplification (LAMP) paired with a microfluidics device to provide point-of-care application to those resource-poor, endemic TB areas without nucleic extraction. RT-LAMP can be used to obtain the quantified gene expression (transcriptomes) of Mtb RNA. Hybridization probes emit different levels of fluorescence during the test, which quantifies the gene expression level through the measuring of mRNA. The quantified gene expression of Mtb RNA is then fed into the DRonA leveraged DST to determine a CVS.



(Srinivas et al., 2021)

LAMP assays are also involved in the simple diagnosis of TB. Previous research has shown LAMP as a promising new assay able to rapidly diagnose viral, bacterial, and parasitic diseases by finding whether the pathogen's DNA or RNA is present. LAMP is able to amplify

DNA/RNA rapidly and effectively at a constant temperature (isothermal), using loop primers to accelerate the process. Four to six primers that identify specific regions of target DNA are needed to perform a LAMP assay. Amplification begins with strand invasion by one of the inner primers (step 1). Then a strand-displacing DNA polymerase extends the primer and separates the two strands of target DNA (step 2). An outer primer that anneals to an upstream target region (step 3) displaces the end of the product formed by the primer (step 4) to form a self-hybridizing loop structure (step 5/6). The annealing and displacing process is repeated on the opposite end of the target sequence, creating a dumbbell structure, which forms multiple sites for initiation of synthesis, creating a seed for exponential LAMP amplification (step 6/7/8). In our project, we are using RT-LAMP, which has an added step of reverse transcription (RT) of RNA to DNA using reverse transcriptase to allow for the amplification process to proceed.

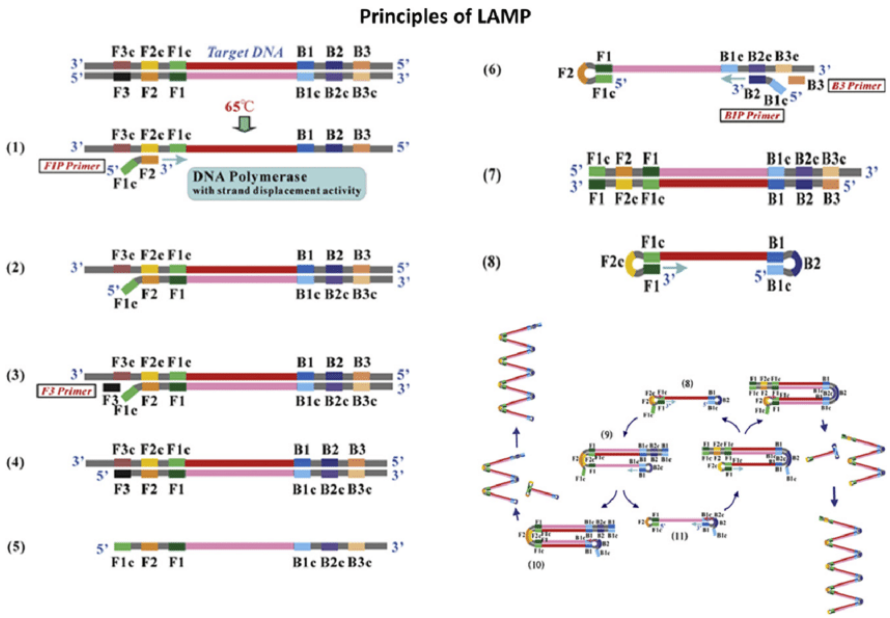


Fig. 5 e Principle of RT-LAMP

LAMP assays do not have the restrictions of many current assays, making them favorable new candidates for diagnosis. PCR-based assays are promising new methods but are also expensive and technically demanding, making them inaccessible in developing countries which have the majority of TB cases. Current conventional methods of diagnosis are often unreliable, time-consuming, and technically demanding as well, leading to the demand for a new method. Because LAMP reactions are performed at a fixed temperature (60-65 degrees Celsius), both time and money can be saved. LAMP reactions result in a highly efficient synthesis of amplified products, around 50 times higher than PCR reactions. Most notably these reactions result in high amounts of insoluble salt of magnesium pyrophosphate as a by-product. This allows a visual detection of positive reactions (turbidity) with the naked eye, saving time and money as there is no requirement for expensive machinery. However, the turbidity is only used for diagnosis purposes. In our project we use RT-LAMP to generate quantified gene expression values for DRonA to calculate a CVS.

Overall, the aim of our project is to redesign primers for specific genes in *Mycobacterium tuberculosis*, optimizing them for a more effective and specific LAMP assay. This in turn will contribute to the proposed DST utilizing DRonA, through more accurate quantified gene expression values. We will assess the effectiveness* of our primers through mean Ct values for the primer sets.

*Effectiveness could also be observed through a stronger correlation between already known RNA-seq gene expression values and calculated gene expression values from the LAMP assay. However, we cannot calculate these correlation values in our project due to our RNA being pooled from 4-6 T0 no drug samples from previous RNA extractions. The correlation needs to come from RNA-seq data for the exact RNA samples.

Methods/Process

In previous work, LAMP primer sets were designed to target 46 genes of Mtb with the highest weights (gene impact on viability). These primer sets were analyzed through their correlation of already known RNA-seq gene expression values and the calculated gene expression value from the LAMP assay. A correlation closer to one or negative one shows a more effectively designed primer set since the LAMP assay is able to generate values closer to the more expensive RNA-seq. Additionally, the effectiveness of the designed primers was analyzed by cycles to threshold (Ct) values, the amount of cycles of amplification needed until the Mtb DNA is detected. The lower the threshold, the more effective the primer is since less time is needed to detect Mtb.

Ranking Genes

Because our aim was to optimize LAMP primers for these genes, we first needed to determine which primer sets were performing the worst. To do this, the 46 sets were given two ranks: one for their correlation values and one for their Ct values. Correlation values closest to positive one or negative one and lower Ct values were given a lower rank. The higher ranks showed primers that had poor correlation and higher Ct values, demonstrating the primers' ineffectiveness. We took the average of the correlation and Ct value ranks, which gave us a list of the least effective of the 46 designed primers that we should redesign. When the average of two ranks were the same, we would place the rank with the correlation value further from one or negative one higher, as correlation demonstrates ineffectively designed primers better. In the end, we had a list of the 18 most ineffective primer sets out of the 46.

Designing Core Primers

In order to redesign the primers, we found the gene sequence the ineffective primers were targeting in the National Center for Biotechnology Information (NCBI) gene bank. If the sequence was over 2000 base pairs (bp), we used the software *LAMP Designer.* This software

can analyze large sequences, making it optimal for those genes. After putting in the sequence, it generates many sets of primers (including loop primers) and automatically selects the best set for that gene. If the gene sequence was under 2000 bp, we downloaded the FASTA gene sequence and pasted it into the software *Primer Explorer V5*. *Primer Explorer* generates around 5 sets of core primers (F3, B3, FIP, and BIP) for that gene. In order to narrow down the core primer sets, we first eliminated the set used to generate the correlation number from previous research. We then looked for the set with the highest change in free energy, dG(dimer), value. This indicates “the propensity for dimer formation. A low value of the change in free energy results in a higher likelihood of dimer formation and thus the primer set is unacceptable” (Primer Explorer V5 Manual). A primer dimer is a structure formed when two primers anneal to each other (or one to itself). They occur when primers are designed with complementary bases, leading the primer molecules to hybridize to each other instead of the DNA *(Rebecca and John Moores UCSD Cancer Center DNA Sequencing Shared Resource*, n.d.). This can potentially impede the amplification during the LAMP reaction and quantification after. For most genes we ended up choosing the core primer set with the highest dG value to minimize the potential of dimer formation (need to compare relative importance with other factors). We then checked the stability at the ends of specific primers in that set. The 3’ end of F2/B2 and F3/B3 and the 5’ end of F1c/B1c should have a change in free energy (3’ dG or 5’dG) of −4.0 kcal/mol or lower. These specific areas are starting points for gene amplification by primers so their end stability is important. The lower this value (⊿G (stability)) is, the more stable the primer will be. If the core primer set we chose before based on dG(dimer) values does not have a ⊿G (stability) value of -4.0 kcal/mol or lower for those areas, we chose a new primer set (second largest dG value, etc.). Lastly, we ran the core primers in the set through Basic Local Alignment Search Tool (BLAST) on the NCBI website to check the specificity of the primers. Ideally, each primer should only align with the *Mycobacterium tuberculosis* species, targeting multiple strains. We then saved the core primers to generate loop primers.

EXAMPLE:

Rv3219/whiB1 Core Primers:

2 ID:45	dimer(minimum)dG=-2.16									
label	5'pos	3'pos	Tm	5'dG	3'dG	GCrate	Sequence			
F3	54	72	60.76	-7.12	-5.39	0.58	CCCGGTAGGAAACAGTGGT			
B3	231	247	59.33	-5.51	-5.35	0.65	CGGTACGGGCTTTCGTG			
FIP							TGGTGACCGGGCACCGAT-CCGGCACTTGCGCAGAT			
BIP							GCACTGAATACCGGCCAGGA-GCGTTGCGACGCTTCA			
F2	73	89	62.31	-7.53	-5.14	0.65	CCGGCACTTGCGCAGAT			
F1c	113	130	65.67	-5.42	-5.57	0.67	TGGTGACCGGGCACCGAT			
B2	212	227	59.75	-6.34	-5.26	0.63	GCGTTGCGACGCTTCA			
B1c	148	167	64.04	-5.9	-5.7	0.6	GCACTGAATACCGGCCAGGA			

Alignment of Core Primers to **Rv3219/whiB1** Sequence

```
  1 ATGGATTGGC GCCACAAGGC GGTCTGTCGT GACGAGGATC CGGAACTGTT CTTCCCGGTA GGAAACAGTG GTCCGGCACT   80
    ********** ********** ********** ********** ********** ********** ********** **********
                                                          <====== ==F3====== =><=======

 81 TGCGCAGATC GCTGACGCGA AACTGGTCTG TAATCGGTGC CCGGTCACCA CAGAGTGCCT CAGCTGGGCA CTGAATACCG  160
    ********** ********** ********** ********** ********** ********** ********** **********
    F2======>                       <======== F1=======>                  <== ======B1==

161 GCCAGGACTC GGGCGTCTGG GGAGGCATGA GCGAAGACGA GCGGCGCGCG CTGAAGCGTC GCAACGCCCG CACGAAAGCC  240
    ********** ********** ********** ********** ********** ********** ********** **********
    ======>                                               <======B2 ======>    <=======B3

241 CGTACCGGGG TCTGA                                                                       255
    ********** *****
    ======>
```

Designing Loop Primers

Loop primers, though not an essential requirement for LAMP, are highly encouraged as they provide an increased number of starting points for DNA synthesis, resulting in shorter amplification time and higher specificity. To generate loop primers, we inserted the core primer set we made for the gene into *Primer Explorer*. The software then automatically aligns the core primers to the target gene sequence and generates numerous loop primer sets. Often too many sets were generated, requiring us to narrow down the selection. First in detailed settings, we set the GC content range to between 50%-60%. When this range is implemented, “favorable amplification performance will be obtained experimentally” (Primer Explorer V5 Manual). This usually narrowed down the selection to around 20 sets. We then used the same process used for the core primer sets in assessing the loop primer sets, looking at the dG(dimer) values and 3’ end of LF/LB. We additionally evaluated their specificity using BLAST. For every gene, except Rv1872c, we managed to generate two loop primers while optimizing the other features. As a visual aid, we manually aligned the loop primers to the target sequence with the other core primers.

EXAMPLE:

Rv3219/whiB1 Loop Primers:

2 ID:26 dimer(minimum)dG=-2.95								
label	5'pos	3'pos	Length	Tm	5'dG	3'dG	GCrate	Sequence
LF	92	110	19	60.14	-5.35	-5.68	0.58	CAGACCAGTTTCGCGTCAG
LB	182	200	19	61.34	-6.1	-5.43	0.58	GAGGCATGAGCGAAGACGA

Alignment of Loop Primers to **Rv3219/whiB1** Sequence

```
  1 ATGGATTGGC GCCACAAGGC GGTCTGTCGT GACGAGGATC CGGAACTGTT CTTCCCGGTA GGAAACAGTG GTCCGGCACT   80
    ********** ********** ********** ********** ********** ********** ********** **********
                                                          <====== ==F3====== =><=======
               <======== =LF=======>
 81 TGCGCAGATC GCTGACGCGA AACTGGTCTG TAATCGGTGC CCGGTCACCA CAGAGTGCCT CAGCTGGGCA CTGAATACCG  160
    ********** ********** ********** ********** ********** ********** ********** **********
    F2======>                          <======== F1=======>                   <== ======B1==
                        <======== =LB=======>
161 GCCAGGACTC GGGCGTCTGG GGAGGCATGA GCGAAGACGA GCGGCGCGCG CTGAAGCGTC GCAACGCCCG CACGAAAGCC  240
    ********** ********** ********** ********** ********** ********** ********** **********
    ======>                                              <======B2 ======>    <=======B3

241 CGTACCGGGG TCTGA                                                                       255
    ********** *****
    ======>
```

LAMP PCR Specificity Test

After our ordered primers arrived, we resuspended them by rehydrating the dehydrated primers. This was necessary to perform a PCR protocol to test the specificity and functionality of our F3 and B3 primers. These primers should amplify their target regions on the template Mtb DNA during the PCR reaction. To proceed with the reaction we added water, buffer, dNTPs, DNA polymerase, and our 10uM/uL F3 and B3 primers to a 96 well plate. We then added Mtb DNA or water (blank) to each well. Each pair of F3 and B3 primers was tested with DNA and water. With our 18 primer sets, we had a total of 36 reactions. We then followed the thermal cycling protocol for the PCR reaction.

Materials:

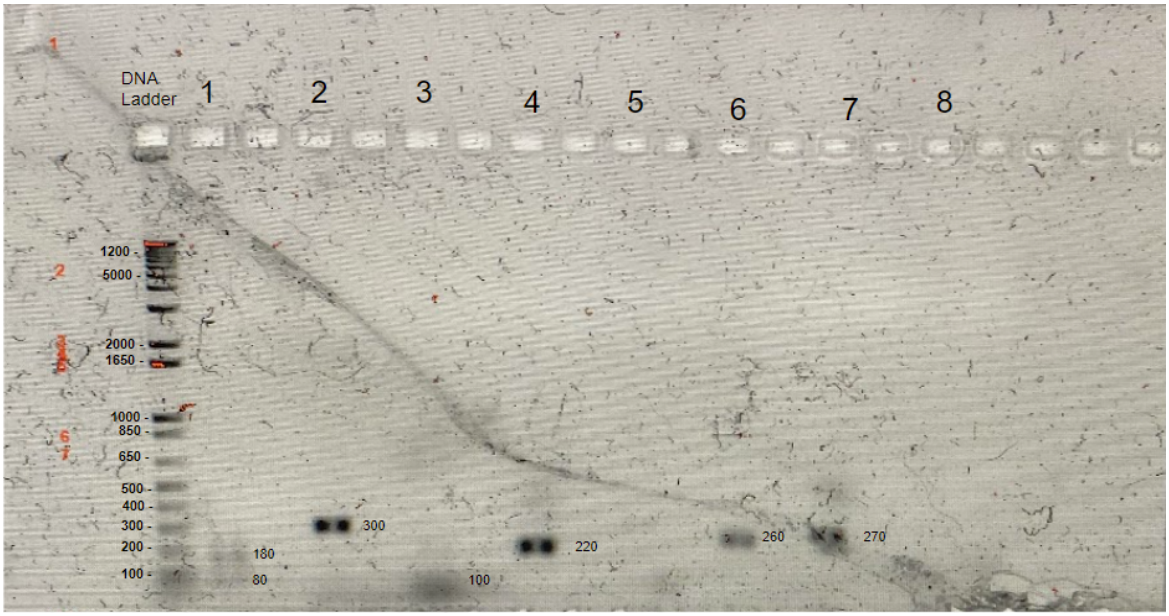
- 5x Phusion Buffer
- 25mM dNTPs
- DNA polymerase
- dH2O
- Mtb DNA template
- F3 and B3 primers, 10uM/uL

PCR Cycling

98°C	30sec	
98°C	10sec	
50°C	30sec	>30x
72°C	60sec	
72°C	10min	
4°C	Hold	

Gel Electrophoresis for Specificity of Primers

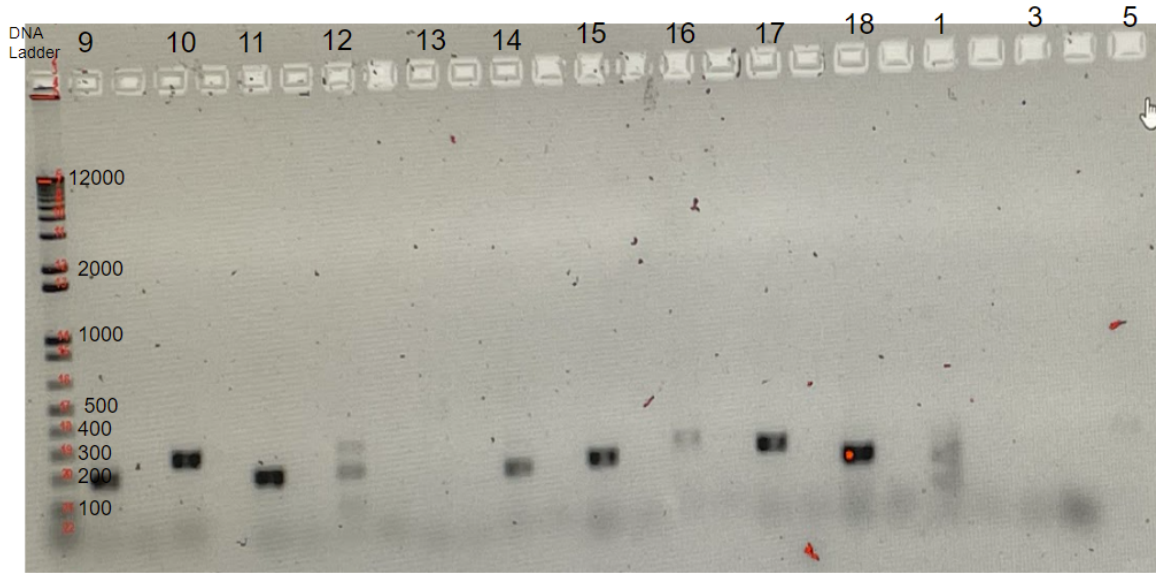
After the PCR amplification finished, we needed to view our products, so we used gel electrophoresis. We know our primers are specific if amplified products are only one length. This means the single correct region was amplified, not multiple regions. We also know the primers are functional if the blank wells (no DNA) didn't generate any bands. To make the gel, we used agarose in 1xTAE buffer. After the gel set, we put 8 primer sets and their blanks on the top half and the last 10 sets and their blanks on the bottom half.



(Figure 1.)
*DNA ladder added to first well to determine product length

These are the top half results (Fig. 1) with the products from the first 8 primer sets: Rv0667 (#1), Rv0824c (#2), Rv0932c (#3), Rv1094 (#4), Rv1297 (#5), Rv1308 (#6), Rv1398c (#7), and Rv1872c (#8). We can observe that Rv0667 (#1) generated multiple bands, indicating the unspecificity of the primer set. The band for Rv0932c (#3) is blurry and no bands showed up for Rv1297 (#5) and Rv1872c (#8). The rest of the products from the primer sets each generated a strong, clear band, indicating the specificity and functionality of those primers. Also no bands showed up for the blank wells with no Mtb DNA, indicating no dimer formation.

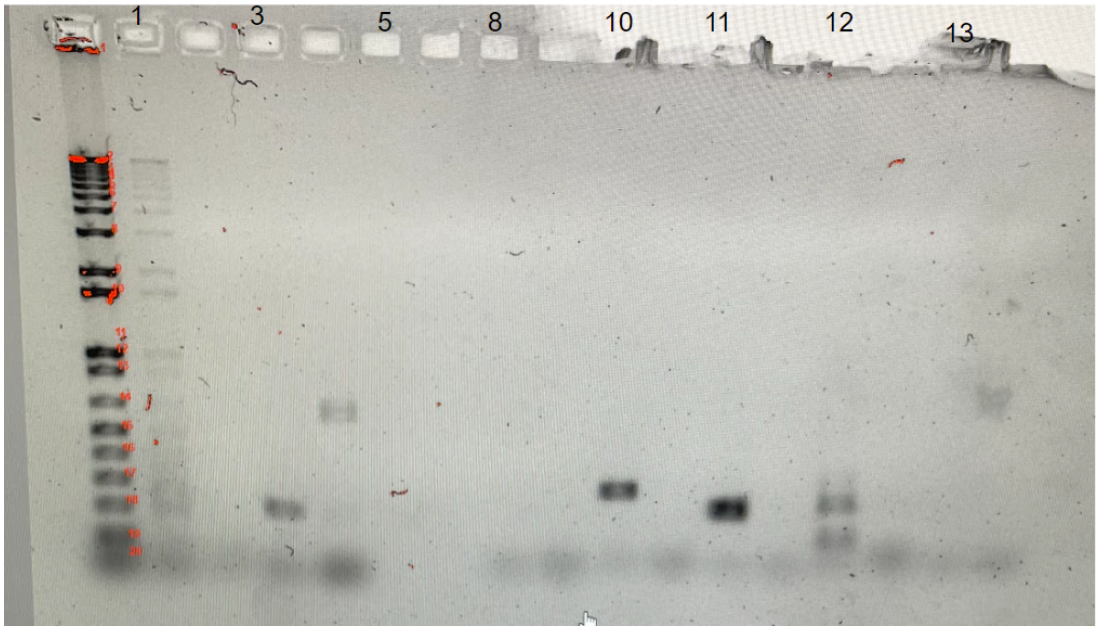
The bottom half of our first run was unreadable because we accidentally dropped the gel. So we had to rerun the last 10 primer set products on a new gel. Because we had room on the new gel, we also reran the first 3 defective primers from the top half.



(Figure 2.)

These are the bottom half rerun results (Fig. 2) with the products from last 10 primers: Rv2196 (#9), Rv2444c (#10), Rv3219 (#11), Rv3583c (#12), Rv3800c (#13), Rv3801c (#14), Rv3804c (#15), Rv3825c (#16), Rv3859c (#17), and Rv3875 (#18), as well as Rv0667 (#1), Rv0932c (#3), and Rv1297 (#5) from the top half. The gel shows that the Rv0667 (#1) and Rv3583c (#12) primers generated multiple bands, indicating the need to rerun them again and possibly redesign them. The Rv0932c (#3), Rv1297 (#5), Rv1872c (#8), and Rv3800c (#13) primers generated blurry results or no bands, again indicating the need to rerun them and possibly redesign them. The rest of the primers generated a strong, clear single band, showing their specificity.

The next steps were to obtain more PCR products and rerun the products from defective primers through the gel one more time. We repeated the PCR protocol and gel electrophoresis, placing Rv0667 (#1), Rv0932c (#3), Rv1297 (#5), Rv1872c (#8), Rv3583c (#12), and Rv3800c (#13) amplified products into the wells. We also included the primer sets Rv2444c (#10) and Rv3219 (#11) we know are specific for comparison.



(Figure 3.)

Figure 3 shows our results from the rerun. There was contamination with the ladder for Rv0667 (#1), as numerous bands were generated, leading to the results being inaccurate. The blank well for Rv0932c (#3) generated one band, indicating dimer formation because there was no Mtb DNA added, yet a product was generated. Bands for Rv1297 (#5), Rv1872c (#8), and Rv3800c (#13) products did not show up again on the gel. Rv3583c (#12) product continued to generate multiple bands. This indicates that there is a need to redesign those 6 primers, including Rv0667 (#1). Even though there were not accurate results for Rv0667 (#1) on this gel because of contamination, on the other two gels, it consistently generated multiple bands. Lastly, Rv2444c (#10) and Rv3219 (#11) products continued to each generate one clear band, demonstrating the specificity and functionality of the primers.

Addressing Errors

For the defective primers, we double checked the input sequences and length of product relative to the gel results. We reviewed the plate specs of the ordered primers and ensured the primer sequences were identical to the ones generated by each software. All of the ordered primers were the correct sequence. We then analyzed the product length on the gel, using the ladder for measurement. We compared the approximate length with the length from F3 and B3 primers on the sequence. They all were approximately the same. Overall, we tried to find patterns in the 6 primers that were nonspecific during the PCR reaction. We didn't find any major trends between the 6 primers, but we did discover that the Rv0667 B3 primer generated by the LAMP Designer software did not match the sequence alignment also generated by the software. This could have been a source of error for that primer set as seen below.

Rv0667

Name	Primer	Concentration	Position	Length	Tm	GC%	Rating
F3	GGAAGAGGTGCTCTACGA	4.8	240	18	60.1	55.6	93.1
B3	GGTCCCGTTGATGATGAAC	4.8	495	19	60.6	52.6	83.3
FIP(F1c+F2)	GCGTACGTCATGTCCTTGTCTTCGTTGTCGTTCTCTGACC	2.6		40			
BIP(B1c+B2)	CGCCGAGTTCATCAACAACAACCTTCTCGGTCATCATCGG	2.4		40			
LoopF	GTGCCTTGACATCGTCGA	5.3	334	18	62.1	55.6	88
LoopB	CAAGAGTCAGACGGTGTTCA	4.4	423	20	62	50	93.3
F2	CGTTGTCGTTCTCTGACC	5.8	293	18	60.1	55.6	93.1
F1c	GCGTACGTCATGTCCTTGTCTT	4.6	371	22	65.2	50	88.8
B2	CTTCTCGGTCATCATCGG	5.6	471	18	59.3	55.6	87
B1c	CGCCGAGTTCATCAACAACAAC	4.1	390	22	65.1	50	93.2

```
141    CCTTGACGTC CAGACCGATT CGTTCGAGTG GCTGATCGGT TCGCCGCGCT GGCGCGAATC CGCCGCCGAG
211    CGGGGTGATG TCAACCCAGT GGGTGGCCTG GAAGAGGTGC TCTACGAGCT GTCTCCGATC GAGGACTTCT
281    CCGGGTCGAT GTCGTTGTCG TTCTCTGACC CTCGTTTCGA CGATGTCAAG GCACCCGTCG ACGAGTGCAA
351    AGACAAGGAC ATGACGTACG CGGCTCCACT GTTCGTCACC GCCGAGTTCA TCAACAACAA CACCGGTGAG
421    ATCAAGAGTC AGACGGTGTT CATGGGTGAC TTCCCGATGA TGACCGAGAA GGGCACGTTC ATCATCAACG   B3
491    GGACCGAGCG TGTGGTGGTC AGCCAGCTGG TGCGGTCGCC CGGGGTGTAC TTCGACGAGA CCATTGACAA
561    GTCCACCGAC AAGACGCTGC ACAGCGTCAA GGTGATCCCG AGCCGCGGCG CGTGGCTCGA GTTTGACGTC
631    GACAAGCGCG ACACCGTCGG CGTGCGCATC GACCGCAAAC GCCGGCAACC GGTCACCGTG CTGCTCAAGG
701    CGCTGGGCTG GACCAGCGAG CAGATTGTCG AGCGGTTCGG GTTCTCCGAG ATCATGCGAT CGACGCTGGA
```

PCR Specificity Test Rerun: Rv0667 and Rv3583c (Generated Multiple Bands)

As a last effort to confirm the primers we needed to redesign, we did PCR reruns of the 2 primer sets Rv0667 and Rv3583c that generated multiple bands. The anneal stage of previous runs were all performed at 50°C, which could have been too low, leading to the primers to anneal incorrectly. Also, the previous runs all used 10 uM/uL concentration of the F3 and B3 primers, so we wanted to test multiple concentrations in the new reruns (2.5uM/uL, 5uM/uL, and 10uM/uL) to see if it makes a difference in the gel. We used two different PCR cycling protocols, with the annealing stage at 55°C and 60°C.

Plate 1 Protocol

PCR Cycling	98°C	30 sec	
	98°C	10 sec	
	55°C	30 sec	>30x
	72°C	60 sec	
	72°C	10 min	
	4°C	Hold	

Plate 2 Protocol

PCR Cycling	98°C	30 sec	
	98°C	10 sec	>30x
	60°C	30 sec	
	72°C	60 sec	
	72°C	10 min	
	4°C	Hold	

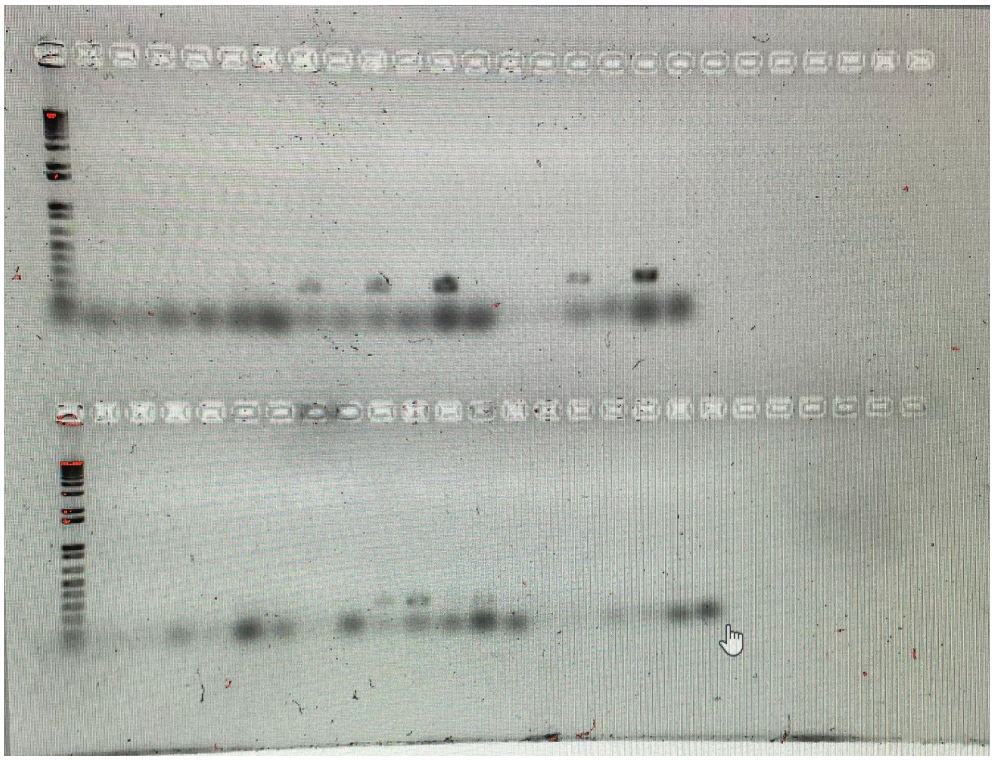
Plate 1 and 2 Set up

						Blank, No DNA Template					
	1	2	3	5	6	7	8	9	10	11	12
A	2.5	5	10		2.5	5	10				
B	2.5	5	10		2.5	5	10				
C	2.5	5	10		2.5	5	10				
D											
E											
F											
G											
H											

1. Rv0667	
12. Rv3583c	
17. Rv3859c	control (already generates single band) -> to compare to

Primer Dilutions

Primer dilution for F3 primer * 3						
Start Con	Need Con	Need Vol	Final Vol	H2O		Total Vol
10	5	9	4.5	4.5		6
5	2.5	6	3	3		6
Primer dilution for B3 primer * 3						
Start Con	Need Con	Need Vol	Final Vol	H2O		Total Vol
10	5	9	4.5	4.5		6
5	2.5	6	3	3		6



Our results from the gel shown in the image above. The primer for Rv0667 at various primer concentrations at both PCR cycling temperature of 55 degrees Celsius and 60 degrees Celsius had no bands show up, suggesting that we should test this primer at a lower temperature than 55, but higher than the usual 50 degrees, or redesign the primer. The primer for Rv3583c produced a single band at all primer concentrations, with 10x forming the clearest band, at 55 degrees celsius. This shows that the primer is specific, and that previously the temperature was not high enough for it to anneal to the target sequence specifically. We then added this primer to test in our fifth RT-LAMP assay because it was deemed specific by this gel. At 60 degrees Celsius, the bands were faint for all the primers, including the control primer that worked in our previous gels, suggesting the temperature was too warm.

LAMP Assay Protocol and Process

To perform our RT-LAMP assays, we first made 10x stocks of all of our primer sets. We followed the NEB LAMP Protocol, combining all the primers in each set.

WarmStart LAMP Kit (DNA and RNA) Protocol
10X LAMP Primer Mix

Primer	10X Concentration (stock)
FIP	16uM
BIP	16uM
F3	2uM
B3	2uM
LOOP F	4uM
LOOP B	4uM

To run the RT-LAMP assays we needed the materials down below (Image 1). We performed the assays with the stocks of primers that were confirmed to be specific by the gel tests. We diluted the 10x primer stocks to various concentrations for our assays. We also diluted either RNA or DNA to various concentrations for our reactions. For each assay, we made Master Mixes that contained all the materials needed for the LAMP reaction. We pipetted 18uL of the Master Mix into the wells of a 96 well plate. Depending on the assay, we either added the primers or DNA/RNA directly into the Master Mix, and then added 2uL of the other material not in the Master Mix afterwards. We then followed the LAMP heating protocol, which takes about an hour and 15 minutes total.

Materials (Image 1):

- Nuclease-free water
- WarmStart LAMP 2x Mix
- Mtb RNA
- 10X LAMP Primer Mix
- Fluorescent Dye 50x

Protocol:
65°C - 1 min
65°C - 15 sec
65°C - 1 min
x60 (60 cycles)

The RT-LAMP assay will obtain Ct values of the Mtb RNA by measuring the fluorescent levels in the assay. These will then be compared to Ct values of the old primers. By doing this, we can evaluate the efficiency of the new primers at varying concentrations of Mtb RNA compared to the old primers. Furthermore, these Ct values could be used to calculate gene expression values for DRonA in the future.

Results and Analysis

First LAMP assay

Our first LAMP assay used primers for Rv1308, Rv2196, Rv2444c, Rv0824c, Rv1094, and Rv1398c genes. These were the first 7 primer sets confirmed to be specific from the PCR/Gel specificity test. We also included IS6110 primers, ones we know are functional, for comparison. We diluted the 10x primer stocks to 2.5x stocks for those primer sets. For this assay, we used RNA dilutions of 0.25, 2.5, 25, and 250ng/20uL reaction (0.125, 1.25, 12.5, 125 ng/uL). Before performing the assay, we made a plate set up to designate primer location and calculate the amount of materials needed.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
B	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
C	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
D	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
E	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
F	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
G	0.25	0.25	0.25	2.5	2.5	2.5	25	25	25	250	250	250
H	NTC	NTC	NTC									

Each row from 1-12 across is one primer set - the blue highlight is the IS6110 primer that is known to work effectively to ensure the assay is working. Each row is testing the primer set at various Mtb RNA concentration levels in triplicates. As Mtb RNA concentration increases, the Ct value should decrease because less cycles should be needed to detect the Mtb RNA. A graph of log Mtb RNA vs. Ct Mean of the primers should show a decreasing linear line to

demonstrate an effective assay. NTC are the negative test controls without the RNA to check for primer dimers and make sure no other materials are affecting the test.

Once we had the plate set up we calculated the RNA dilution volumes as seen below. The Mtb RNA we started with was at the concentration 150ng/uL.

Start Con	Need Con	Need Vol	Final Vol	H2O		Total Vol
150	125	160	133.3333	26.66667	125	160
125	12.5	140	14	126	12.5	140
12.5	1.25	140	14	126	1.25	140
1.25	0.125	140	14	126	0.125	140

We first wanted to have our concentration of RNA to be 250ng/20uL reaction. For our assays we added 2uL volume of RNA, to end with a total volume of 20uL for each reaction when combined with the other materials. This means our concentration of RNA needed to be 250/2 = 125 ng/uL since 2uL of this concentration will make the ending 250ng/20uL we wanted. Because it was a 10-fold dilution, we used each dilution for the subsequent dilution. The “Final Vol " in the image above is the amount of RNA in microliters we take from the start concentration column. The “H2O" column in how much water we add to the RNA.

For the first RT-LAMP assay, we made Master Mixes that contained the RNA dilutions and added the primers separately as seen below. We pipetted18 uL of the Master Mix shown below into the wells of a 96 well plate. Then, we added 2 uL of a 2.5x primer stock across each row, matching the plate set up.

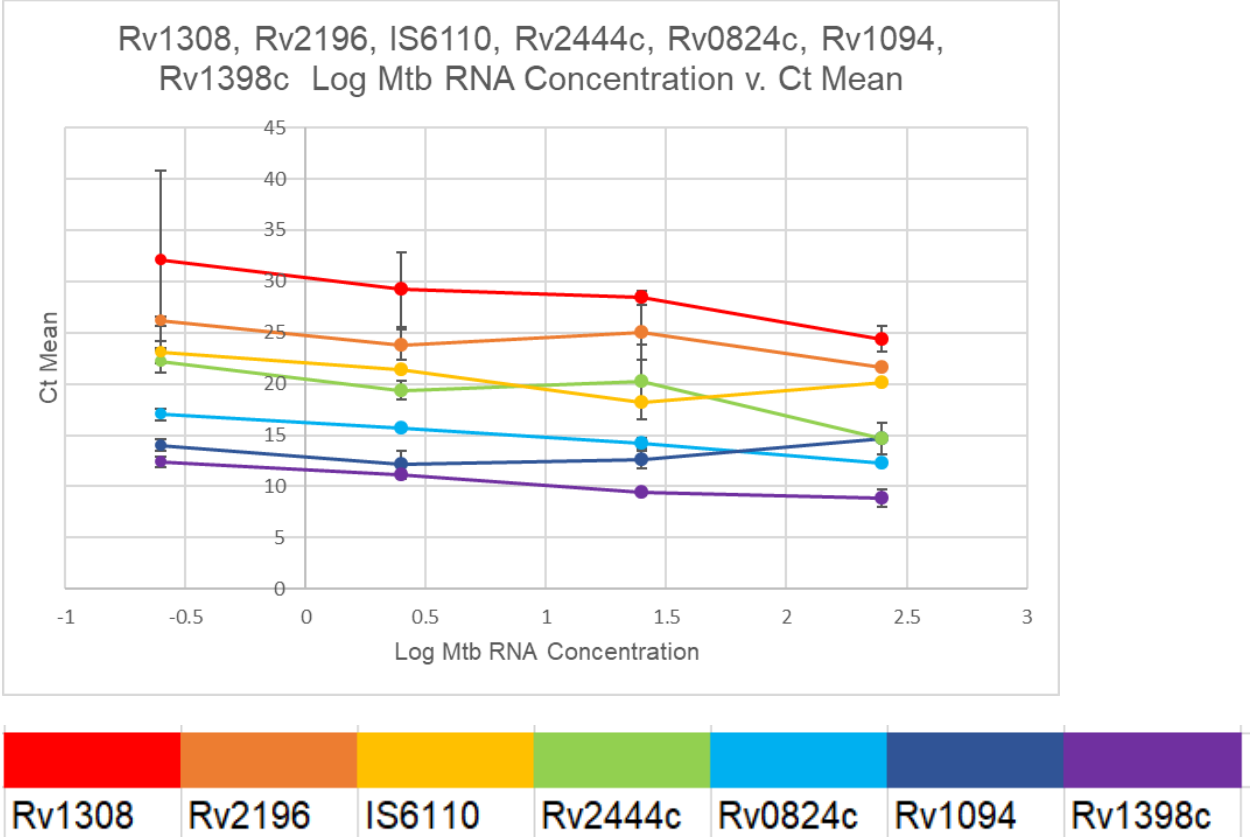
Mtb RNA: 0.25ng, 2.5ng, 25ng, 250ng/20uL reaction

	Master Mix 0.25			Master Mix 2.5			Master Mix 25			Master Mix 250		
	1x	24x			1x	24x		1x	24x		1x	24x
WarmStar	10	240	WarmStar	10	240	WarmStar	10	240	WarmStar	10	240	
Fluorescer	0.4	9.6	Fluorescer	0.4	9.6	Fluorescer	0.4	9.6	Fluorescer	0.4	9.6	
Mtb RNA (	2	48	Mtb RNA (	2	48	Mtb RNA (	2	48	Mtb RNA (	2	48	
H2O	5.6	134.4	H2O	5.6	134.4	H2O	5.6	134.4	H2O	5.6	134.4	

*Add 2.0uL of 2.5x Primer Mix

After the LAMP assay was finished, we collected data from the PCR machine used to perform the LAMP assay. The LAMP assay gave us the Ct values of all the primers with different concentrations of Mtb RNA. We then created a graph of the log of Mtb RNA concentration (to make the graph linear) v. the Ct mean values of all the primers. We graphed the standard deviations as well (how much the triplicates we tested deviated from the Ct mean). As Mtb RNA concentration increases, the Ct mean should decrease, forming a negative linear relationship. This is because as there is more RNA in the assay, it should take less cycles to detect the RNA. The trend of a decreasing linear graph should be seen for all of our primers to consider them as effective. However, in our graph, not all of the primers formed a linear

relationship. IS6110 and Rv1094 look problematic; as Mtb RNA increases, the Ct increases for certain RNA concentrations. Primer sets Rv2196 and Rv2444c are also not forming clear linear graphs either. A potential cause of these issues is primer-dimer formation. But since IS6110 (the baseline) was also problematic, there was likely an issue in the process not related to the primers. One possibility was that there was an error in our RNA dilutions or LAMP protocol, leading to the weak relationships.



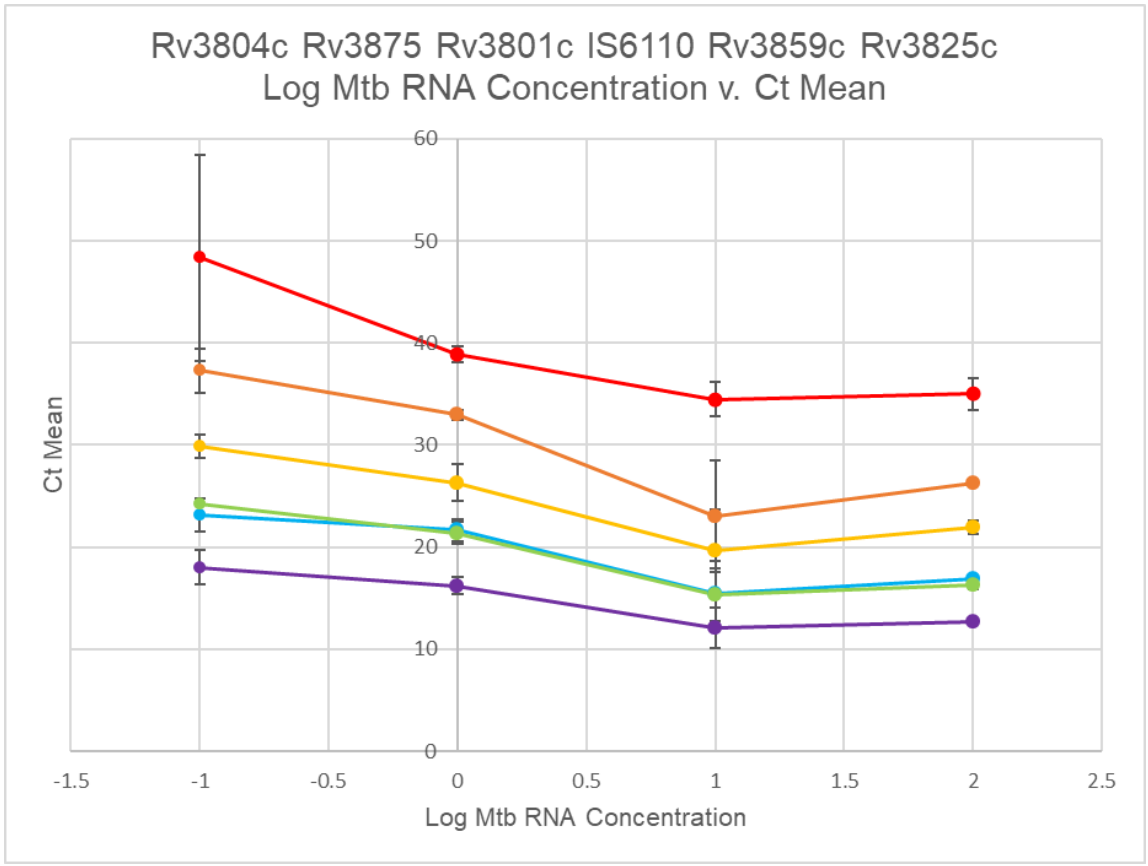
Slope and R-squared Values

We calculated the R^2 coefficient of determination values between the Mtb RNA concentration and Ct mean for each of the primer concentrations to demonstrate how effective each primer set was. The closer the coefficient value is to one, the stronger the linear relationship is between the Ct mean and Mtb RNA concentration. Coefficient values that are far from one illustrate a weak linear relationship, suggesting the need for a redesign or an error in the LAMP assay. Since the IS6110 correlation was extremely weak, there was likely an error in the LAMP assay, as assumed using the graph. We also calculated the slopes to show the negative linear correlation between the two variables. The positive slope of Rv1094 primers, also was an indicator of an error with the assay.

Gene	slope	R^2
Rv1308	-2.4102	0.944264
Rv2196	-1.2239	0.672233
IS6110	-1.2026	0.561018
Rv2444c	-2.1588	0.771055
Rv0824c	-1.5713	0.991616
Rv1094	0.2628	0.08155
Rv1398c	-1.2278	0.969365

Second LAMP Assay

For our second LAMP assay, we tested the rest of the primers that were shown as specific from the gel. We tested primers Rv3219, Rv3801c, Rv3804c, Rv3825c, Rv3859c, Rv3875, and IS6110 as base using the same plate set up as the first LAMP assay. To deduce whether our error from the first plate set up could be related to dilutions, we used different RNA dilutions than the first LAMP assay: instead of 0.25, 2.5, 25, and 250 ng/20uL reaction, we used 0.1, 1, 10, 100 ng/20uL reaction for easier measurements and less room for dilution errors. Also the 250ng/20uL reaction concentration may have been too high and caused errors. Additionally, in order to discover if human error messed up our first plate, we tried using an automatic pipette. However, the automatic pipette was having calibration issues and incorrectly pipetted our primers in the A1, F1, and F2 wells. We graphed the Log Mtb RNA concentration vs. Ct means with standard deviation of our second LAMP assay run. There was a pattern of the 100ng/20uL RNA concentration being higher than 10ng/20uL RNA concentration. This could be from another dilution error or primer issues, leading us to run a third assay with primer titrations.



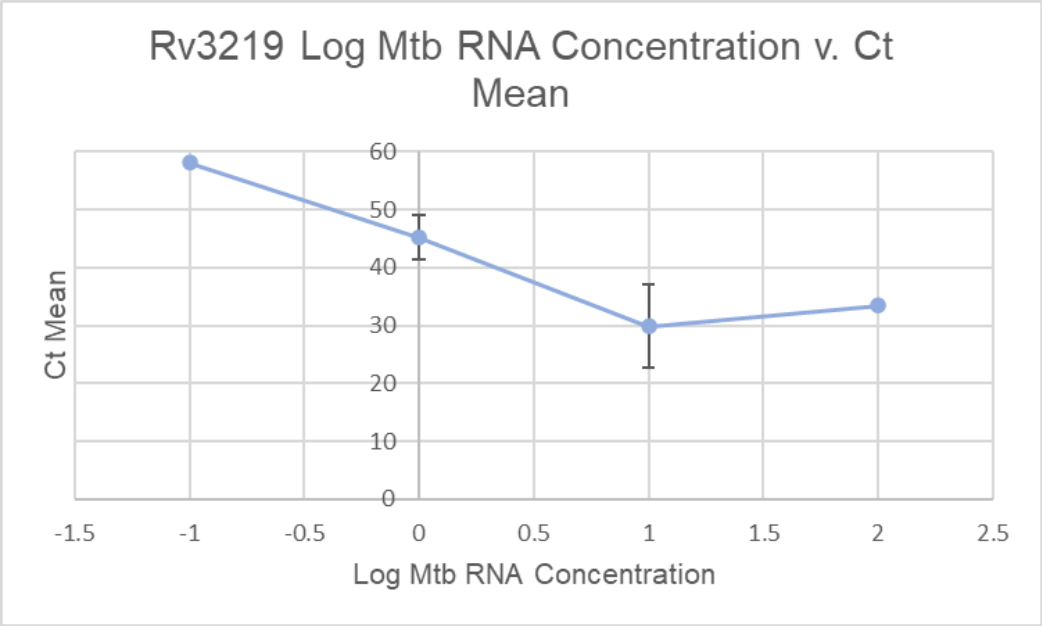
Rv3804c	Rv3875	Rv3801c	IS6110	Rv3859c	Rv3825c

Slope and R-squared Values

Gene	slope	R^2
Rv3804c	-4.4412	0.799891
Rv3875	-4.2868	0.742849
Rv3801c	-3.0381	0.739196
IS6110	-2.9726	0.836047
Rv3859c	-2.4809	0.76243
Rv3825c	-2.0078	0.840907

We also calculated the slope and R^2 values to assess the strength of the negative linear relationships between Log Mtb RNA Concentration and the Ct Means. There was more consistency with R^2 values in the second assay than the first assay, however the values are a bit low. The slopes were all negative, indicating the negative relationship.

The Rv3219 primers generated erroneous results, so we omitted them from the analysis with all primers. The Ct mean values were very high for all concentrations of RNA. The Ct mean for the 100ng/20uL reaction RNA concentration increased from the previous Ct value for the 10ng/20uL RNA concentration. This was most likely due to an error with the primers or specific wells in the plate.



Slope and R-squared Values

Gene	slope	R^2
Rv3219	-8.95736	0.81602

<u>Third LAMP Assay: Primer Titrations</u>

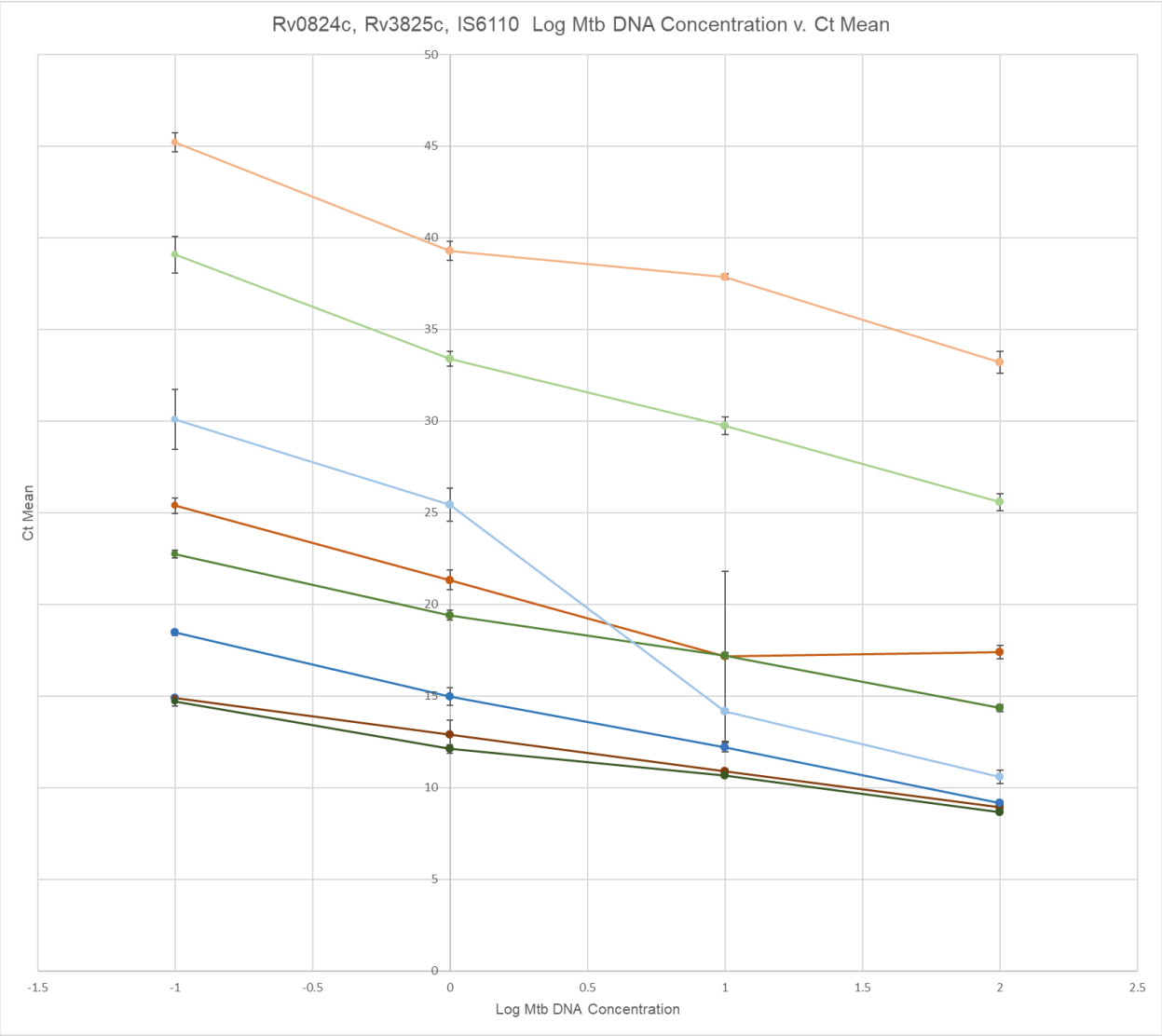
We ran our third LAMP assay test with various primer concentrations and Mtb DNA concentrations in order to discover if our primer concentration amount that we were using before is not the most effective. We also used DNA in this test to check if synthesizing RNA to DNA was causing the issues in the previous LAMP assays. We tested two primers (Rv0824c and Rv3825c) that were shown to be most promising in our previous LAMP assay runs because of their low CT values and negative linear relationship, as well as the IS6110 primers as a baseline.The primer concentrations we ran were 2.5, 1.25, 0.625 uM/uL. For each primer we also had various Mtb DNA concentrations of 100, 10, 1, 0.1 ng/20uL reaction. Since there was not enough room on the well plate, we only ran the IS6110 primer with 2.5 and 1.25 uM/uL concentrations. Below are our calculations for Mtb DNA and primer dilutions in order to figure out how much water and extracted Mtb DNA we should use to achieve the correct concentration.

	Dilution of DNA					
	119 ng/ul					
	Dilution for DNA		Final Vol: volume DNA needed			
Start Con	Need Con	Need Vol	Final Vol	H2O		Total Vol
119	50	70	29.41	40.59	50	64
50	5	60	6	54	5	54
5	0.5	60	6	54	0.5	54
0.5	0.05	60	6	54	0.05	54
	Primer Dilution for one primer					
Start Con	Need Con	Need Vol	Final Vol	H2O		Total Vol
2.5	1.25	80	40	40		55
1.25	0.625	50	25	25		50
2.5x primer already diluted						

Our plate set up for the primer titrations is below. We ran Rv0824c and Rv3825c at the three primer concentrations in triplicates for each Mtb DNA concentration and IS6110 in triplicates for 2.5 and 1.25 uM/uL primer concentrations for each Mtb DNA concentration.

2: Rv0824c		1	2	3	5	6	7	8	9	10	11	12
16: Rv3825c	A	0.1 P2.5	0.1 P2.5	0.1 P2.5	1 P2.5	1 P2.5	10 P2.5	10 P2.5	10 P2.5	100 P2.5	100 P2.5	100 P2.5
IS6110	B	0.1 P1.25	0.1 P1.25	0.1 P1.25	1 P1.25	1 P1.25	10 P1.25	10 P1.25	10 P1.25	100 P1.25	100 P1.25	100 P1.25
	C	0.1 P0.625	0.1 P0.625	0.1 P0.625	1 P0.625	1 P0.625	10 P0.625	10 P0.625	10 P0.625	100 P0.625	100 P0.625	100 P0.625
	D	0.1 P2.5	0.1 P2.5	0.1 P2.5	1 P2.5	1 P2.5	10 P2.5	10 P2.5	10 P2.5	100 P2.5	100 P2.5	100 P2.5
	E	0.1 P1.25	0.1 P1.25	0.1 P1.25	1 P1.25	1 P1.25	10 P1.25	10 P1.25	10 P1.25	100 P1.25	100 P1.25	100 P1.25
	F	0.1 P0.625	0.1 P0.625	0.1 P0.625	1 P0.625	1 P0.625	10 P0.625	10 P0.625	10 P0.625	100 P0.625	100 P0.625	100 P0.625
	G	0.1 P2.5	0.1 P2.5	0.1 P2.5	1 P2.5	1 P2.5	10 P2.5	10 P2.5	10 P2.5	100 P2.5	100 P2.5	100 P2.5
	H	0.1 P1.25	0.1 P1.25	0.1 P1.25	1 P1.25	1 P1.25	10 P1.25	10 P1.25	10 P1.25	100 P1.25	100 P1.25	100 P1.25

As primer concentration increased and Mtb DNA concentration increased, the Ct mean decreased, showing a more effective assay. The 2.5x primer concentration had the lowest Ct values, demonstrating its effectiveness compared to the other concentration levels. This informed us that the 2.5x primer concentration we used in our previous LAMP assays was likely not an issue that caused our defective results. It is possible that the RNA used in the other assays was causing challenges with the extra step of reverse transcription, as the DNA used in this assay produced more consistent results for the 2.5x primer concentrations.



Primers	Primer Concentration							
Rv0824c	2.5x		Rv3825c	2.5x		IS6110	2.5x
Rv0824c	1.25x		Rv3825c	1.25x		IS6110	1.25x
Rv0824c	0.625x		Rv3825c	0.625x			

Slope and R-squared Values

Gene	slope	R^2
Rv0824c	-1.99431	0.999943
Rv0824c	-2.81428	0.875871
Rv0824c	-3.73795	0.952978
Rv3825c	-1.95756	0.987494
Rv3825c	-2.74485	0.994294
Rv3825c	-4.41667	0.991147
IS6110	-3.0663	0.997699
IS6110	-6.98153	0.95846

The data from the third assay was also used to calculate the slope and R^2 values. The slopes are all negative, indicating a negative relationship between the Mtb DNA concentration and the Ct means. All of the R^2 values are close to 1, demonstrating the strength of the negative, linear relationship for all the primer concentrations.

Fourth LAMP assay: Primer Titrations with DNA and RNA

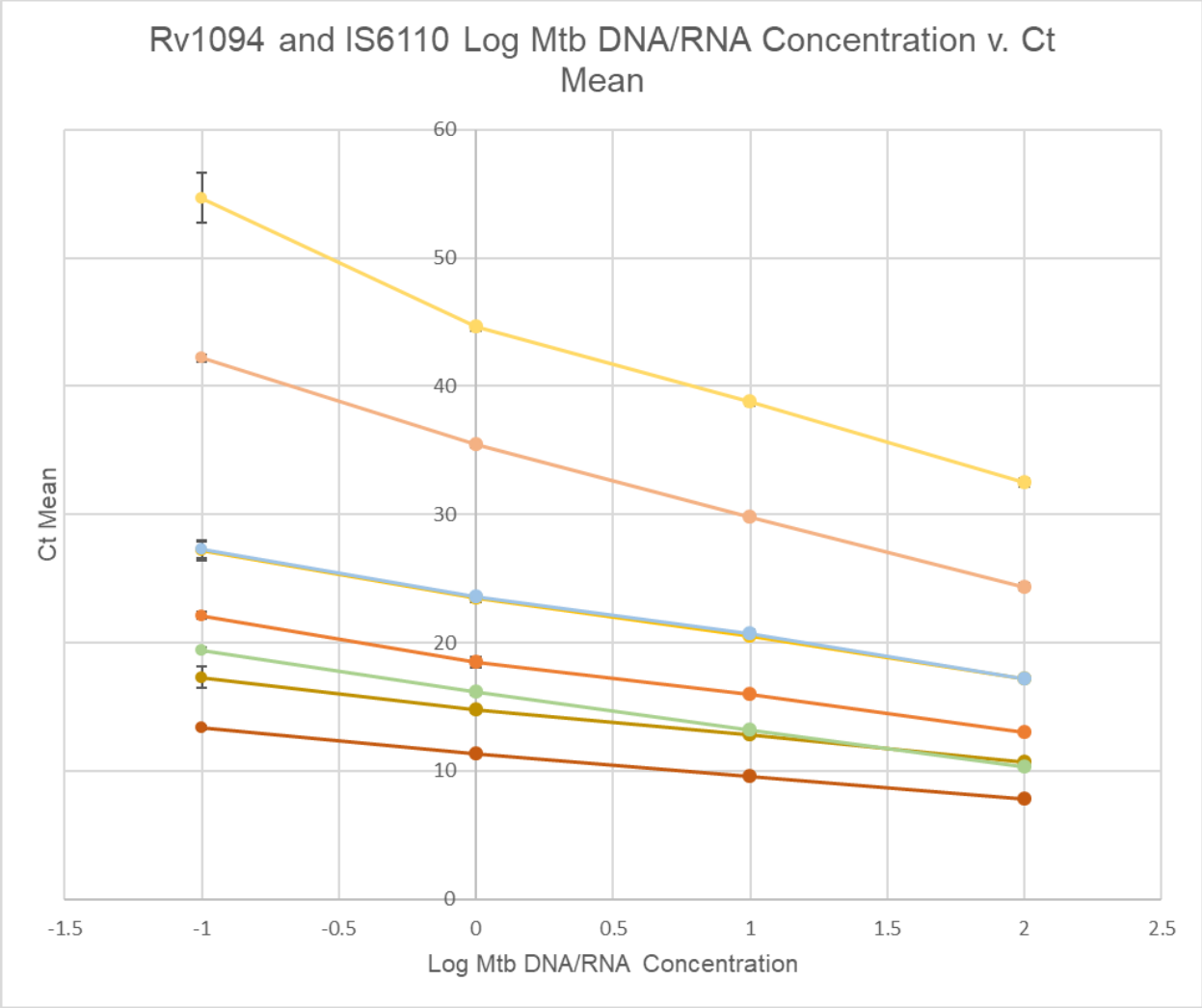
In the fourth LAMP assay, we ran more primer titrations. This time we chose primers that had an obvious curve issue to see if the concentration of primers would make a difference or if the primer set needed to be redesigned. Primers targeting gene Rv1094 had severely defective results in the first assay, so we chose to test them again. We had three primer concentrations for the primers: 2.5, 1.25, and 0.625 uM/uL. Additionally, for each triplicate of primer concentration, we tested both DNA and RNA at 0.1, 1, 10, and 100 ng/20uL reaction concentrations, so we could analyze whether the extra step of reverse transcription may be causing the issues in our first few RT-LAMP assays.

Below is a screenshot of our plate setup. We also added the IS6110 primers (blue) as a baseline to compare to.

	1	2	3	5	6	7	8	9	10	11	12	
A	0.1 P2.5	0.1 P2.5	0.1 P2.5	1 P2.5	1 P2.5	10 P2.5	10 P2.5	10 P2.5	100 P2.5	100 P2.5	100 P2.5	DNA
B	0.1 P1.25	0.1 P1.25	0.1 P1.25	1 P1.25	1 P1.25	10 P1.25	10 P1.25	10 P1.25	100 P1.25	100 P1.25	100 P1.25	DNA
C	0.1 P0.625	0.1 P0.625	0.1 P0.625	1 P0.625	1 P0.625	10 P0.625	10 P0.625	10 P0.625	100 P0.625	100 P0.625	100 P0.625	DNA
D	0.1 P2.5	0.1 P2.5	0.1 P2.5	1 P2.5	1 P2.5	10 P2.5	10 P2.5	10 P2.5	100 P2.5	100 P2.5	100 P2.5	RNA
E	0.1 P1.25	0.1 P1.25	0.1 P1.25	1 P1.25	1 P1.25	10 P1.25	10 P1.25	10 P1.25	100 P1.25	100 P1.25	100 P1.25	RNA
F	0.1 P0.625	0.1 P0.625	0.1 P0.625	1 P0.625	1 P0.625	10 P0.625	10 P0.625	10 P0.625	100 P0.625	100 P0.625	100 P0.625	RNA
G	0.1 P2.5	0.1 P2.5	0.1 P2.5	1 P2.5	1 P2.5	10 P2.5	10 P2.5	10 P2.5	100 P2.5	100 P2.5	100 P2.5	DNA
H	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	0.1 P2.5	RNA

Below are our calculations for the amount of water, DNA, RNA, and primers we needed to use to obtain the correct dilution amounts.

	Dilution for DNA		Final Vol: how much of DNA needed	
Start Con	Need Con	Need Vol	Final Vol	H2O
119	50	40	16.81	23.19
50	5	35	3.50	31.50
5	0.5	35	3.50	31.50
0.5	0.05	35	3.50	31.50
	Dilution for RNA		Final Vol: how much of RNA needed	
Start Con	Need Con	Need Vol	Final Vol	H2O
150	50	40	13.33	26.67
50	5	35	3.50	31.50
5	0.5	35	3.50	31.50
0.5	0.05	35	3.50	31.50
	Primer Dilution for Rv1094			
Start Con	Need Con	Need Vol	Final Vol	H2O
2.5	1.25	80	40	40
1.25	0.625	52	26	26



Warm	Rv1094		
			Primer Concentration
Orange	DNA		2.5x Rv1094
			1.25x Rv1094
			0.625x Rv1094
Yellow	RNA		2.5x Rv1094
			1.25x Rv1094
			0.625x Rv1094

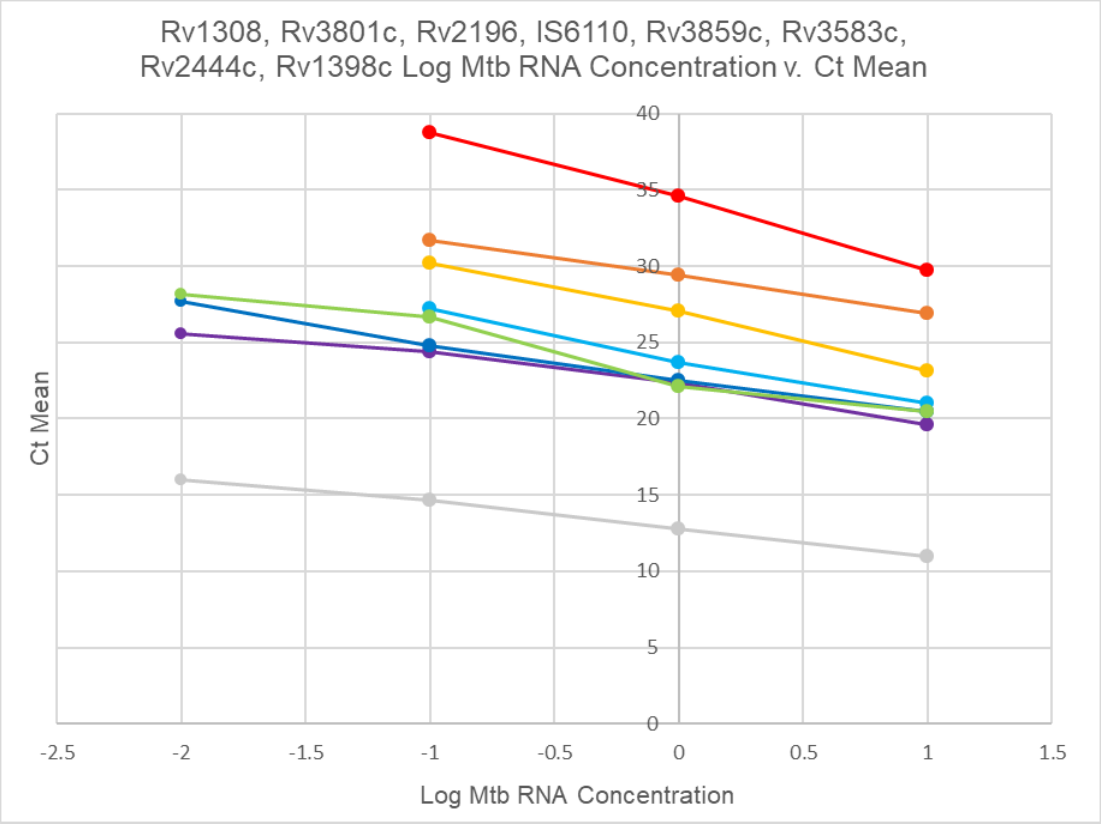
Cool	IS6110		
			Primer Concentration
Green	DNA		2.5x IS6110
Blue	RNA		2.5x IS6110

Above is the graph and key of our results. We noticed a pattern that the higher the primer concentration, the lower the Ct values for both DNA and RNA. DNA Ct values were slightly lower overall than RNA values. These results suggest that the highest primer concentration (2.5x) and using DNA allow the primers to work most effectively, though RNA still

works successfully. The Ct values decreased linearly as Mtb DNA/RNA concentration increased for the gene Rv1094, suggesting that the primer worked effectively and the LAMP assay was able to detect increased amounts of Mtb as the DNA/RNA amplified. Our results also allude to the issues with our first LAMP assay. In that assay, the primers for Rv1094 at 2.5x concentration appeared severely defective, as the Ct values increased as the RNA concentration increased. In this fourth LAMP assay, the Rv1094 primers at 2.5x concentration were functional as the graph shows a strong linear negative relationship with low Ct values. This implies a larger underlying issue with our first LAMP assay that led to inaccurate results.

Fifth LAMP Assay: Rerun Specific Primers

In our first and second RT-LAMP assays, certain Mtb RNA dilution amounts produced faulty results. Therefore, we decided to rerun some primers that were promising (mean Ct value was under 30) from those assays. We chose primers targeting Rv1308, Rv1308c, Rv2196, Rv2444c, Rv1398c, and Rv3859c. Additionally, in this RT-LAMP assay, we added the new primers deemed specific from our last gel rerun (primers for Rv3583c, #12) as well as IS6110 primers. Since higher Mtb RNA concentrations (100 ng/20uL reaction and 250 ng/20uL reaction) produced our faulty results, we reduced our dilutions by tenfold, using 0.01, 0.1, 1, and 10 ng/20uL reaction to try and produce the decreasing linear graph the assay should create if it works effectively. This fifth RT-LAMP assay will allow us to find out whether we should redesign certain primers. We tested our primers at the 2.5x concentration because this concentration worked best, as observed in the third and fourth LAMP assays.



Rv1308	Rv3801c	Rv2196	IS6110	Rv3859c	Rv3583c	Rv2444c	Rv1398c

A graph of our results is above. For some of the primers, the 0.01 ng/20uL reaction RNA concentration created erroneous results. We interpreted this as the concentration amount being extremely low and difficult to detect. We omitted the results for the four primers with those issues at the 0.01ng/20uL reaction concentration in our analysis. For all the primers, a strong decreasing linear graph was formed, showing an effective RT-LAMP assay since the more Mtb RNA there is, it takes fewer cycles to detect it.The highest Mtb RNA concentration we used in this assay (10ng/20uL reaction) produced the lowest Ct scores, and was consistently lower than the smaller concentrations, demonstrating 10 ng/20uL reaction as an effective concentration of RNA to use for the RT-LAMP assay. At 10 ng/20uL of RNA, all of our primers were below the Ct mean of 30, showing they are all effective. However, Rv1308 and Rv3801c are on the higher end and could possibly require redesigning.

Slope and R-squared Values

Gene	slope	R^2
Rv1308	-4.54471	0.997698
Rv3801c	-2.42125	0.998714
Rv2196	-3.55569	0.995454
IS6110	-2.76793	0.955699
Rv3859c	-3.10149	0.992449
Rv3583c	-2.40133	0.993736
Rv2444c	-1.98695	0.96992
Rv1398c	-1.67838	0.995719

The data from the fifth assay was also used to calculate the slope and R^2 values. The slopes are all negative, indicating a negative relationship between the Mtb RNA concentration and the Ct means. All of the R^2 values are extremely close to 1, demonstrating effective primers because of the negative linear relationship for all the Mtb RNA concentrations.

Conclusions

We noticed from our fourth LAMP assay that DNA worked better than RNA in the primers we tested, but RNA was still effective at producing low Ct means. Furthermore, RNA must be used for RT-LAMP, so we will continue to use various RNA concentrations for future RT-LAMP assays.

Ct Mean Comparison (Our Primers v. Old Primers)

For the most part, we cannot directly compare the Ct means from our primers to the old primers because the concentration of RNA/DNA used was not identical and many of the assays with the old primers included a spike in. The Ct means used to rank the primers that needed to be redesigned at the beginning were from an assay using different concentrations of RNA/DNA. However, by using data from different assays using the old primers we were able to generally assess the effectiveness of our new primers. The Ct threshold we set for our RT-LAMP assays was 1,500,000. The old primers had a threshold of 400,000. This difference does not need to be taken into account when comparing Ct means of old and new primers because the threshold should be placed at a point where the amplification plot is most linear, which can be different for various RT-LAMP assays.

	Old Primers	**New Primers**
	RNA 25 ng/sample	RNA 25 ng/sample
Gene	Ct Mean	Ct Mean
Rv0667	25.07	REDESIGN
Rv0824c	20.42	14.211
Rv0932c	29.5	REDESIGN
Rv1094	26.57	12.646
Rv1297	55.65	REDESIGN
Rv1308	25.22	28.389
Rv1398c	9.39	9.414
Rv1872c	56.05	REDESIGN
Rv2196	36.67	25.053
Rv2444c	15.56	20.231

	Old Primers	**New Primers**
	RNA 25 ng/sample	10ng/20ul reaction
Gene	Ct Mean	Ct Mean
Rv3219	32.36	29.888
Rv3583c	11.42	REDESIGN
Rv3800c	30.1	REDESIGN
Rv3801c	23.12	19.607
Rv3804c	34.28	34.488
Rv3825c	18.81	12.126
Rv3859c	20.6	15.478
Rv3875	14.75	23.041

Primers with ‘REDESIGN’ mean we did not run LAMP assays with them because they were confirmed unspecific by the gel tests. These primers need to be redesigned before use in a RT-LAMP assay. We can directly compare Ct values for the primers tested with the same concentration of RNA (25ng/sample). However, for some of the primers, we did not test them with 25ng/sample. The closest concentration tested was 10ng/sample. As previously mentioned, the assays that generated the Ct mean values for the old primers we were comparing to added

spike-ins. This addition could have changed the Ct results for the old primers, so there is some nuance to our entire comparison.

The left table shows the Ct means for Rv0824c, Rv1094, and Rv2196 at the 25ng/sample RNA concentration were lower than the previous primers, suggesting that our primers were more effective. Rv1308 and Rv1398c at the 25ng/sample RNA concentration Ct means were about the same as the old values, suggesting the primers were effective in the assay. Rv2444c at the 25ng/sample RNA concentration Ct value is slightly higher than the old values, though it is still relatively low (under 30), suggesting the need to retest.

The right table compares Ct means of the old primers with 25ng/sample RNA and Ct means of our primers at 10ng/sample RNA. Every primer set except Rv3875 (and REDESIGN primers) had a lower Ct value. At a lower RNA concentration, most of our primers still generated lower Ct values, implying the effectiveness of them. Rv3875 had consistently high Ct values, demonstrating the need to redesign. However, as stated before, this comparison is relative as the assays were not performed identically.

Overall Conclusions

Our primers for Rv0667 were deemed not specific in our first gel because multiple bands were generated. When we reran the gel with the primers at 55 degree Celsius instead of the usual 50 degrees Celsius, no product appeared. We could test a gel again with a temperature higher than 50 and lower than 55 degree Celsius to see if the multiple bands will disappear and that the temperature is the cause of unspecificity. However, an error in the generation of the primers occurred in software LAMP Designer, as the sequence for the B3 primer and the sequence of the B3 primer in the sequence alignment do not match for this particular gene. This suggests the primers should be redesigned.

Our primers for Rv0824c developed a single band in our gel, showing that they are specific to their target sequence. When we ran the primers in our first and third LAMP Assays, they consistently produced low Ct values and a clear decreasing linear relationship with a correlation of around 0.99 between Mtb RNA concentration and Ct mean, suggesting that the primers do not need to be redesigned.

Our primers for Rv0932c did not produce bands in our gel through two runs, so these primers will need to be redesigned.

Our primers for Rv1094 produced a single band in our gel run, showing that they are specific to their target sequence. In the first LAMP assay, the Ct mean went up while Mtb RNA concentration increased, which should not happen. But, when we reran the primers in our fourth LAMP assay with primer titrations and Mtb DNA and RNA, they formed a clear decreasing linear graph for Ct mean values when Mtb DNA/RNA increased. The Ct values at primer concentration of 2.5x were low as well, suggesting that these primers were effective and do not need to be redesigned.

Our primers for Rv1297 did not produce bands in our gel through two runs, so these primers will need to be redesigned.

Our primers for Rv1308 developed a single band in our gel, showing that they are specific to their target sequence. In the first LAMP assay, as Mtb RNA concentration increased, the Ct mean decreased, showing an effective assay. However, a clear linear line was not formed, so we retested them in our fifth LAMP assay. The results formed a clear linear line with a correlation between Mtb RNA concentration and Ct mean of 0.99, showing the primers are functional. However, the Ct mean for these primers are quite high (above 30) at various concentrations, so these primers could be redesigned, depending on preference.

Our primers for Rv1398c developed a single band in our gel, showing that they are specific to their target sequence. In the first LAMP assay, as Mtb RNA concentration increased, the Ct mean decreased, showing an effective assay. We also retested these primers in our fifth LAMP assay and a clear decreasing linear graph was formed with a correlation of 0.99. Additionally, the Ct values for these primers were one of the lowest, suggesting that these primers do not need to be redesigned as they are already effective.

Our primers for Rv1872c did not produce bands in our gel through two runs, so these primers will need to be redesigned.

Our primers for Rv2196 developed a single band in our gel, showing that they are specific to their target sequence. In our first LAMP assay, as Mtb RNA concentration increased, the Ct mean varied, making a correlation value around 0.67, demonstrating that either an error occurred in the LAMP assay, or the primers needed to be redesigned. We reran the primers in our fifth LAMP assay, and a clear decreasing linear graph with a correlation of 0.99 was formed, showing an effective assay. The Ct values of these primers were under 30, suggesting that these primers do not need to be redesigned and that the first LAMP assay may have had an error not related to the primers, such as dilutions.

Our primers for Rv2444c developed a single band in our gel, showing that they are specific to their target sequence. We tested these primers in our first LAMP assay and as Mtb RNA concentration increased, the Ct mean varied, making a correlation value around 0.77. We reran the primers in our fifth LAMP assay, and a clear decreasing linear graph with a correlation of 0.97 was formed, showing an effective assay. The Ct values of these primers were under 30, suggesting that these primers do not need to be redesigned and that the previous LAMP assay may have had an error not related to the primers.

Our primers for Rv3219 developed a single band in our gel, showing that they are specific to their target sequence. We tested the primers in our second LAMP assay. As Mtb RNA increased, the Ct mean increased for the highest RNA concentration of 100 ng/20uL reaction, creating a poor correlation value of around 0.82. The Ct values were additionally very high, so we decided that these primers need to be redesigned.

Our primers for Rv3583c were deemed not specific in our first gel because multiple bands were generated. When we reran the gel with the primers at 55 degree Celsius instead of the usual 50 degrees Celsius during PCR cycling, a single band appeared for every primer concentration we tested, showing that the primers are specific to their target sequence and just need a certain annealing temperature. We then tested these primers in our fifth LAMP assay run, where they formed a clear decreasing linear graph demonstrating their effectiveness. They also had Ct mean values under 30, suggesting that these primers do not need to be redesigned as they are already effective.

Our primers for Rv3800c did not produce bands in our gel through two runs, so these primers will need to be redesigned.

Our primers for Rv3801c developed a single band in our gel, showing that they are specific to their target sequence. We tested the primers in our second LAMP assay. As Mtb RNA increased, the Ct mean increased for the highest RNA concentration of 100 ng/20uL, creating a poor correlation value of around 0.74. To determine if an error occurred in the LAMP assay, we reran the primers in our fifth LAMP assay because the Ct values of these primers in the second assay were low and had potential for an effective assay. The primers formed a clear decreasing linear graph in the fifth assay demonstrating their effectiveness. They also had Ct mean values under 30, suggesting that these primers do not need to be redesigned.

Our primers for Rv3804c developed a single band in our gel, showing that they are specific to their target sequence. We tested the primers in our second LAMP assay. As Mtb RNA increased, the Ct mean increased for the highest RNA concentration of 100 ng/20uL, creating a poor correlation value of around 0.80. The Ct values for these primers were above 30, even for the middle Mtb RNA concentration of 10 ng/20uL, so the primers do not have much potential to be effective, so we did not rerun and decided the primers need to be redesigned.

Our primers for Rv3825c developed a single band in our gel, showing that they are specific to their target sequence. We tested the primers in our second LAMP assay. As Mtb RNA increased, the Ct mean increased for the highest RNA concentration of 100 ng/20uL, creating a poor correlation value of around 0.74. However, the whole assay had this error, so we decided to rerun the primers in our third LAMP assay as they had low Ct mean values, demonstrating their potential to be effective. As primer concentration increased to 2.5x and Mtb RNA concentration increased to 100 ng/20uL, the Ct mean decreased, forming a linear line with good correlation values around 0.98-99 for each primer concentration, showing the effectiveness of the primers. Additionally, the Ct means were under 30 for both the 1.25x and 2.5x primer concentrations, suggesting the primers do not need to be redesigned as they are shown to be effective.

Our primers for Rv3859c developed a single band in our gel, showing that they are specific to their target sequence. We tested the primers in our second LAMP assay. As Mtb RNA increased, the Ct mean increased for the highest RNA concentration of 100 ng/20uL,

creating a poor correlation value of around 0.76. However, the whole assay had this error, so we decided to rerun these primers in our fifth LAMP assay as they had low Ct mean values, demonstrating their potential to be effective. In the fifth LAMP assay, as Mtb RNA concentration increased, Ct mean decreased, creating a correlation value around 0.99, showing an effective LAMP assay. Additionally, the Ct means were under 30, suggesting the primers do not need to be redesigned as they are shown to be effective.

Our primers for Rv3875 developed a single band in our gel, showing that they are specific to their target sequence. We tested the primers in our second LAMP assay. As Mtb RNA increased, the Ct mean increased for the highest RNA concentration of 100 ng/20uL, creating a poor correlation value of around 0.74. Most of the Ct values for these primers were above 30, so the primers do not have much potential to be effective, so we did not rerun the primers and decided that they need to be redesigned.

Next Steps

In the future, we will need to redesign the primers that were not effective from our RT-LAMP assays and shown not specific by our gels. For the effective primers, we can move on to develop correlation results and quantitative gene expression values to then use in DRonA.

Correlation Results

In theory, we would calculate how effective our LAMP assay primer results are compared to RNA seq results to generate correlation values to compare with values from the old primers. The closer the correlation between our LAMP assay gene expression values and the RNA seq gene expression values are to one or negative one, the more effective the LAMP assay was. This means RT-LAMP is closer to being able to replace the more expensive RNA seq to obtain gene expression values to feed into DRonA. However, as stated earlier, we do not have the RNA-seq data of the pooled RNA samples we used in our RT-LAMP assays to compare to.

Gene Expression Values

As we wrote about earlier, LAMP assays can obtain the quantified gene expression values (transcriptomes) to feed into DRonA to calculate the cell viability score in order to predict treatments for patients with Mtb.

Below are the instructions of how we would calculate the quantified gene expression values from our LAMP assay.

Use IS6110 data as control: Calculate gene expression with equation –

$$GE_g = 2^{-(CT_{DNA-control} - CT_g)}$$

(CT values are the Ct mean data from LAMP assay)
Calculate normalized gene expression with equation –

$$nGE = \frac{(GE_{Spike-in2} - GE_{g})R}{(GE_{Spike-in2} - GE_{Spike-in1})}$$

Spikes are used in the LAMP assays to calculate the normalized gene expression. However, we did not use them in our assays because we were focused on optimizing the primers' Ct values for various RNA concentrations. The normalized gene expression equation is multiplied with R = 3000. The difference of 10^3 reads/ log2 dye intensity, i.e. difference in amount of spike-in1 and spike-in2 corresponds to the rank difference of 3000 in the compendium DRonA was trained on.

DRonA/DUST

The RT-LAMP gene expression values will be fed into DRonA to predict cell viability scores. To ensure the accuracy of the algorithm, these results will then be compared with gold standard genotypic and phenotypic DST.

By using RT-LAMP assays to obtain gene expression values (transcriptomes) of Mtb after exposure to a panel of drugs to then feed into DRonA to predict the cell viability score and determine treatments for patients with Mtb, the DST is cheaper and faster, meeting WHO's requirements for a Mtb DST. DUST has been shown to detect survivability, allowing it to differentiate dead and non-cultivable cells and be agnostic to Mtb mechanism of killing and genetic background of strain, allowing DUST to be used for determining Mtb susceptibility to any TB drug. DUST's methods can be generalizable to other DST for other pathogens as well. The final step would be to test DUST in a high burden clinical TB setting of South Africa.

Acknowledgements/References

We would like to thank Dr. Eliza Peterson and Julie Do for mentoring us during our internship at the Institute for Systems Biology. We are so grateful for all your help in answering our questions and guiding us!

We would also like to thank Claudia Ludwig, Miranda Johnson, and the Systems Education Experiences department for giving us the opportunity to participate in this amazing internship and teaching us so much about science!

Lastly, we would like to thank Nitin Baliga and all the ISB staff and interns for being so welcoming and helping us throughout this process!

References:

Eliza's Proposal document (not yet published)

Aryan, E., Makvandi, M., Farajzadeh, A., Huygen, K., Bifani, P., Mousavi, S.-L., Fateh, A., Jelodar, A., Gouya, M.-M., & Romano, M. (2010). A novel and more sensitive loop-mediated isothermal amplification assay targeting IS6110 for detection of Mycobacterium tuberculosis complex. *Microbiological Research*, *165*(3), 211–220. https://doi.org/10.1016/j.micres.2009.05.001

Bennett, J. E. (2015). *Mycobacterium Tuberculosis - an overview*. Sciencedirect.com. https://www.sciencedirect.com/topics/medicine-and-dentistry/mycobacterium-tuberculosis

NCBI. (2017). *Polymerase Chain Reaction (PCR)*. Nih.gov. https://www.ncbi.nlm.nih.gov/probe/docs/techpcr/

PrimerExplorer V5 Manual. (n.d.). https://primerexplorer.jp/e/v5_manual/pdf/PrimerExplorerV5_Manual_2.pdf

Rebecca and John Moores UCSD Cancer Center DNA Sequencing Shared Resource. (n.d.). Moores.ucsd.edu. https://moores.ucsd.edu/Research/Shared/dna/chr_pdimer.asp#:~:text=Primer%20dimers%20occur%20when%20primers%20are%20accidentally%20designed

Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for diagnosis of dengue - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/e-Principle-of-RT-LAMP_fig1_260560059

RT-LAMP (open access). (n.d.). RTLAMP. https://www.rtlamp.org/get-started/rt-lamp-open-access/

Srinivas, V., Ruiz, R. A., Pan, M., Immanuel, S. R. C., Peterson, E. J. R., & Baliga, N. S. (2021). Transcriptome signature of cell viability predicts drug response and drug interaction in Mycobacterium tuberculosis. *Cell Reports Methods*, *1*(8), 100123. https://doi.org/10.1016/j.crmeth.2021.100123

WarmStart LAMP Kit (DNA & RNA) Protocol (E1700) | *NEB*. (n.d.). Www.neb.com. https://www.neb.com/protocols/2016/08/15/warmstart-lamp-kit-dna-rna-protocol-e1700