Development of a DNA Library Synthesis Protocol for Illumina-Compatible Sequencing of Human Genomic DNA

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Abstract

DNA sequencing is a technique used to determine the nucleotide sequence of DNA. There has been a vast advancement in sequencing over the past years, such as employing faster sequencing methods. One of these methods is Next Generation Sequencing, where millions of small DNA fragments are sequenced simultaneously. While several equipment platforms exist with different sequencing methods, one of the most popular systems is that of Illumina, based in San Diego, California (http://www.illumina.com). Illumina sequencing is cost efficient per base-pair of DNA, and sequences the genome faster than previously used methods i.e Sanger's method. This is a result of the extremely high throughput of the number of bases read over time. A library containing sequenceable DNA is made in order for the DNA to be sequenced via Illumina sequencing. DNA sequencing is widely used to study the cause of genetic disorders like Familial Exudative Vitreoretinopathy (FEVR) and Norrie Disease. For our experiment, we made two duplicate libraries using protocols provided by manufacturers. Lastly, three quality control tests were done during this experiment in order to confirm that sequenceable DNA was synthesized.

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Introduction

DNA sequencing is used to determine the exact order of nucleotide bases in a DNA strand. DNA sequencing allows scientists to determine the genome sequence. This technique is widely used to search for genetic variations and mutations of a gene. These variations or mutations play a role in the development and progression of a disease; hence, it is important to study them.[1] The evolution of DNA sequencing can be split into three major generations. The first generation includes Sanger and Gilbert sequencing, the second generation involves pyrosequencing, and the third generation is Illumina sequencing. Illumina sequencing falls into the category known as Next Generation Sequencing, which is widely used today along with Sanger's method, or gold-standard PCR + thermal cycling sequencing.[2]

The Human Genome Project (HGP) cost $2.7 billion using first generation sequencing.[3] This project, sequencing a single person's genome, would cost approximately $10,000 using Next Generation Sequencing.[2] Additionally, it would conclude in a timespan of days - compared to the years spent on the first Human Genome Project - because the accurate human genome reference sequence created by the HGP is used as a template to quickly align the hundreds of millions of DNA fragment reads generated by NGS systems. Illumina sequencing utilizes several different sequencing machines, with each sequencer using a different number of reads per sequenced genome, comprising a range of 130-400 million reads per genome sequenced.[4] High end sequencing machines use more reads per genome compared to low end sequencers.

The molecular requirements for Illumina sequencing depend upon the library preparation kit utilized. THruPLEX DNA-seq kit from RUBICON Genomics in Ann Arbor, MI (http://rubicongenomics.com), works ideally with a DNA input of 50 ng, but it can work within a range of 50 pg to 50 ng. The optimal DNA fragment size is less than 1,000 bp and the max input sample volume is 10 microliters. Genomic DNA is sonicated before being utilized in this kit to ensure it is less than 1,000 bp. The three major steps in this protocol are Template Preparation, Library Synthesis, and Library Amplification. During template preparation, the fragmented dsDNA is repaired by DNA ligase and the overhangs are cleaved so both strands of DNA are the same length. During library synthesis, stem loop adaptors are added and ligated onto both ends of the dsDNA fragments. Library amplification occurs after blunt end ligation of the adaptors. In this step, the adaptors are cleaved and extended on the DNA fragments. This allows for primers and barcode sequences to be on the DNA strands. The product of this reaction is an indexed Illumina NGS library.

Illumina sequencing works for a 200-800 base pair range DNA.[5] DNA strands that are input into the sequencer are cleaved into short sections (100-150 base pairs). This allows them to cluster and amplify more efficiently on the Illumina flow cell. Longer fragments are ligated by DNA ligase. Each read is amplified by PCR. On a slide, the nucleotides are fluorescently labeled and added one at a time by a fluorescence signal. There are four reversible terminator bound dNTPs. A terminator is placed after a nucleotide is added, and the terminator is removed to add

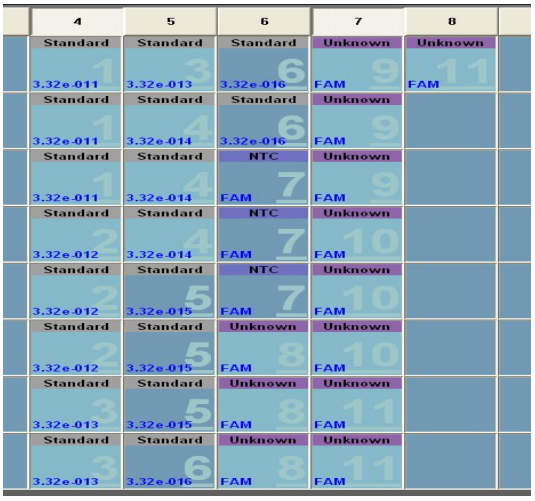
another nucleotide. This ensures that only one nucleotide is added at a time, allowing for highly accurate base-by-base sequencing. The sequence is constructed and displayed on a computer.

Quality control tests are used to avoid sequencing failure and the time/financial costs associated with failure. These tests determine whether the output of a reaction is as expected. Three quality control (QC) tests were utilized in this experiment. The first QC test was to run sonicated DNA through gel electrophoresis. Through the process of gel electrophoresis (Appendix A), the sonicated DNA is compared to a 100 bp and 1kb ladder. The base pair range of sonicated DNA is found by this comparison. The second QC test was the input of EvaGreen dye during library amplification (Appendix B) to visualize actual amplification of the library. Fluorescence data was provided by real time PCR as shown in Figure 1, Appendix D. This QC test shows that there is DNA in the Library. The third QC test was the use of SYBR Green in a qPCR reaction (Appendix C). The results for this test are in Appendix D. This QC test shows that the amount of sequenceable DNA by comparing the Library DNA to standards from KAPA biosystems. Quality control tests are important in any laboratory setting to avoid economic loss, as well as avoiding wasted time.

Materials and Methods

We followed protocols from various manufacturers (Appendix E) in order to make protocols specific to our research laboratory needs. Three protocols were made for this experiment. Appendix A shows the protocol for "Sonication of Genomic DNA and Gel Electrophoresis." Appendix B shows the protocol for "Library Synthesis and Amplification" using Rubicon's ThruPLEX DNA-seq kit. Appendix C shows the protocol for our qPCR QC test.

Two duplicate sonicated samples were prepared and ran through the "Library Synthesis and Amplification" reaction. Fluorescence data is provided for both samples in Figure 1, Appendix D. After Library Synthesis and Amplification, both duplicate samples were run through a QC test (qPCR). This protocol is outlined in Appendix C. The lanes used in the real-time qPCR machines are detailed in the figure below. Preparation of these lanes is also detailed in the appendices and shown in the table below.



qPCR Lanes	
Sample 1	Standard 1: 20 pM
Sample 2	Standard 2: 2 pM
Sample 3	Standard 3: 0.2 pM
Sample 4	Standard 4: 0.02 pM
Sample 5	Standard 5: 0.002 pM
Sample 6	Standard 6: 0.0002 pM
Sample 7	NTC (dH_2O)
Sample 8	DNA Sample 1: 1/1,000 Dilution
Sample 9	DNA Sample 2: 1/1,000 Dilution
Sample 10	DNA Sample 1: 1/100,000 Dilution
Sample 11	DNA Sample 2: 1/100,000 Dilution

Results

We found the best sonication time by using a previously made ERI program in the sonicator. We modified this program by changing the process time from 10 minutes to 12 minutes in order for the DNA to be sonicated more. This change output sonicated DNA mainly within a bp range of 300-600, which less than 1000 bp and within 200-800 bp range. ThruPLEX required the sonicated DNA to be under 1,000 base pairs (Appendix E) and Illumina sequencing works ideally under a 200-800 base pair range, so our sonicated DNA fulfills the ideal conditions for library preparation and Illumina sequencing. The base pair range of sonicated DNA was found by using the QC test of gel electrophoresis.

Fluorescence data via real time PCR was compiled during library amplification of both libraries. The data is shown in Figure 1. These figures can also be found for reference in Appendix D.The data provided by this QC test shows that DNA in each library was successfully amplified.

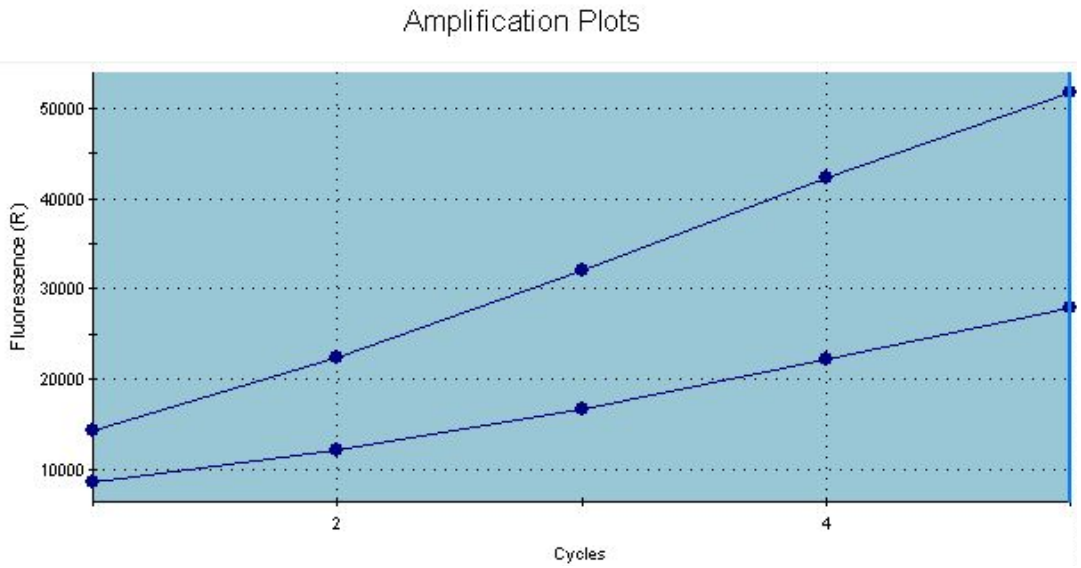


Figure 1: Library Amplification Fluorescence Data Results

Results from real time qPCR are the remaining figures in Appendix D. Each sample was diluted and triplicated in this process. Each sample was diluted in a 1/1,000 ratio and a 1/100,000 ratio. This quality control test showed that there is ample sequenceable DNA in both libraries as shown by the amplification plot in Figure 3. Primers 5 and 7 are on each end of sequenceable DNA. Unknown 1 and 2 (Figure 2) are the duplicated libraries and they were compared to standards provided by KAPA biosystems. Six standards were used and they vary in concentration by a power of ten as shown in the table (Materials and Methods). Standards 5 and 6 are not included in the figures because they were too small and caused significant error in the standard curve. Sample 7 is our non-template control (NTC) sample (deionized H_2O); however, through experimental error, some of the prepared library ended in the NTC. This was shown in

the dissociation curve - the NTC had a similar curve as the libraries (Figure 4). The dissociation curve is an indicator of whether there is a PCR product, so it states if the DNA is amplified with the primers.[6] Table 1 shows the concentrations of our unknown before dilutions, which is 33.2 nM. Lastly, the standard curve in Figure 5, shows the PCR amplification efficiency by real time qPCR. This is shown by the slope of the standard curve, and we got 97.5% efficiency.

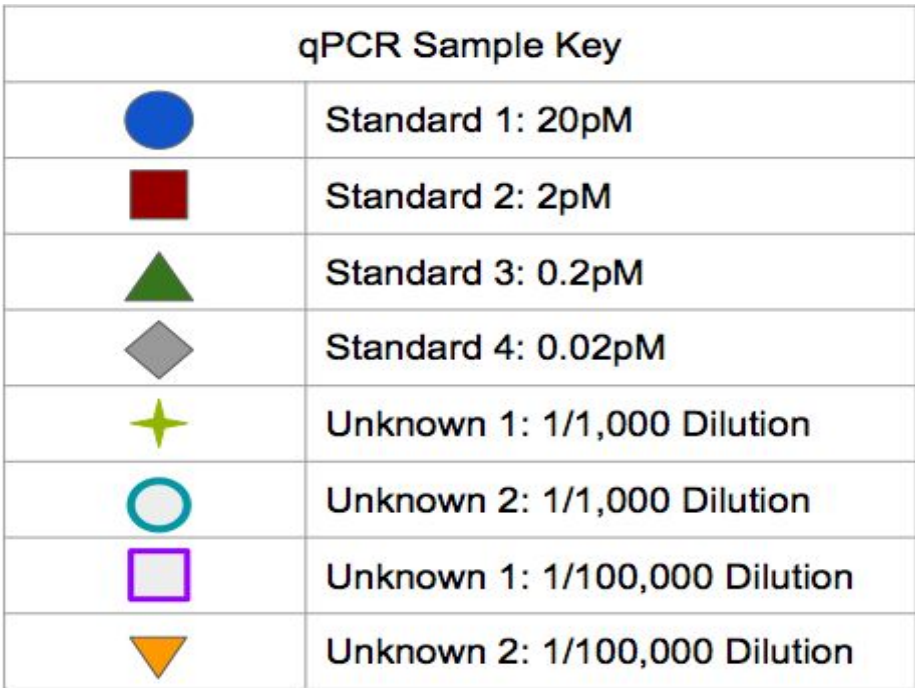


Figure 2: Sample Key for qPCR Graphs

	Concentration (M)
Standard 1	2.0 E-11
Standard 2	2.0 E-12
Standard 3	2.0 E-13
Standard 4	2.0 E-14
Unknown 1: 1/1,000 Dilution	3.32E-11
Unknown 2: 1/1,000 Dilution	3.32E-11
Unknown 1: 1/100,000 Dilution	3.32E-13
Unknown 2: 1/100,000 Dilution	3.32E-13

Table 1: Numerical values of standard curve (Figure 5)

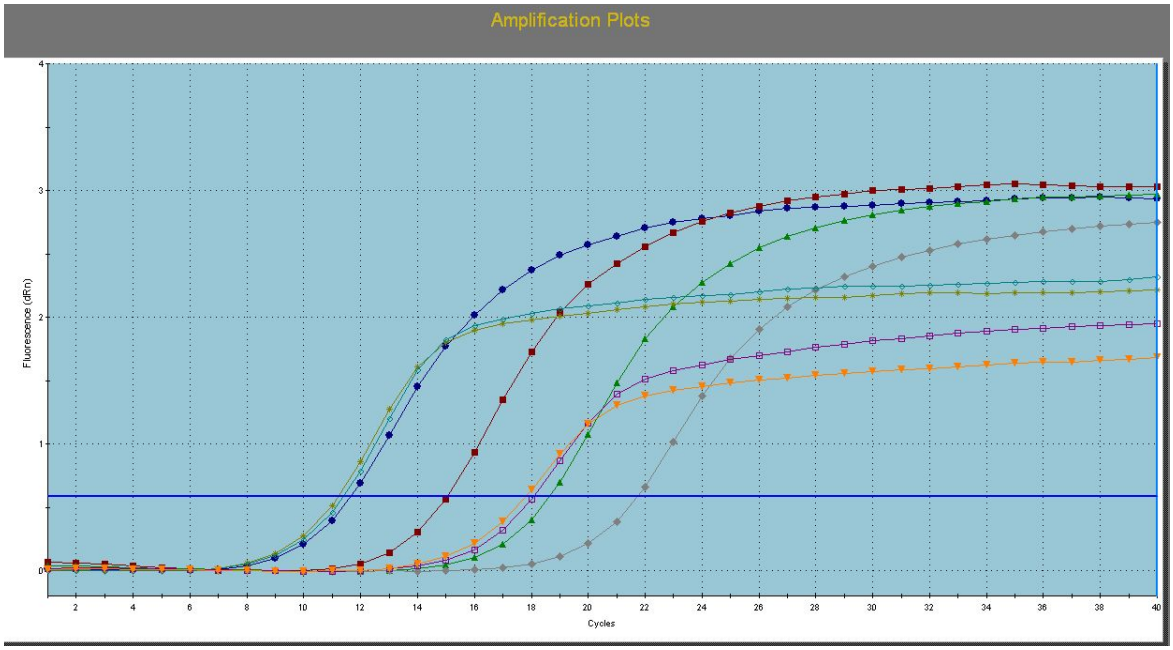


Figure 3: qPCR Amplification Plot

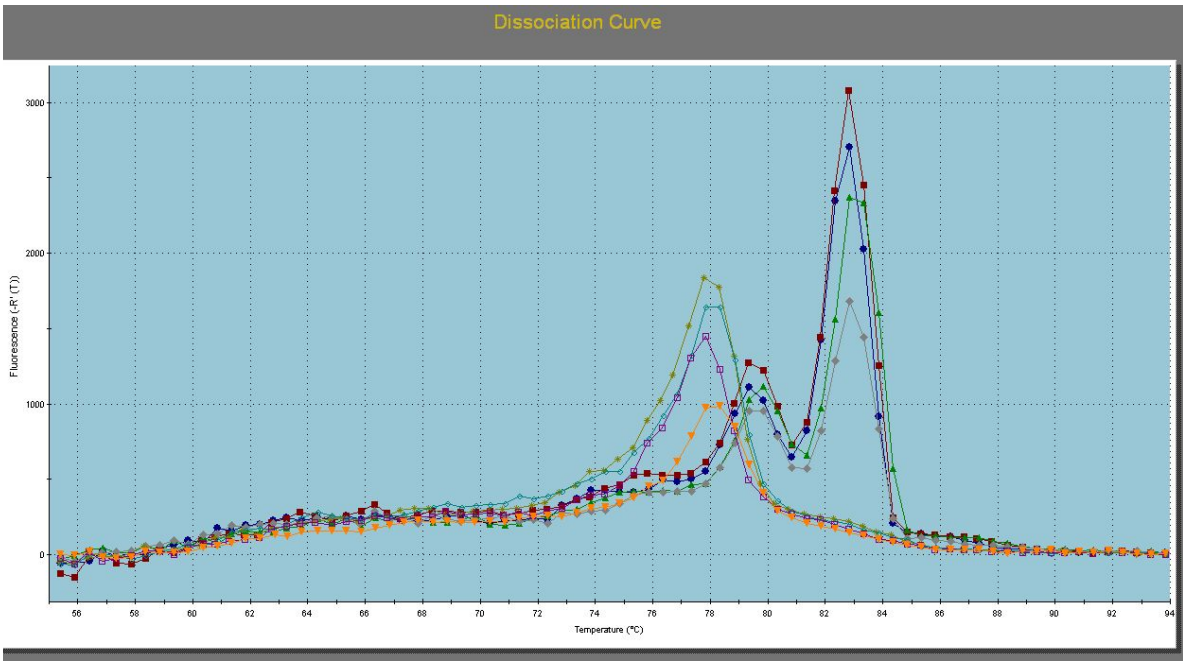


Figure 4: qPCR Dissociation Curves

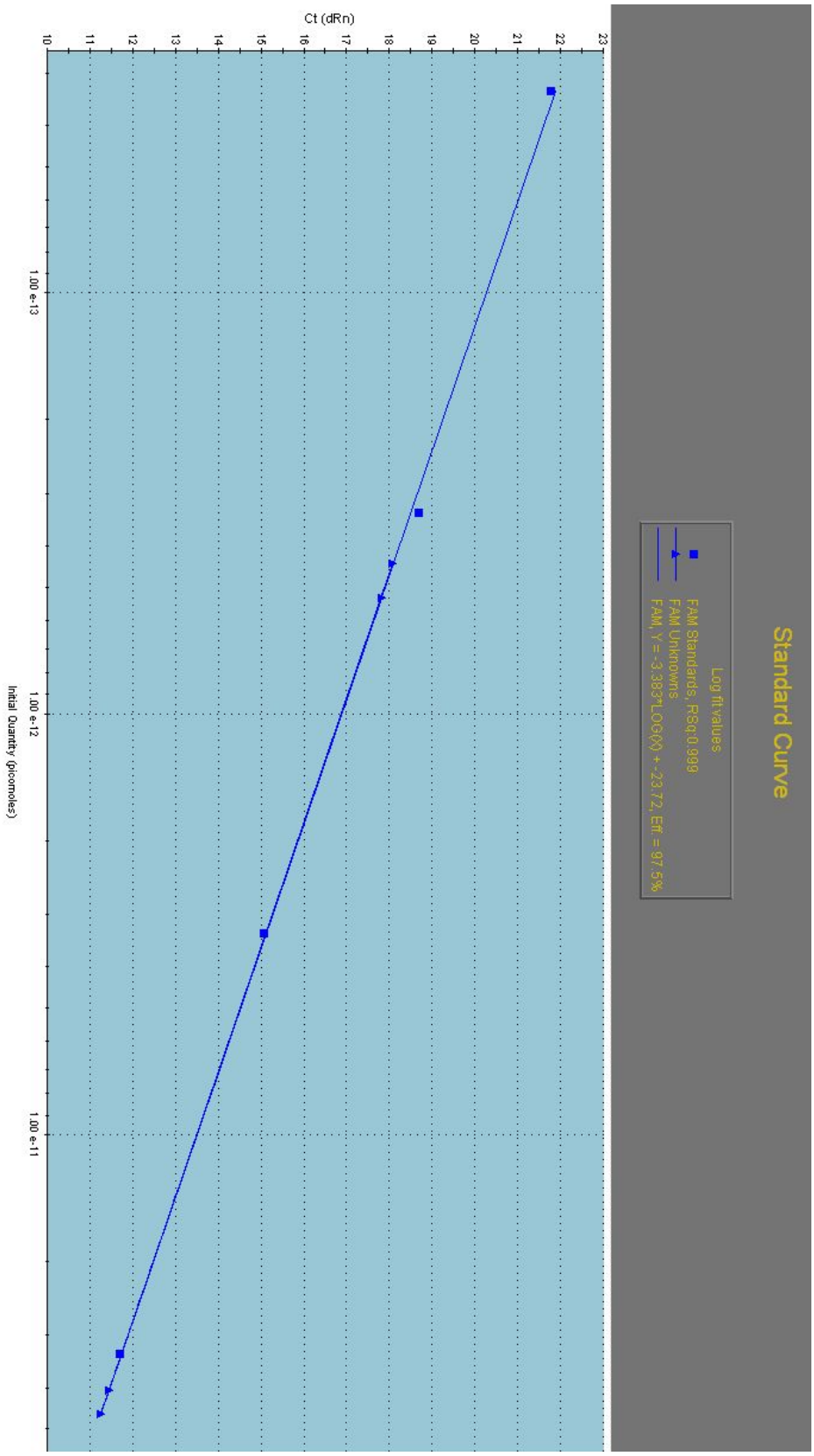


Figure 5: Standard Curve

Discussion

The purpose of this experiment is to design a protocol to prepare a library for Next Generation Sequencing in the Eye Research Institute at Oakland University. This protocol will be used for understanding genetic disease, such as FEVR, by genetic sequencing. Illumina Sequencing was picked because it is the most widely used form of Next Generation Sequencing, and it is both time/cost efficient compared to other sequencing techniques. We succeeded in making the protocols (Appendix A-C) by using the reference of various manufacturer protocols (Appendix E). We implemented our protocols by making two duplicate libraries and tested these libraries through three quality control tests: gel electrophoresis, fluorescence data obtained during library amplification, and qPCR data. The ERI can now do Next Generation Sequencing by using the protocols in Appendix A-C, which are specific to their equipment and reagents.

As mentioned earlier, genetic sequencing can be used to determine what can cause a disease by comparing an individual's DNA with the disease to an individual's DNA without the disease. For future studies, xGen lockdown probes can be used along with the protocols made in this experiment. xGen Lockdown probes are used in capture of specific genes by target enrichment. A library, specific to the captured genes, can be made by ThruPLEX DNA-seq kit. This will allow for a reduced target pool size instead of making a library of a whole genome.[7] A smaller library is more cost and time efficient, and will be a fraction of the cost of whole genome sequencing. Familial Exudative Vitreoretinopathy (FEVR) is a rare eye disease that affects the retina, clear vitreous layer, and blood vessel.[8] This is a progressive disease, so it gets worse over time. The ERI has compiled ten genes possibly associated with FEVR: NDP, FZD4, LRP5, TSPAN12, ZNF408, TGFBR2, LGR4, Wnt 3a, Wnt 5a, and Wnt 7a. When there is a mutation in the NDP gene, the symptoms are similar to that of FEVR, but it is called Norrie Disease (named after the person who discovered the relevant protein: Norrie Disease Protein).[9] These genes follow the Wnt pathway, and a mutation in this pathway can lead to many diseases.[10] The ERI can have a better understanding of this disease through the implementation of capture probes and library preparation by Next Generation Sequencing.

Our project holds significance for the Eye Research Institute. Additionally, we were able to learn laboratory techniques along the way and the importance of quality control checks. We succeeded in creating a protocol for sonication of DNA in preparation of a DNA library. We confirmed this success by running gel electrophoresis to confirm we had DNA of a fairly uniform and relevant length. Another success we had was in creating a DNA library. We took our sonicated strands of DNA and attached primers and indices to the ends, which will allow them to be sequenced. Again, we confirmed this success by running a real-time fluorescence test to ensure that the DNA was properly amplified. Finally, we proved that our experiment as a whole was a success by performing real-time qPCR to quantify the amount of sequenceable DNA contained in our libraries (33.2nM concentration). The next step in checking our successes would be to pay for our DNA sample to be sequenced. If it covers the genome fairly well, we can

label our protocols as a definitive success. However, this is still expensive and may not be feasible. Regardless, we have enough definitive results to reasonably conclude that our protocols are well-designed.

Appendices

Appendix A - Sonication/Fragmentation of DNA, and Quality Control by Gel Electrophoresis

I. Sonication

1. Dilute DNA to 50 ng/µL. Use the equation $M_1V_1 = M_2V_2$. Prepare these dilutions in a 1.5 mL Eppendorf tube - the final diluted volume should be 100 µL.
2. Ensure that the "Sonicator S-4000" Cup Horn sonicator is properly functional.
    a. The o-rings should be flexible and uncracked. Ensure that they are properly lubricated and maintained.
3. Assemble the cup horn sonicator inside the soundproof box (Fig. 1, 2, 3). Fill up the cup with water and ice. This water level should be at a point where the Eppendorf tubes, when suspended in the water, should be no more than 5 mm above the metal horn (Fig. 4).
4. Run the following program on the cup horn sonicator (Fig. 5):

Program 3	
Amplitude	90
Process Time	00:12:00
Pulse-ON Time	00:00:15
Pulse-OFF Time	00:00:45

   Periodically as the process goes on, open the box during "Pulse-OFF Time" to add ice to the water and take some water out - otherwise, the sonication will warm the water.
5. After the final pulse, remove the Eppendorf tubes. You may either proceed to gel electrophoresis or freeze the fragmented DNA at this point.

II. Examples of how the Cup Horn Sonicator should look

Figures 1, 2, 3: The general setup of the Cup Horn Sonicator in the soundproof box

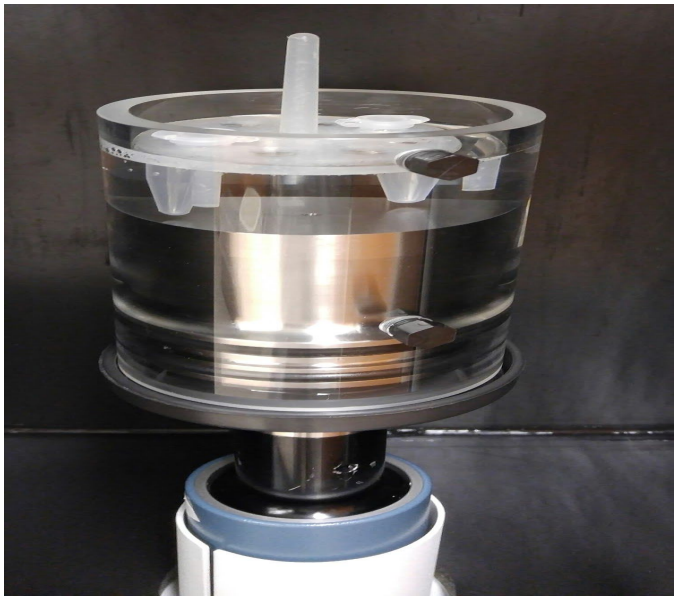


Figure 4: Proper setup of the Eppendorf tubes inside the sonication cup

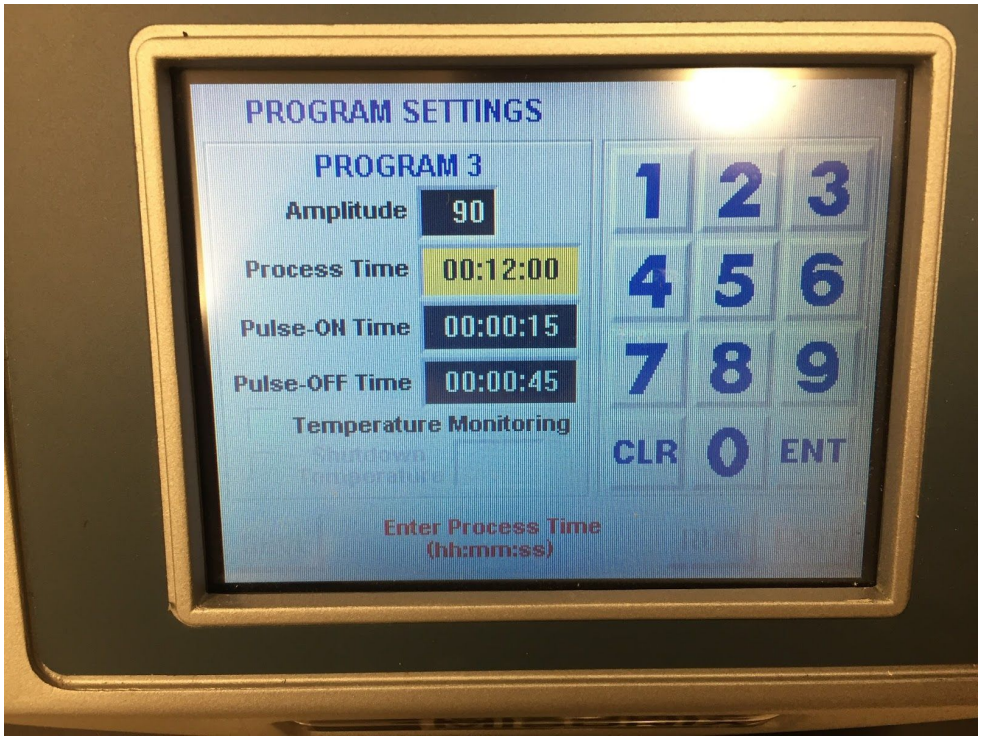


Figure 5: Program settings for this procedure (Program 3). Amplitude = 90, Process Time = 00:12:00, Pulse-ON Time = 00:00:15, Pulse-OFF Time = 00:00:45

III. Gel Electrophoresis (Quality Control)

1. Prepare a 1.5% agarose gel.
2. Thaw and vortex the fragmented DNA. Additionally, prepare the 100 bp + 1kb ladders, the TE, sybr green dye, and loading dye.
3. Prepare 10 µL mixes in the following volumes. To prepare, secure a portion of parafilm on the work-bench. Add the volumes to a droplet and mix thoroughly with a pipette. Avoid excess bubbles.

Ladders (100 bp and 1kb)	
Ladder mix	5 µL
Sybr Green	1 µL
TE	4 µL

Genomic DNA sample	
DNA	1 µL (50 ng)
Sybr Green	1 µL
6x Loading Dye	1 µL
TE	7 µL

4. The final volume for each drop should be 10 µL.
5. Ensure that the gel chamber is filled to the proper level with TE.
6. Carefully pipette each drop into separate lanes (mark in your lab book what is in each lane).
7. Run the gel through electrophoresis.
8. Remove gel from the chamber and photograph under UV light.
9. Sonicated samples should fall mostly in the range of 200-800 bp.

Appendix B - Library Synthesis and Amplification

The following protocol is based on "THruPLEX DNA-seq KIT" data sheets.

Prior to use, transfer enzymes to ice and centrifuge briefly. Thaw buffers, vortex briefly and centrifuge prior to use. Keep buffers & enzymes on ice until being used.
Indexing reagents can be frozen/thawed no more than four times. **Put a hash mark to count thaws on each indexing reagent tube use. Make sure to date/initial all materials used.**

The following steps should be done sequentially.

For these steps there are two methods that may be used. Each of the 3 main processes include a step that prepares a master mix. If there is a sufficiently small amount of libraries to prepare, you may add the components directly to the PCR tubes. If there are a large number of libraries being prepared, you should create a separate master mix and add 5% for loss due to pipetting.

I. Template preparation (for using 50 ng of sheared human genomic DNA)

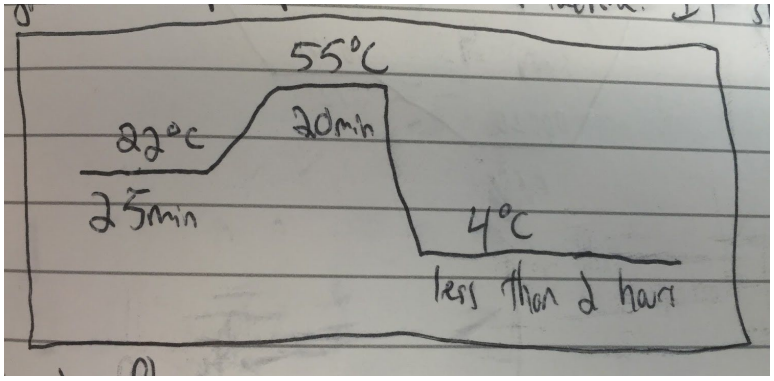
1. Add 1.0 µL of fragmented 50 ng/µL DNA to a PCR tube (this should be 50 ng of DNA). Add 9.0 µL of deionized water to bring total volume to 10 µL.
2. Per reaction**,** prepare the Template Preparation Master Mix as described below.

Template Preparation Master Mix per reaction mixture		
Component	Cap Color	Volume
Template Preparation Buffer	Red	2.0 µL
Template Preparation Enzyme	Red	1.0 µL

3. To the 10 µL DNA sample from step #1, add 3.0 µL (per reaction) of Template Prep Master Mix made in step #2. (Total volume is now 13.0 µL per reaction)
4. Tightly cap tube.
5. Vortex tube.
6. Centrifuge briefly to collect contents at the bottom of tube - ensure to centrifuge with proper counterweighting.

7. Place the tubes in the PCR machine. Prepare this template using the preset program called “TempPrep” (it is the following program):

Template Preparation Reaction	
Temperature	Time
22°C	25 min
55°C	20 min
4°C	Hold ≤ 2 hours



Check to ensure the program looks similar to the one above.

8. Remove tube from PCR machine and centrifuge briefly.

II. Library Synthesis Step

1. Reagents must be thawed and vortex mixed before using them to make the master mix. Add the reagents below to the master mix.

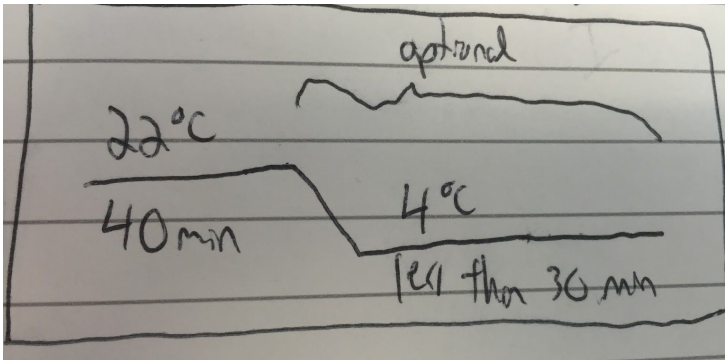
Library Synthesis Master Mix per reaction mixture		
Component	Cap Color	Volume
Library Synthesis Buffer	Yellow	1.0 µL
Library Synthesis Enzyme	Yellow	1.0 µL

(If this master mix was prepared, ensure to vortex before pipetting into separate reactions).

2. Add 2.0 µL of Library Synthesis Master Mix to each tube (Total volume per reaction should now be 15.0 µL).
3. Mix thoroughly by vortexing.

4. Centrifuge briefly to collect contents at the bottom of tubes.
5. Place the tubes in the PCR machine. Prepare this library through the preset program called “LibSynth” (Make certain it is the following conditions):

Library Synthesis Reaction	
Temperature	Time
22°C	40 min
4°C	Hold ≤ 30 min



Check to ensure that the program looks similar to the one above.

6. Remove tubes from the PCR machine, vortex, and centrifuge briefly.

III. Library Amplification Step

1. Remove the desired indexing reagents from freezer and thaw. Prior to use, vortex and centrifuge the indexing reagents to collect the contents at the bottom.
2. Prepare Library Amplification Master Mix using the following volumes per reaction. Vortex thoroughly.

Library Amplification Master Mix		
Component	Cap Color	Volume
Library Amplification Buffer	Green	25.0 µL
Library Amplification Enzyme	Green	1.0 µL
Nuclease Free Water	Clear	1.5 µL
EvaGreen Dye	---	2.5 µL

We used EvaGreen dye for real-time quantification of amplified DNA.

3. Transfer contents of PCR tubes used in library synthesis to strip PCR tubes used for real-time quantitative PCR.
4. Add 30.0 µL of Library Amplification Master Mix to each reaction.
5. Add 5.0 µL of Indexing Reagent to each reaction. MAKE SURE that if you are performing reactions with multiple subjects to use multiple DIFFERENT indexing reagents.
   ex. We are creating two libraries: Patient A and Patient B. We will use indexing reagent 1 for Patient A's library and indexing reagent 2 for Patient B's library.
6. Final volume per reaction should be 50.0 µL.
7. Firmly close the strip tubes and briefly vortex and centrifuge to collect contents at the bottom.
8. Place the strip tubes in the real-time PCR machine.
9. Start the real-time PCR computer and open the program to run Windows. Choose to connect at USB to the real-time PCR machine. Open MxPro and select EvaGreen as the fluorescent dye.
10. Amplify this library through the preset program in the folder: My Documents->Mitton_LibraryPrep->EvaGreen program. It should be the following program, set to work for 50ng of DNA input.

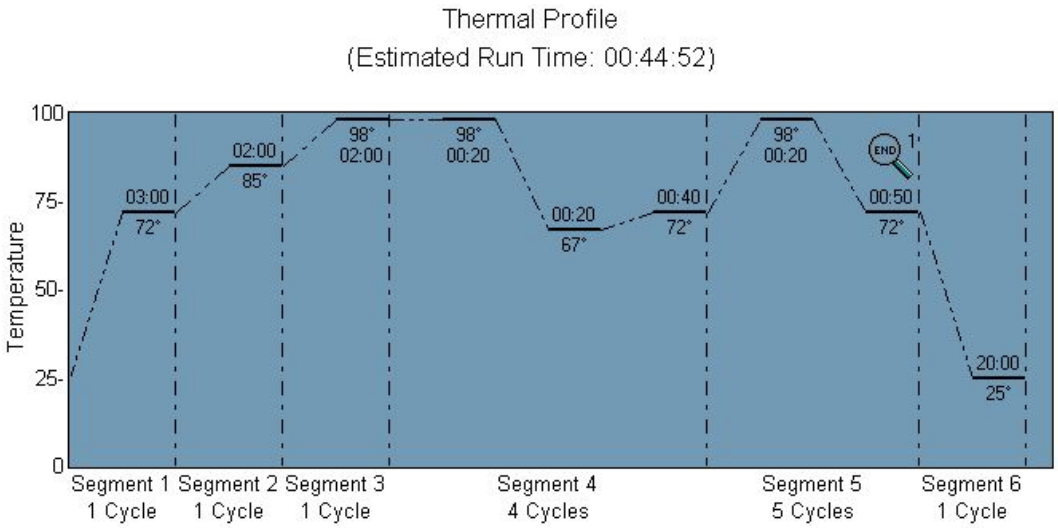
Library Amplification Reaction

	Stage	Temperature	Time	Number of Cycles
Extension & Cleavage	1	72°C	3 min	1
	2	85°C	2 min	1
Denaturation	3	98°C	2 min	1
Addition of Indexes	4	98°C	20 s	4
		67°C	20 s	
		72°C	40 s	
Library Amplification	▲ 5	98°C	20 s	▲ 5 to16 (see table on right)
		*72°C	50 s	
	6	4°C	Hold	1

*Acquire fluorescence data at this step, if monitoring amplification in real-time.

▲ Stage 5 Amplification Guide

DNA Input (ng)	Number of Cycles
50	5
20	6
10	7
5	8
2	10
1	11
0.2	14
0.05	16



Check to ensure that the program looks similar to the one above.

Also, in Step 5 as shown in the table above, you should notice amplification in the real-time fluorescence data. This is a quality control test.

11. Remove tubes from cycler and centrifuge briefly.
    i. At this point, samples are ready for qPCR and can be stored at -20°C.

Appendix C - qPCR and Quality Control Tests

The following protocol is based on "Fast SYBR Green Master Mix" and KAPA Library Quantification Kit" data sheets.

I. Preparation

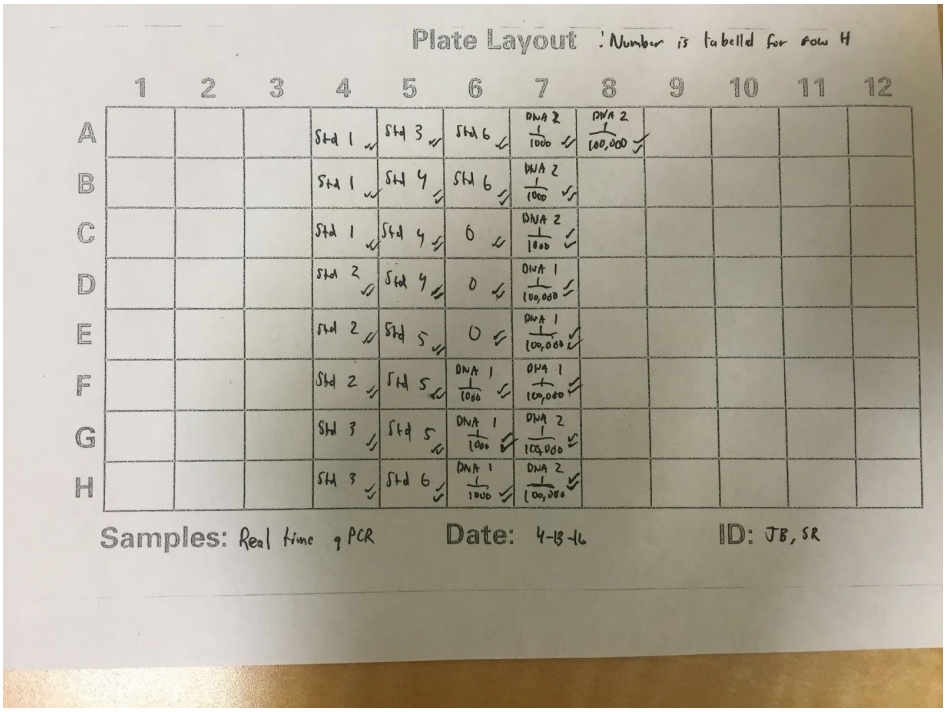
1. Thaw and vortex KAPA quantification kit, prepared DNA library, and SYBR Green Master Mix
    a. Ensure that you mark when primers/kits were thawed and used
2. Aliquot a portion of your library. This should be enough so that you are able to perform the following experiment in triplicate. Dilute it so that it falls within the range of the standards (20-0.0002 pM).
    a. NOTE: The dilution should be 1/1,000-1/100,000. We performed both 1/1,000 and 1/100,000 dilutions
3. Dilute primers to 15 pmol/µL.
    a. There is an Excel spreadsheet in ERI documents that will assist in doing the calculations for primer dilutions
4. The following table will serve as your template for each reaction tube. Ensure that you prepare enough of the following reagents.

qPCR Mix Per Reaction Tube		
Concentration	Component	Volume per reaction
n/a	SYBR Green Master Mix	10 µL
15 pmol/µL of EACH primer	Primer P5 + P7	1 µL
n/a	Deionized H_2O	5 µL
Variable	Diluted DNA library **OR** Standard **OR** Nontemplate control (NTC)	4 µL

5. Prepare a master mix of SYBR Green Master Mix + Primers + deionized H_2O. Calculate the volume necessary for your number of reactions. This master mix should be 16 µL per reaction.
    a. NOTE: when you make a master mix of SYBR Green + Primers + deionized H_2O, make sure to add 5% to your calculations to account for pipetting error.

II. Tube Preparation

1. Prepare the number of strip tubes necessary for the number of reactions.
    a. It is good procedure to lay out which standards/NTCs/samples are going in which tubes beforehand
2. Pipette 4 µL of standards into their respective PCR tubes. Pipette 4 µL of deionized H_2O into the NTC tubes. Pipette 4 µL of library sample into their respective PCR tubes.

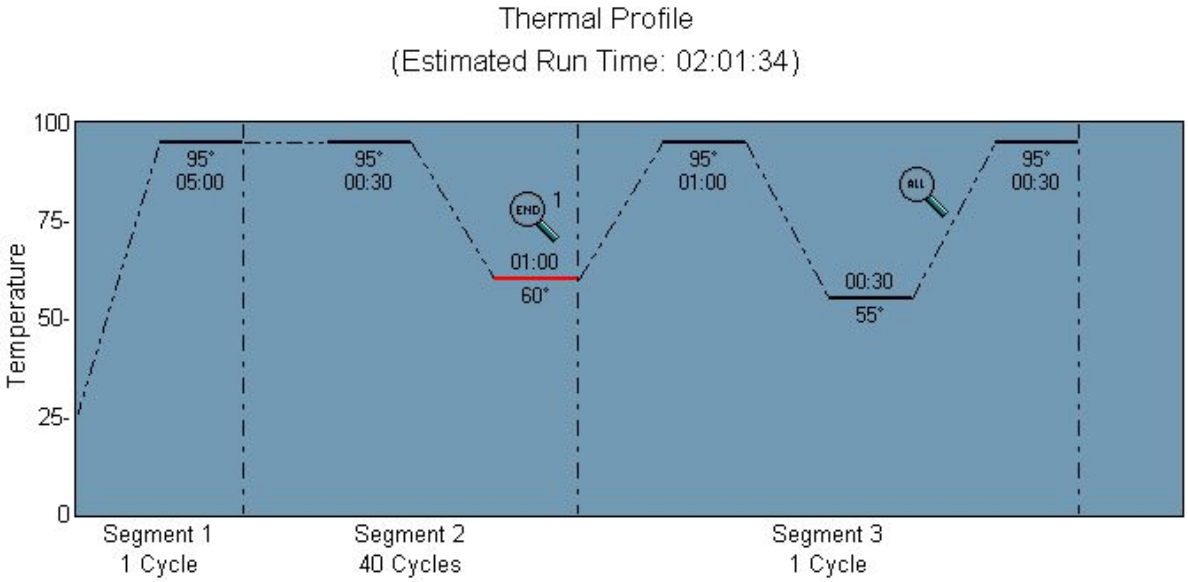


This is an example of a plate layout used in this experiment.

3. To each PCR tube, add 16 µL of the master mix prepared in step I.5.
    a. The total volume per tube should now be 20 µL.
4. **<u>Thoroughly</u>** vortex and centrifuge the PCR tubes to collect reagents at the bottom.

III. qPCR

1. Prepare the following program in the real-time PCR machine:



2. Allow real-time PCR machine to warm up.
3. Place PCR tubes in the machine in a known order and orientation.
4. Allow program to run.
5. After program is finished, retrieve data and utilize for Quality Control.

Appendix D - Graphical Results

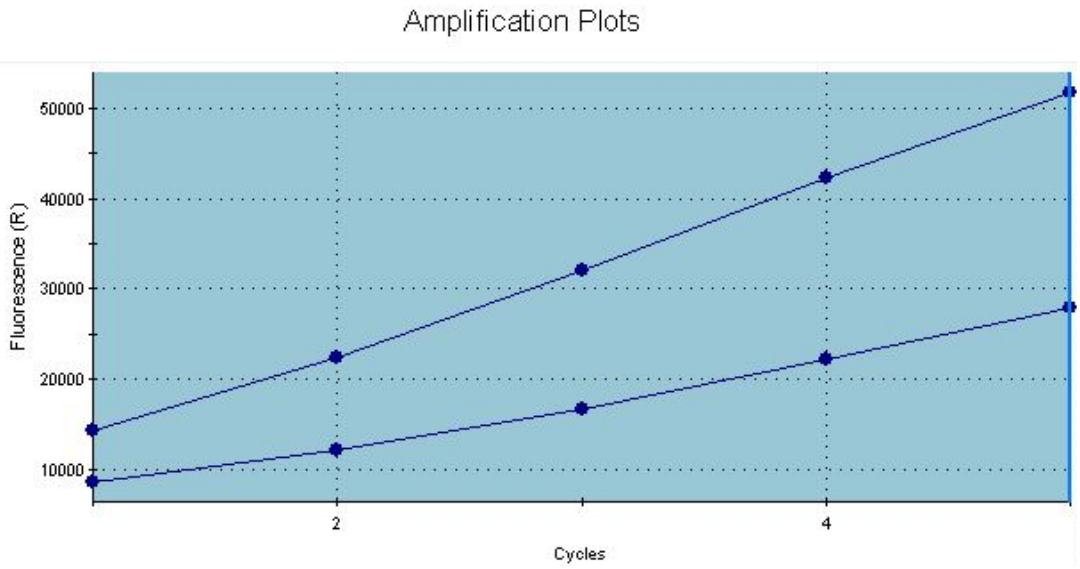


Figure 1: Library Amplification Fluorescence Data Results

qPCR Sample Key	
●	Standard 1: 20pM
■	Standard 2: 2pM
▲	Standard 3: 0.2pM
◆	Standard 4: 0.02pM
✦	Unknown 1: 1/1,000 Dilution
○	Unknown 2: 1/1,000 Dilution
□	Unknown 1: 1/100,000 Dilution
▼	Unknown 2: 1/100,000 Dilution

Figure 2: Sample Key for qPCR Graphs

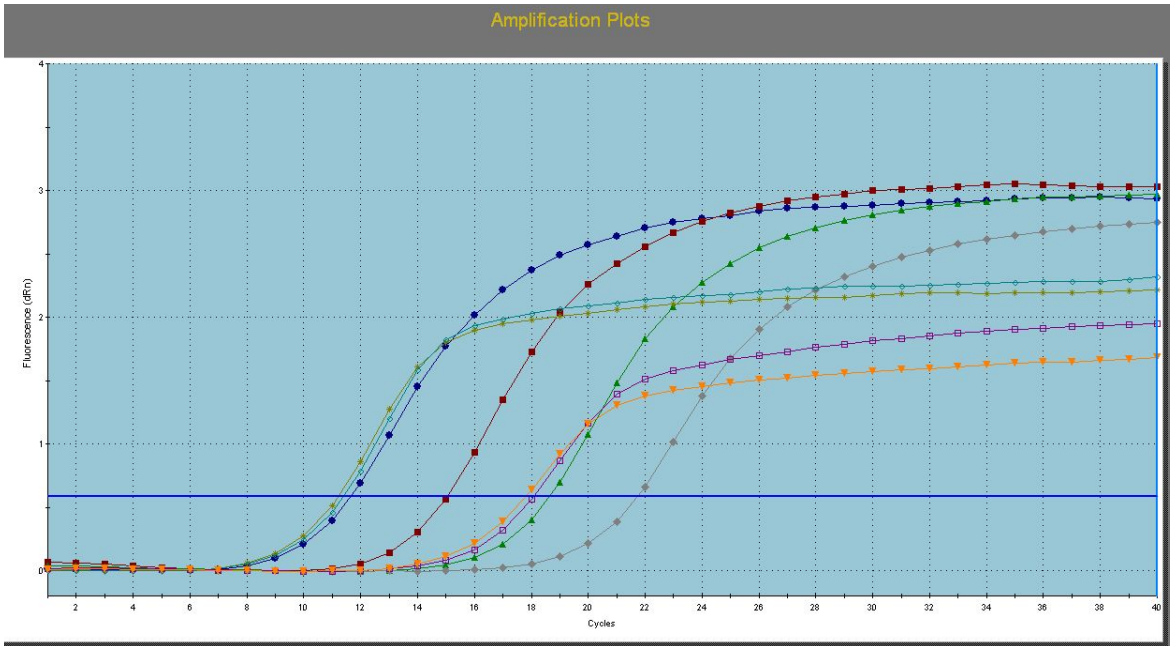


Figure 3: qPCR Amplification Plot

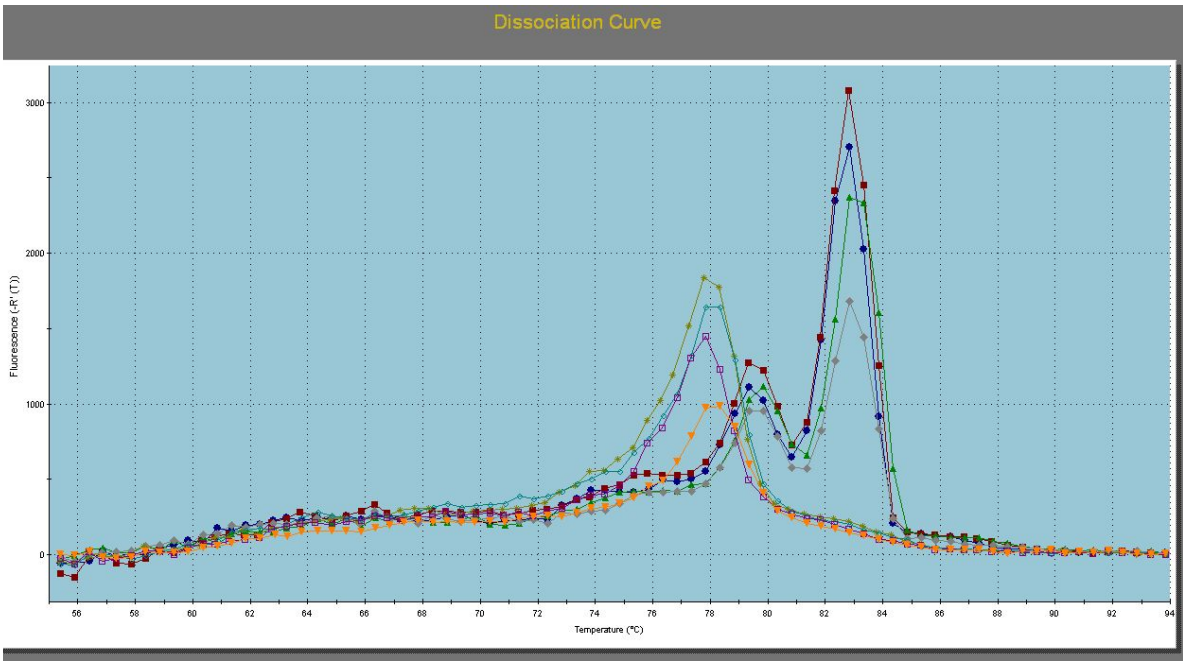


Figure 4: qPCR Dissociation Curves

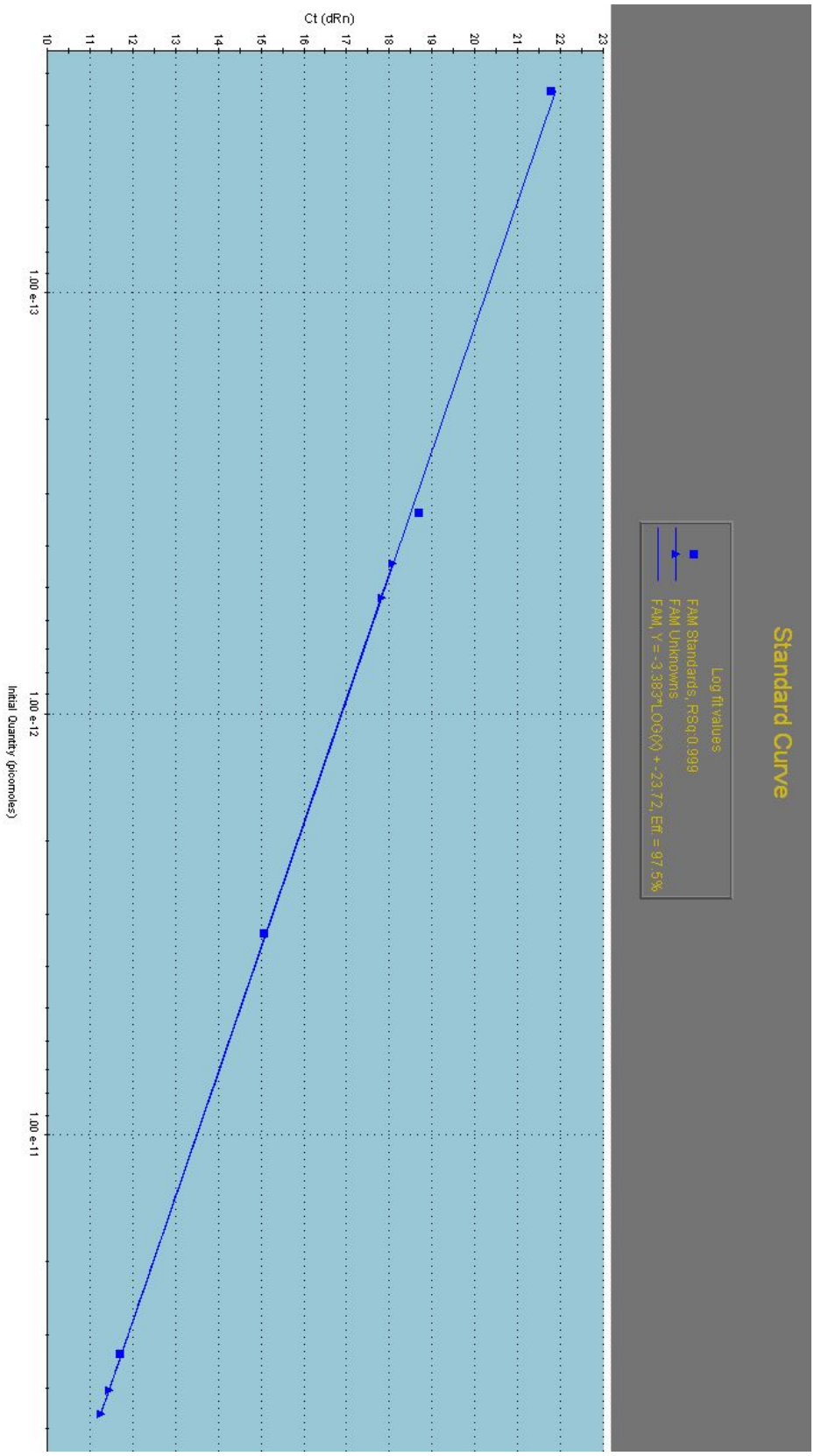


Figure 5: Standard Curve

Appendix E - Manufacturer Protocols

Manufacturer's protocols are in the Google Docs Folder and can be found online via references listed below.

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Manufacturer Protocols

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