BACKGROUND TO THE LAB

Human Immunodeficiency virus (HIV-1) has caused a global epidemic of concern to us all and in the thirty years since its discovery, has resulted in the death of an estimated 30 million people. Approximately 40 million individuals world-wide are currently infected with this virus and many of them will also die of AIDS, the syndrome that results when the virus has ablated their CD4+ T cells. Progression to AIDS is influenced by a variety of host and viral factors, including the copy number of virus particles in the blood of an infected individual. In general, the higher the number of virus particles, the faster progression occurs. The level of virus in the blood can be measured using antibody- and PCR-based methods, especially real-time qPCR.

Real-time qPCR techniques have revolutionised diagnostic virology labs. This method is similar to conventional PCR, which you will also have the opportunity to perform and compare, in that it amplifies specific regions of DNA from a sample of nucleic acid. The product is monitored as the reaction proceeds. During a real-time qPCR, the PCR product is quantified using a fluorescent dye called SYBR Green, which binds to double stranded DNA. Therefore, the intensity of the fluorescence increases as more PCR product is produced with each cycle of the PCR. You can therefore accurately measure viral copy number within a sample.

You will be using conventional and real-time qPCR techniques in this lab to help diagnose whether any of the given patient samples are infected with the HIV virus. This combination of techniques is useful to perform and provide the basis for their comparison. These techniques are based on methods currently used in diagnostic labs to detect this virus and should allow you to transform PCR from a theoretical concept into a real life application.

Lab Plan

Briefly, you will be doing the following:

AM

- Introductory talk
- Set up conventional PCR
- Set up real-time qPCR

PM

- Run agarose gel using PCR samples

Results from the real-time qPCR run are gathered and collated to be analysed in a computing session.

Additional Resources

Suggested reviews:

Wong M.L. and Medrano J.F. (2005) Real-time PCR for mRNA quantitation. *Biotechniques* **39**, 1-10

Fraga D. *et al*. (2008) Real-time PCR. *Current Protocols Essential Laboratory Techniques* **10.3.1-10.3.34.** (http://onlinelibrary.wiley.com/doi/10.1002/9780470089941.et1003s00/pdf)

Valasek M.A. and Repa J.J. (2005) The power of real-time PCR (2005) *Advan. In Physiol. Edu.* **29**, 151-159.

DAY 5 (AM) - CONVENTIONAL HIV PCR

The DNA sequence below shows a partial sequence of the HIV-1 envelope glycoprotein (env) gene. The forward and reverse primers were designed against the underlined sequences. A successful PCR in the presence of HIV-1 env template sequence should amplify the region in bold and the amount of product generated will be directly proportional to the concentration of HIV in the original sample.

ATATAAAGTA ATAAAAATTG AACCATTAGG AATAGCACCC ACCAAGGCAA AGAGAAGAGT
GGTGCAGAGA GAAAAAAGAG CAGTGGGAAT AGTAGGAGCT ATGTTCCTTG GGTTCTTGGG
AGCAGCAGGA AGCACTATGG GCGCAGTGTC ATTGACGCTG ACGGTACAGG CCAGACAATT
TTTGCTGAGG GCTATTGAGG TCTGTTGCAA CTCACAGTCT GGGGCATCAA GCAGCTCCAG
GCAAGAGTCC TGGCTGTGGA AAGATACCTA AGGGATCAAC AGCTCCTAGG GATTTGGGGT
TGCTCTGGAA AACTCATTTG CACCACTGCT GTGCCTTGGA ATGCTAGTTG GAGTAATAAA
TCTCTGGAAG ACATTTGGGA

Figure 1 Part of the HIV-1 envelope glycoprotein (env) sequence. The region in bold will be amplified during the PCR in the lab.

Reagents

You will require the following reagents (with red dots) to run this reaction. Check everything that you need is present before you start.

- 1 x 500µl Qiagen Sterile Nuclease Free Water
- 1 x 15ul PCR HIV F-primer (10µM)
- 1 x 15ul PCR HIV R-primer (10µM)
- 1 x 10ul PCR Blood cDNA Sample 1
- 1 x 10ul PCR Blood cDNA Sample 2
- 1 x 10ul PCR positive control cDNA
- 1 x 150ul PCR Master Mix (green reagent)

Method

1. Check that everything has defrosted. You may have to briefly centrifuge your tubes to ensure all the liquid is at the bottom.
2. Make up a PCR Master Mix for 5 reactions in a 1.5ml eppendorf:

Reagent	**Master Mix for 5 reactions (µl)**
PCR Master Mix	125
10µM PCR HIV F-primer	10
10µM PCR HIV R-primer	10
H_2O	95

3. Using a marker pen, write your initials on the top of each PCR tube and label them 1-4 so that you can distinguish which tube contains which sample. Place 48 µl of the Master Mix into each PCR tube and then add 2µl of the following into the appropriate tube:

   Tube 1: PCR Blood cDNA sample 1
   Tube 2: PCR Blood cDNA sample 2
   Tube 3: PCR positive control cDNA
   Tube 4: Water (Negative control)

4. Close the tubes, mix the contents by flicking the tube and spin **very briefly at low speed** (less than 4K for a few seconds only) to collect the contents to the bottom of the tubes.

5. Place the tubes in the rack at the front of the lab to be put into the PCR machine. The reaction conditions will be as follows:

Start		95°C	5 min
5 X	denaturing	95°C	1 min
	annealing	70°C	30 sec
	extension	72°C	1 min
25 X	denaturing	95°C	30 sec
	annealing	70°C	30 sec
	extension	72°C	1 min
1 X		72°C	5 min

Once all the class samples are in the PCR machine, the demonstrator will start the cycling reaction. This will take around 2 hours. You will run these samples on an agarose gel in the afternoon.

Questions

Q1. The template you will be using in the PCR is in the form of cDNA. Briefly, describe how cDNA is generated?

Q2. Why is it important to use cDNA for these experiments?

Q3. The primers for this reaction have been designed to the sequences underlined in the sequence shown in Figure 1. They are referred to in this lab as HIV F-primer (forward primer) and HIV R-primer (reverse primer). Using your knowledge of PCR primer design, write out below what the forward and reverse sequences would be (hint: write out the sequence 5' to 3').

HIV F-primer:

HIV R-primer:

Q4. What size is the PCR product you would amplify and expect to see if you ran this on a gel?

Q5. Calculate the T_m of these primers (T_m = 2°C x (A+T) + 4°C x (C+G)).

Q6. Look carefully at the chosen sequence of both primers. Do you think the choice is a good one? Qualify your answer using your knowledge of primer design.

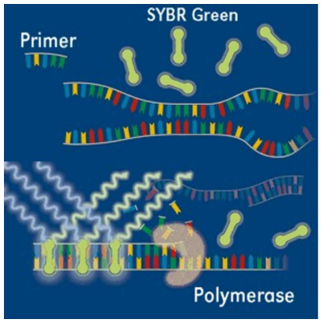
Q7. When designing PCR primers for use in a real-time qPCR, the final PCR product size should be between 50 and 150 nucleotides. Why do you think this might be?

DAY 5 (AM)– REAL-TIME QUANTITATIVE PCR

Introduction

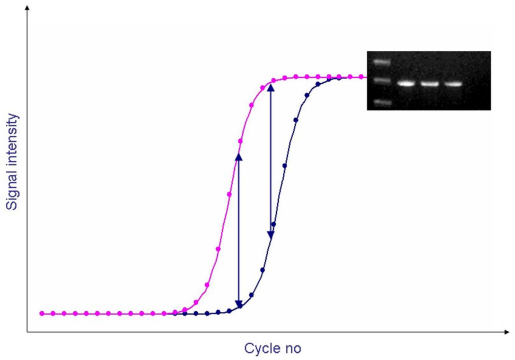
Reading: Dale et al., 3rd Ed: p123–127.

Real-time quantitative PCR (qPCR) is highly suited for a wide range of applications, such as gene expression analysis, determination of viral load, detection of genetically modified organisms (GMOs), SNP genotyping, and allelic discrimination. Real-time qPCR allow accurate quantification of starting amounts of DNA, cDNA, and RNA targets. PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA or fluorescently labelled sequence-specific probes. Fluorescence is measured during each cycle and the amount of this fluorescence is proportional to the amount of PCR product.



The principle of SYBR-based real-time qPCR, which you will be using in this lab practical, is a standard PCR carried out in the presence of a dye, SYBR, which fluoresces when intercalated in the DNA helix. The fluorescence will increase as the amount of the PCR product increases and is quantified after each completed PCR cycle.

The cycle at which the fluorescence exceeds a detection threshold, the **Ct (threshold cycle)** correlates with the number of target cDNA molecules present in the added cDNA. Therefore, by comparison to a calibration curve, it is possible to quantify in absolute amounts the number of target molecules in cDNA samples.



You will be provided with two cDNA samples, each derived from the blood of individuals who are being tested for HIV infection. Using the reagents provided you will be able ascertain whether they are HIV positive or not and assess the copy number of HIV genomes. Quantitative standards have already been run through the real-time qPCR machine for you. Using these, you can compare the results you obtain with your cDNA samples with known concentrations of virus. This will allow you to accurately quantify your samples for viral load.

Reagents

You will require the following reagents (with blue dots) to run this reaction. In addition, you will require 4 real-time fast reaction PCR tubes with caps (both found in strips in a Universal container) and a pink rack, which is used to hold these real-time PCR tubes. Check everything that you need is present before you start.

- 1 x 500µl Qiagen Sterile Nuclease Free Water
- 1 x 20 µl qPCR HIV F-primer (2µM)
- 1 x 20 µl qPCR HIV R-primer (2µM)
- 1 x 10 µl qPCR Blood cDNA sample 1
- 1 x 10 µl qPCR Blood cDNA sample 2
- 1 x 10 µl qPCR positive control cDNA
- 1 x 80 µl SYBR Select Master Mix (in a dark brown Eppendorf)

Method

1. Considerations before starting:

a. Please take your time pipetting and do so as accurately as possible!

b. Real-time qPCR is VERY sensitive to contamination, therefore:
   i. Clean bench area with detergent first
   ii. Use gloves to handle everything and change tips as necessary
   iii. **Use sterile p2, p20 and p200 FILTER TIPS**

c. SYBR Select Master Mix is light sensitive! You must work efficiently, but accurately.

2. Setting up real-time qPCR:

1. Make a qPCR Master Mix for 6 reactions in a sterile 1.5 ml Eppendorf on ice as follows:

Reagent	**qPCR Master Mix for 6 reactions (µl)**
2x qPCR SYBR Select Master Mix	60
2µM qPCR HIV F-primer	12
2µM qPCR HIV R-primer	12
H_2O	24

2. Gently mix the Master Mix tube by flicking the tube and briefly centrifuge the tube for 10 secs at high speed.

3. Place the strip of 4 real-time qPCR tubes in the pink tube holder. The tubes have numbers on them which will be either 1-4 or 5-8. Mark the strip of tubes so the number 1 or number 5 tube has a dot on the side of the tube. **NOTE: Do NOT label the top of the tube**.

4. Accurately pipette 18 µl of qPCR Master Mix into each **real-time qPCR tube** and then add 2µl of the following into the appropriate tube in the correct orientation:

   I. qPCR Blood cDNA sample 1
   II. qPCR Blood cDNA sample 2
   III. qPCR positive control cDNA
   IV. Water (negative control)- NTC (no template control)

The **orientation of the tubes** is as follows:

Tube number	1 or 5	2 or 6	3 or 7	4 or 8
Sample	**Sample 1**	**Sample 2**	**+ve control**	**NTC**

Press the lids on firmly.

5. Take your sample to your lab leader who will position your tubes into the real-time qPCR plate. Please record the number that the lab leader gives you for your analysis.

6. The samples from the group will be run in the ABI Step One Plus Real-time Machine.

The following conditions will be used during the real-time qPCR:

Stage	**Temperature °C**	**Time (min)**	**Cycle number**
Melt/Hot start	95	3 min	1
Amplification	95	3 secs	35
	60	30 secs	
Melt curve	95	15 secs	1
	60	1 min	
	60-95	0.3 secs per temp	

Questions

Q8. Why is it important to prepare a Master Mix?

Q9. Why were you told not to label the top of the real-time qPCR tubes?

Q10. Comment on how real-time qPCR conditions compare to conventional PCR conditions.

Q11. What other types of experiments do you think real-time qPCR could be used for?

DAY 5 (PM) – AGAROSE GEL ELECTROPHORESIS OF CONVENTIONAL HIV PCR

1. Preparation of the Agarose Gel

The gel mix has been prepared for you: note, here you are using a 2% agarose gel. A demonstrator will add 2µl of gel-red into your gel.

- When the gel has set, flood the gel tank with the TAE buffer (there should be a depth of about 5 mm TAE above the gel). Once you have removed the comb and blocks, you are ready to load your samples.

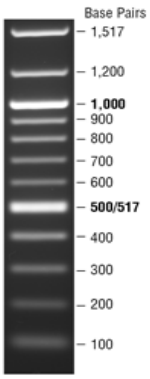
2. Preparation of PCR Samples for Electrophoresis and Running the Gel

- There is one agarose gel for three pairs.
- Collect your 4 PCR samples from the PCR machine. Load 10µl of each sample onto the gel. There is also a DNA Ladder to load – one per gel (15 µl).
- Remember to write your name on the loading sheet so you know where your sample is on the gel.
- After running the gel, please take it to be visualised.

CAUTION: ***potentially lethal voltages. Do not handle gel kits unsupervised, and if you notice a problem in a running gel (like a leak, steam or smoke), seek help but do not touch.***

Interpreting your gel

You used the 100bp DNA ladder below on your gel. Correlate the molecular weight of the bands below with the ladder you ran on your own gel.



Questions

Q12. Why would you use a 2% agarose gel to run this PCR product on?

Q13. Does the size of your PCR product reflect what you predicted?

Q14. Which of your blood cDNA samples is showing a positive PCR result for the HIV *env* gene?

Q15. Have your positive and negative controls worked? If not, what would you do differently next time you ran this experiment?