

How to use the Quantitative PCR lab

In the PCR laboratory you perform genetic tests for both the CYP2D6 *4 allele and CYP2D6 gene copy number, two variants that can have significant effects upon the level of this protein and the ability to metabolise certain prescription drugs.

CYP2D6 gene analysis

The segments of genetic code for the CYP2D6 protein are found in nine exons and the final spliced version of the RNA is called messenger RNA (mRNA). If the bases at or near the sites of splicing are mutated in any way, splicing goes wrong. In this case, portions of RNA that do not code for a protein are left in the mRNA and, in all likelihood, the encoded protein will not be properly synthesised and will be degraded in the cell. One such gene variant that alters splicing of exon 3 to exon 4 is the variant called the CYP2D6*4 (pronounced 'star four') variant and detecting it is one of the genetic tests you will be performing in this investigation. The PCR assay detects the presence of the mutated (non-functional) variant.

The second assay is based upon assessing gene copy number. Analysis of the human genome sequence has led to a surprising and remarkable discovery about the structure of certain regions of the genome. When assembling the genome sequence, long segments on one chromosome appeared to be identical to regions elsewhere in the genome. Such regions are called segmental duplications. These duplicated segments are complete copies of entire segments of DNA sequence with all the genes in the region being duplicated. In fact, almost 5% of the human genome is made up of this type of duplication. Another unexpected observation is that some segments are not simply duplicated, but are present as three or even more copies, and in some cases they can even be completely deleted.

As the number of copies of a gene can vary between individuals, it is perhaps no surprise that it results in differences in the level of RNA being synthesised, with a subsequent increase in the amount of protein being made. For the CYP2D6 gene, it is known that individuals can carry duplications of the entire genes side by side on the same chromosome; chromosome 22 has been shown to carry up to five copies of the gene. On some copies of chromosome 22, the CYP2D6 gene is also completely deleted.

This activity allows you to screen for these two variants in three populations. These data allow you to make comparisons between the populations, predict which individual will have high levels, low levels or no CYP2D6 protein and hence vary in how they metabolise certain drugs.

Instructions

In the PCR laboratory, follow the on-screen instructions. Start by selecting the population you are to study from choices A, B or C. Decide on which tests you are performing (i.e. which

primer pair), on which population and how many replicates of each experiment. Note these down in your laboratory notebook.

You will be required to sequentially add reagents and primer pairs, set-up the PCR machine and selecting the appropriate PCR amplification program.

As the PCR cycles run, you should note the amplification cycle that is visible in the Temperature Profile window and note when fluorescence is detected, increases exponentially and then plateaus for all samples in the Fluorescence detection window (you cannot select individual sample profiles whilst the PCR is running).

As each set of tests is performed you will see a record of the variables chosen in the Experimental Record window. At the end of each experiment, the PCR machine automatically calculates the Ct values for each sample and deposits these data in this window, alongside the record of each control and sample number.

You can examine the data on screen by selecting either individual wells or by selecting individual columns or by selecting all by clicking on 'Show All'. You can hide all data by clicking on 'Clear All'. In each case, you will see the graph of fluorescence in the Fluorescence detection window and the Ct values for those selected wells will appear in the Experimental Record window and on the graph itself.

After each experiment you must record your data as when you reset the PCR machine the experimental record is erased. This is best achieved by copying pasting the data from each individual experiment into a spreadsheet. Ensure that you have selected 'Show' all.

You should collect several images of the PCR screen for your notebook and TMA. This is best achieved using the screen capture shortcut (Shift-Print Screen) followed by pasting the screen grab into a document.

Interpreting your data

In order to interpret the genotypes of each individual in the three populations, you will need to compare the experimental Ct values you obtain with those in the control DNAs. Table 1, below, shows you the copy number and the CYP2D6 *4 status of each of the five control DNAs.

The genotypes of the five control DNAs used in PCR assays. For each sample, the genetic make-up in terms of CYP2D6 gene copy number and which variant is present on each chromosome 22 is indicated.

DNA	Gene copy number	Chromosome 22 CYP2D6 variant present	Chromosome 22 CYP2D6 variant present
Control DNA 1	2	2D6 *4	2D6 *1
Control DNA 2	2	2D6 *4	2D6 *4
Control DNA 3	2	2D6 *1	2D6 *1
Control DNA 4	3	2 × [2D6 *1]	2D6 *1
Control DNA 5	4	2 × [2D6 *1]	2 x [2D6 *1]

Considerations in analysing your data

Data can be analysed in one of two ways:

On screen as each assay is completed, the amplification profile can be examined for each sample and compared to each control. This allows a visual interpretation. You can select individual samples (wells), select all and/or deselect all/each sample.

Ct analysis: Based upon the Ct values for control DNAs 1-5, you should be able to interpret your experimental data obtained for each of the biobank DNA samples 1-90.

You can base your interpretation upon your own control Ct values and on Ct values pooled from control values from your fellow students. This will allow you to establish the range of fluorescence values that represent the copy number for each of the genetic tests.

You should use your data in your spreadsheet and add your interpretation for each sample as a new column to indicate how many copies are present for each test by comparison with Ct values in each control.

For CYP2D6 *4: based upon the Ct value, decide for each sample whether it contains zero, 1 or 2 copies of the CYP2D6 *4 variant.

For CYP2D6 copy number: based upon the Ct value, decide for each sample whether it contains 2, 3 or 4 copies of the gene.

Are there samples for which you have any anomalous results or results that you cannot interpret based upon the control dataset you have? If so, what might you suggest about the genetic make-up of these samples?

Some genetic considerations

Human have two of each chromosome and, therefore, two copies of chromosome 22 and therefore two genetic loci carrying the CYP2D6 gene. Whilst you cannot say with absolute certainty which chromosome 22 carries which configuration of the gene or which might carry the CYP2D6 *4 variant, in the populations you are studying, you have been able to determine for each individual:

- the overall copy number of the CYP2D6 gene present in individuals
- the number of CYP2D6*4 variant genes present.

Whilst this combination of data gives you some important information on those individuals, you must remember that you have only tested for two genetic variants.

For some individuals you do, in fact, have complete genetic data. For example, in individuals who carry two copies of CYP2D6, both of which are the *4 variant, you can confidently state that these individuals will not produce any CYP2D6 protein. In contrast, you will have identified some individuals with 3 or 4 copies of the CYP2D6 gene, none of which are the *4 variant. In this case, we do not, however, know whether each of their 3 or 4 genes are actually functional versions, or whether they carry other variants that inactivate the protein. In order to determine this, further testing is required to determine the DNA sequence of each of their CYP2D6 genes.

Differences exist between world populations as to how frequent each of the 80 known variants of the CYP2D6 gene is. Although all modern human have a common evolutionary origin in Africa over 100 000 years ago, migration across the globe has occurred since then. As migration occurred, breeding populations became relatively isolated from each other and over many thousands of years the relative frequency of the different variants present in each geographically distinct group around the world changed. This, together with the occurrence of random new mutations that generate new variants, means that different world populations carry different frequencies of CYP2D6 gene variants. They will, therefore, also differ in the frequency of the various metaboliser states.

For the group of individuals with three or more functional genes, these are called ultra-metabolisers. Frequencies for these individuals vary from 40% (North Africa) and 26% (Oceania) to 12% (Middle East) and 8% (Americas). Individuals who are considered poor metabolisers are most common in populations of European decent, where as much as 8% of the population are of this status. One obvious implication is that a drug developed for one particular world market may prove potentially either useless or dangerous in other world populations.

The fact that world geographically distinct human populations have differing frequencies of genetic variants means that extrapolating data from one human population onto another is

not possible. In general, when considering genetic data, extrapolations are only valid within the same population that was tested.