Validating candidate regulatory genes using real-time RT-PCR

Once we have identified candidate **regulatory T lymphocyte** specific genes, it is necessary to show that they can be used to distinguish ***inflammatory*** and ***tolerant*** situations in whole tissues. For example, it is possible to graft skin experimentally under conditions such that it is either rejected with an inflammatory cell infiltrate, or it is instead accepted due to the induction of tolerance and the presence of **regulatory T lymphocytes**.

We can then extract the total RNA from all the different cell types in theses tissues, including that from the lymphocytes of interest. The whole pool of mRNA gene transcripts can be purified from the other cell DNA, RNA and proteins by using magnetic beads coupled with **Oligo-dT** that will attach to the polyA tails of just the mRNAs. Using a magnet and a multiwell plate it is possible to extract 96 different tissue samples at the same time.

To measure the amount of the mRNA from the gene of interest we use the ***polymerase chain reaction*** to amplify just the gene transcripts that contain the SAGE **tag** using a pair of primers and a sequence specific probe  that has a fluorescent dye (**FAM**) together with a quencher dye (**TAMRA**). The fluorescence is only released when the probe is bound to the amplified gene product and digested by the polymerase in the next PCR cycle. It is also possible to measure the amount of a second gene transcript as an internal control using further primers and a second probe labelled with a different fluorescent dye (**VIC**).

Real-time Quantitative RT-PCR

The Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is used to detect and amplify minute amounts of mRNA gene transcripts. It works using pairs of primers that are complementary and bind only to the mRNA sequence you wish to detect at 60^{o}C, that then initiate a heat resistant DNA polymerase enzyme to synthesise an exact copy. The samples are then heated to 95^{o}C to separate the original DNA strands from the copies, followed by cooling back to 60^{o}C to initiate another amplification cycle.

At each cycle, a single molecule of the **quenched** FAM or **VIC** labelled probe will bind to the appropriate accumulated product of the PCR reaction, which is then digested by the polymerase to release the active, fluorescent FAM or **VIC**. These reactions take place in a 96 well microtitre plate that is scanned by a laser beam at each cycle by the ***Sequence Detection System (SDS)*** machine. The computer collects all the FAM and **VIC** measurements, together with a reference dye (**ROX**), and calculates the amount of the **test mRNA** against the **control**.

The real time RT-PCR plots show examples of data obtained from the ***Sequence Detection System (SDS)*** machine. Each sample well that contains a detectable amount of the gene transcript of interest generates a coloured line on the plot of flourescence signal (logarithmic Y axis) versus cycle number (X axis). As can be seen from the standard titration curves in the upper two plots, increasing amounts of gene transcript move the parallel lines to the left. In the lower plots, two samples (in duplicate) are shown with different amounts of the **HPRT internal standard mRNA**, but only that with the lower amount has a measurable amplification for the **candidate regulatory gene** transcript.