

<b>UNIVERSITY OF LEICESTER</b> <b>CANCER STUDIES &amp; MOLECULAR MEDICINE</b>  <b>STANDARD OPERATING PROCEDURE</b>  <b>SOP - 646    VERSION - 01</b>	
<b>TITLE:</b> <b>Designing a single set of primers and probe for a genomic region of interest</b>	
<b>Written by:</b> <b>Kevin Blighe</b>	<b>Implementation date:</b> <b>18.12.12</b>
<b>Reviewed by:</b> <b>Karen Page</b>	<b>Review date:</b> <b>18.12.14</b>
<b>Changes introduced:</b>	

**1. PURPOSE**

To design a forward and reverse primer pair and FAM-labelled, MGB, TaqMan® qPCR probe for a genomic region of interest

**2. SPECIAL NOTES**

Be wary of different releases of the human genome reference sequence. As of writing this SOP, the version was Human Genome 19 (hg19) (also known as GRCh37). Also, there are different annotations that apply to the reference sequence. As of writing, the most up-to-date was Annotation Release 104, by RefSeq.

**3. CROSS REFERENCES****4. EQUIPMENT AND MATERIAL**

- 4.1    Computer with Internet access
- 4.2    Primer Express (Applied Biosystems) software

## 5. PROTOCOL

- 5.1 Obtain the start and end genomic co-ordinates for a region of interest
- 5.2 Obtain the DNA sequence for the region of interest using the NCBI's 'Map Viewer' tool.
  - i. Navigate to <http://www.ncbi.nlm.nih.gov/mapview/>
  - ii. Select the latest annotation for *homo sapiens* and then choose the chromosome of interest
  - iii. Click on 'Download/View Sequence/Evidence' on the right
  - iv. Input the genomic co-ordinates, click on 'Change Region/Strand', and then 'Display', beneath
  - v. Copy the DNA sequence that appears
- 5.3 Check that the DNA sequence of interest has little or no homology with other regions in the human genome. Only proceed from this step if there are no other homologous sequences, as these can misrepresent the result of PCR.
  - i. Navigate to <http://blast.ncbi.nlm.nih.gov/> and select 'nucleotide blast'
  - ii. Paste the DNA sequence into the text box, select 'Human genomic + transcript' database, and click 'BLAST'  
*\*The parameters will automatically adjust if the sequence that you've entered is short*
  - iii. On the results page, the alignment score is shown in coloured boxes (black across to red). If the sequence is a true sequence from the human genome, then there will always be a high alignment score (red line) with a query coverage of 100% that matches the true sequence. Any homologous regions elsewhere in the genome will generally have a lower alignment score (and different colour) and lesser query coverage.
- 5.4 Check the level of SNP coverage and other short variations in the DNA sequence of interest. This step is not critical, but the fewer SNPs and short variants, the more precise the qPCR result will be.
  - i. Navigate to [http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST\\_SPEC=SNP&BLAST\\_PROGRAMS=megaBlast&PAGE\\_TYPE=BlastSearch](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=SNP&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch)
  - ii. Paste the DNA sequence into the text box, select 'Homo sapiens' from the drop-down list, and click 'BLAST'  
*\*The parameters will automatically adjust if the sequence that you've entered is short*
  - iii. A graphical display of SNP and short variant hits will display

- 5.5 Design the primer and probe sequences using Primer3
  - i. Navigate to <http://primer3.wi.mit.edu/>
  - ii. Paste the DNA sequence into the text box and ensure that 'Pick hybridization probe' check-box is checked
  - iii. Configure the search using the following parameters:
    - a. 'Primer Tm', 60-66
    - b. 'Primer GC%', 40-60
    - c. 'Product Size Ranges', 50-120

*\*Although these are not the ideal ranges, Primer3 has a tendency to overestimate values, which is why we also overestimate*
  - iv. Click on 'Pick Primers' (also chooses the probe). A new page will appear showing again the DNA sequence that was input; however, the automatically-generated forward (>) and reverse (<) primer and probe (^) will also appear (all in 5'-3' order)

*\*Primer and probe sequences need to follow general guidelines (see Additional Notes, at end)*
  
- 5.6 Keeping Primer3 open, check the primer and probe sequences in Primer Express
  - i. In Primer Express, go to 'Tools' and then 'Primer Probe Test Tool...'
  - ii. Copy the primer and probe sequences from Primer3 and paste into the text boxes in the test tool
  - iii. If the sequences are thermodynamically sound, then the 'Tm' and '%GC' will appear green. If not, then some bases need to be either removed or added from/to either end
  - iv. Check the primers for predicted secondary structures (beneath)
  
- 5.7 Repeat steps 5.5 and 5.6 until a suitable set of primers and probe is designed, changing the parameters at different steps, if necessary.
  
- 5.7 Check which region of the human genome is amplified using *in silico* PCR
  - i. Navigate to <http://genome.ucsc.edu/>, select 'PCR', and ensure that the correct reference sequence is selected in 'Assembly'
  - ii. Copy the primer sequences and paste into the 'Forward Primer' and 'Reverse Primer' text boxes
  - iii. Click on 'submit'. A new page will appear giving details of the hypothetically-amplified regions. Ideally, there should only be one region. If not, then be aware that the probe would add some further level of specificity; however, if not happy, then consider restarting from step 5.1!

## 6. ADDITIONAL NOTES

General guidelines for primer and probe sequences:

### Primers

- i. Optimum amplicon length, 50-120 nucleotides
- ii. Optimum primer length, 20 nucleotides
- iii. Optimal T<sub>m</sub>, 59°C
- iv. 3' end should have no more than 2 Gs or Cs in the final 5 nucleotides
- v. Keep homopolymers (repeating nucleotides) to a minimum; if present, there should be no more than 4 Gs together

### FAM-labelled, MGB probe

- i. Optimum probe length, 13-25 nucleotides
- ii. Optimum T<sub>m</sub>, 68-70°C
- iii. 5' last nucleotide cannot be a G; neither can the penultimate (second-from-last) nucleotide
- iv. 3' end should have few Gs; specifically avoid GGG and GGAG
- v. Keep homopolymers (repeating nucleotides) to a minimum, specifically:
  - a. No more than 4 Gs together
  - b. No more than 6 As together
  - c. No more than 2 CC dinucleotides in the middle of the probe