**Protocol for manual primer design - SDGS**

1. **Navigate to Ensembl**
   * Open your web browser and go to the Ensembl website.
2. **Copy Gene Link**
   * Copy the link from the PDF corresponding to the gene you're designing primers for.
3. **Select Correct Transcript**
   * In Ensembl, navigate to the gene using the copied link.
   * Choose the transcript with the most base pairs, the largest protein, and the most "Flags".
4. **Access Exons**
   * Select the "Exons" button in the "Sequence" section.
5. **Configure Page**
   * Click on 'configure this page'.
6. **Set Parameters**
   * In the text box, set:
     + 300bp of flanking sequence.
     + Full intronic sequence.
     + No line numbers.
     + No variants.
7. **Confirm Settings**
   * Click the tick in the top right corner when parameters are set.
8. **Configure Transcript View**
   * Customize what appears in your transcript by selecting the 'show/hide columns' option.
     + Include Number and Sequence.
9. **Download Sequence**
   * Click on 'download sequence'.
10. **Choose Download Options**
    * In the download text box, select:
      + 'RTF form'.
      + 'Genomic sequence only'.
11. **Download File**
    * Click on 'download'.
12. **Access Primer Design Tools**
    * Open Primer3Plus and SNPCheck in your browser.
13. **Prepare Sequence for Primer3Plus**
    * Copy and paste the exonic sequence (colored and uppercase) along with 25bp of intronic sequence (lowercase & grey) into Primer3Plus.
    * Enclose the entire sequence in [ ].
14. **Expand Intronic Sequence**
    * Add ~300bp of extra intronic sequence on both sides of the brackets, ensuring no bases are duplicated.
15. **Clean Up Sequence**
    * Remove any spaces between exonic and intronic sequences without altering the sequence itself.
16. **Generate Primers**
    * Click on 'pick primers' in Primer3Plus.
17. **Record Primer Sequences**
    * Copy and paste the primer sequences generated by Primer3Plus into the primer design spreadsheet.
18. **Keep Primer3Plus Open**
    * Maintain the Primer3Plus tab open for further use.
19. **Enter Additional Information**
    * Fill in the primer design spreadsheet with:
      + Gene name.
      + Exon number.
      + Version of primer used.
      + Chromosome the gene is on.
20. **Analyze Primers for SNPs**
    * Copy and paste the result from the primer design excel form into SNPCheck.
21. **SNPCheck Analysis**
    * Click on the SNPCheck button.
22. **Repeat for Additional Primer Sets**
    * If no SNPs are detected, save PDF and excel files of SNP results and add to your Primer design – primer list excel document.
    * Repeat the process for the next set of primers for the same exon, then proceed to the next exon.

**Options for Handling SNPs in Primer Pairs:**

1. **Try Alternative Primer Pairs**
   * If SNPs are detected, try using other primer pairs generated by Primer3Plus.
     + If these pairs have no SNPs and meet the criteria, use them instead.
2. **Modify Primer Sequences**
   * If SNPs persist, follow these steps:
     + Identify the location of the SNP using SNPCheck.
     + Replace the SNP base with 'N' in Primer3Plus to avoid generating primer pairs covering this area.
     + Repeat the primer design process.
     + If new primer pairs also contain SNPs, replace the SNP base with 'N' and retry.

**Handling Other Results from SNPCheck:**

1. **All Filtered Out**
   * If SNPCheck indicates 'All filtered out':
     + Try another primer pair, using it if it has no SNPs.
     + If all options have SNPs or have been filtered out, use the first 'filtered out' option.
     + Record this result on the primer ordering sheet.
2. **Maps to Multiple Regions**
   * If primers match to multiple regions:
     + Try other primer pairs for fewer matches.
     + Exercise judgment based on the number of matches; avoid pairs matching to numerous regions to minimize nonspecific binding.
     + Repeat the process to find pairs with fewer matches.