# Stratagene's High-Specificity Primer Design Guidelines

***Note: This information is provided free of charge to our customers in order to provide consistent quality service and allow for the greatest specificity possible for miRNA detection.***

## Step 1. Obtain miRNA Sequence Information

- Download the most recent update of mature miRNA sequences (mature.fa "**Fasta** format sequences of all mature miRNA sequences") from the Sanger database at http://microrna.sanger.ac.uk/sequences/ftp.shtml. The following screen will appear:

**miRBase Sequence Download**

Go to the FTP site
Previous releases

| | |
|---|---|
| README | Release notes - read these first! |
| miRNA.dat | all published miRNA data in EMBL format |
| hairpin.fa | Fasta format sequences of all miRNA hairpins |
| mature.fa | Fasta format sequences of all mature miRNA sequences |
| maturestar.fa | Fasta format sequences of all minor miR* sequences |
| miRNA.diff | Changes between the last release and this |
| miRNA.dead | List of entries that have been removed from the database |
| miFam.dat | Family classification of miRNA hairpin sequences |

- Extract the file and then open with Microsoft Word

## Step 2. Identify Species Specific Information

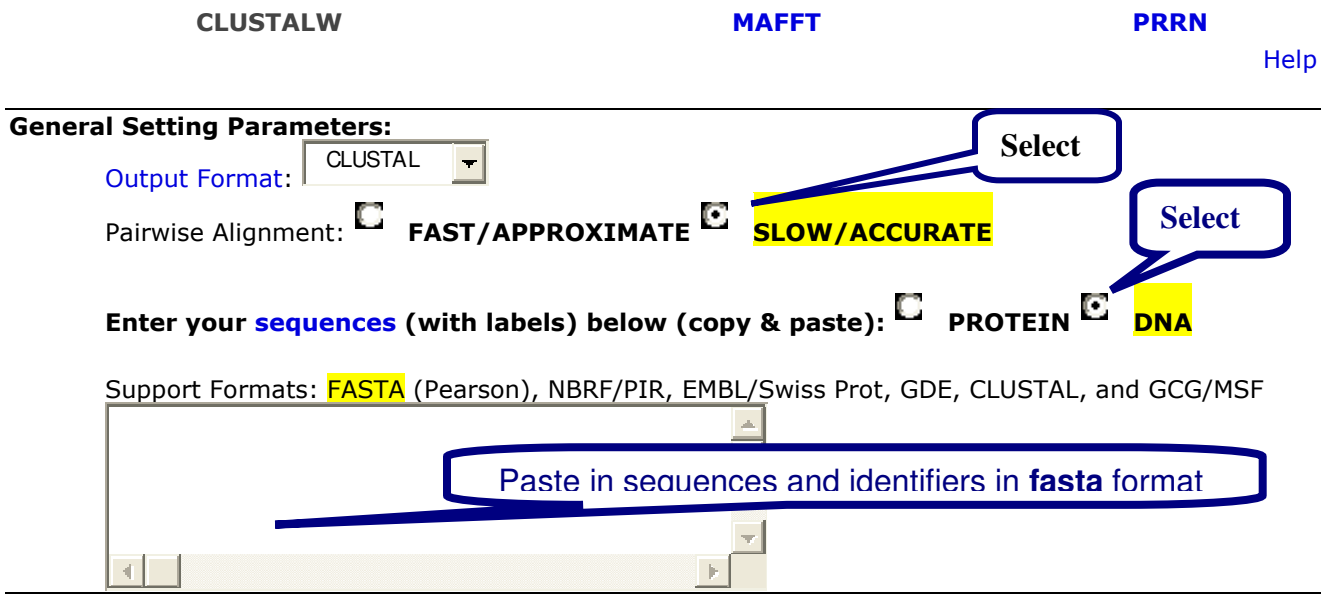
- Remove all non-specific target species information

**For example:**

For human sequence (hsa) specific information, delete all non-human miRNA information

## Step 3. Align all sequences using ClustalW

- Go to http://align.genome.jp/ The following screen will appear:




  - Use default settings
  - Push **Execute Multiple Alignment** (at the bottom of the web page)
  - After program has finished alignment
    - select **clustalw.aln** (at top left hand corner of screen)
    - aligned sequences will appear
    - highlight and copy into a Microsoft Word file

## Step 4. Compare Aligned miRNA

- Use the 'Find' function in Microsoft Word to locate your desired miRNA.
- You can highlight the desired miRNA sequence in a different color and add blank lines above and below it to make it easier to see.
- Compare aligned miRNA above and below to the desired miRNA – **especially at the 3' end** – and identify those with the **highest** sequence homology.
- Cut and paste those with the **highest** sequence homology to a separate file.
- You can now close the original file without saving changes.

## Step 5. Convert RNA to DNA

- Change the **U** in the desired miRNA sequence to **T** (convert RNA to DNA).
- We recommend that you highlight the nucleotides that are not the same as the desired miRNA in red or some other distinguishable color for easier identification.

## Step 6. Identify Nucleotides

- Identify a nucleotide position close to the 3' end that has the lowest nucleotide identity with the other miRNA – make sure that there is at least **1** nucleotide in the remaining portion of the sequences that is not the same – delete the nucleotides 3' of this position.

  ***Note:*** *Nucleotides do not have to be deleted – this is only performed to increase specificity when needed*
- Avoid relying upon a **G:T** or **T:T** mismatch for specificity as they are not easily discriminated
- **C:A** mismatches are most easily discriminated

## Step 7. Calculate Tm

- Determine the estimated Tm of the remaining desired miRNA.

  ***Note:*** *You can use the standard Tm calculators of your oligo house. For example, if using Integrated DNA Technologies website use the default settings to estimate the Tm.*

## Step 8. Adjust the Tm

- You now need to adjust the Tm of the sequence in order to obtain a Tm in the appropriate range of **50℃ to 55℃**. This temperature range provides for greater

specificity.

- Add one or more **G** to the 5' end of the remaining desired miRNA to adjust the Tm to **50℃ -55°C.**
- The minimum length before adding G's should not be less than 15 nucleotides.

***Note:*** *You may determine that there may be more than one sequence for your desired miRNA primer.*

## Step 9. Order Sequences

- Design and order your primer(s). Order more if needed. In some cases you will determine one or more sequences that are projected to provide the desired specificity.

  ***Note:*** *HPLC purification is required for highest consistency and sensitivity*
- Order synthetic miRNA templates of the desired miRNA(s) from your standard oligo supplier and any other closely related miRNA(s). You will want to test empirically for specificity.