

User Manual For Assembly PCR Oligo Maker

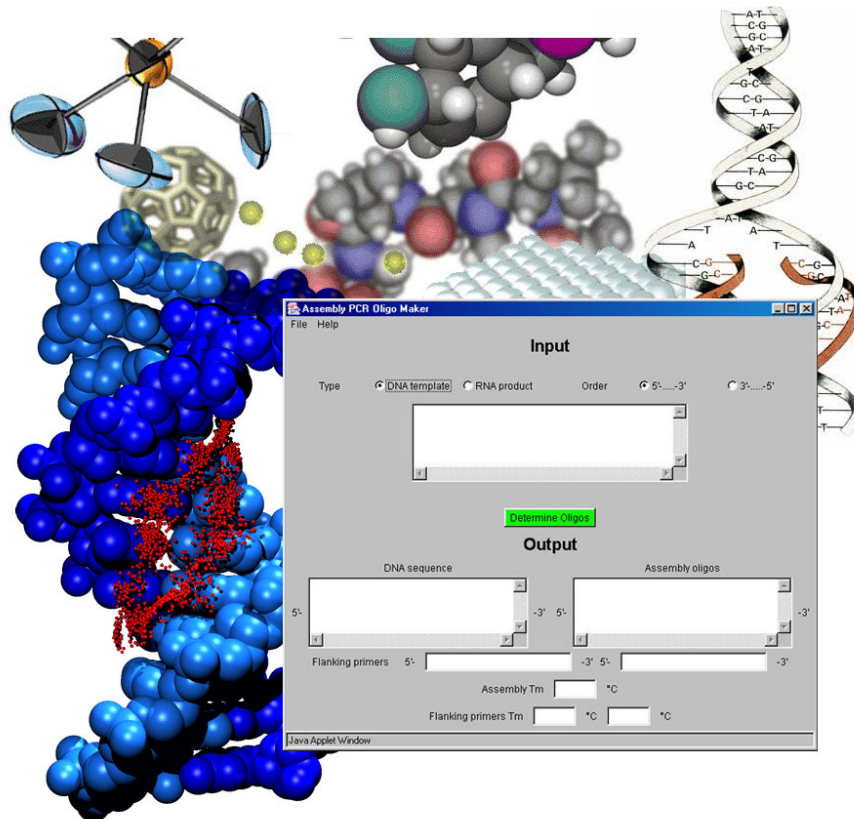


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GETTING STARTED

ASSEMBLY PCR OLIGO MAKER

Assembly PCR Oligo Maker has been created to automate the design of oligodeoxynucleotides for the PCR-based construction of long DNA molecules. This application is designed specifically to aid in the design of DNA molecules that are to be used for the production of RNA molecules by *in vitro* synthesis with T7 RNA polymerase.

The input for Assembly PCR Oligo Maker is either the desired DNA sequence to be made or an RNA sequence. If RNA is the input, the program first determines the DNA sequence necessary to produce the desired RNA molecule. The program then determines the sequences of all the oligodeoxynucleotides necessary for a two-step assembly, PCR-based synthesis of the desired DNA molecule.

The output oligodeoxynucleotide sequences are designed to have a uniform melting temperature and are checked for regions of overlap outside of the desired priming regions necessary for the PCR.

Assembly PCR Oligo Maker has several advantages over similar applications that are currently available. These advantages are in addition to the ability of Assembly PCR Oligo Maker to handle RNA as an input and add the top17 sequence to determine the DNA sequence needed for RNA production. While this is a trivial process, the use of Assembly PCR Oligo Maker does remove the human element from this task, which can inadvertently introduce sequence errors into the final product.

The major advantage of using Assembly PCR Oligo Maker is that you have the option of determining the maximum length of oligodeoxynucleotides for assembly PCR that are created by the program. This option is not available in the other programs. You thus have the option of choosing to create fewer but longer oligodeoxynucleotides than determined by other programs. Working with fewer oligodeoxynucleotides means that the process of dissolving and diluting them and setting up the assembly PCR reaction is simplified and that fewer opportunities for human error are available during the experiment.

Finally, the output of Assembly PCR Oligo Maker is very clear; it lists the oligodeoxynucleotides needed for the assembly PCR reaction and the two oligodeoxynucleotides needed for the second PCR reaction as well as providing

the predicted melting temperature values. All the oligodeoxynucleotides in the output are listed 5' to 3', which is a form that is suitable for cutting and pasting the sequences into an email or a web form for ordering oligodeoxynucleotides from the vendor of your choice.

TARGET AUDIENCE

The intended audience of this user manual is any person wishing to synthesize a DNA template or an RNA product for research purposes and wants to simplify the work by using a computer application such as Assembly PCR Oligo Maker. Assembly PCR Oligo Maker is freely available at <http://publish.yorku.ca/~pjohnson/AssemblyPCROligomaker.html>. It is assumed you have prior knowledge of biology and DNA.

POLYMERASE CHAIN REACTION, PCR

The construction of long DNA oligonucleotides, such as synthetic genes, is useful for a number of reasons. For protein expression it is possible to improve the yield of a desired protein by optimizing codon usage to reflect the relative codon abundance in the host organism used for protein production. Additionally, oligodeoxynucleotides can be used to produce large amounts of RNA for structural or biochemical studies by *in vitro* transcription methods. Many RNA molecules studied to date are under 100 nucleotides in length, and the DNA oligonucleotides needed for their production are readily synthesized and are commercially available (up to 100-150 nucleotides long). As larger RNA molecules are studied, and if methods such as the use of cis3' and/or 5' ribozymes to facilitate RNA production or RNA-based affinity tags to aid in RNA purification are used, it is not possible to purchase DNA oligonucleotides of sufficient length to encode the desired RNA product. To produce these long DNA molecules (over 120-150 nucleotides), it is necessary to use techniques originally developed for gene synthesis, such as assembly PCR.

The polymerase chain reaction (PCR) is a commonly used method to amplify DNA sequences. PCR methods can be used to construct a synthetic gene through the use of a two-step PCR method referred to as assembly PCR (Figure 1).

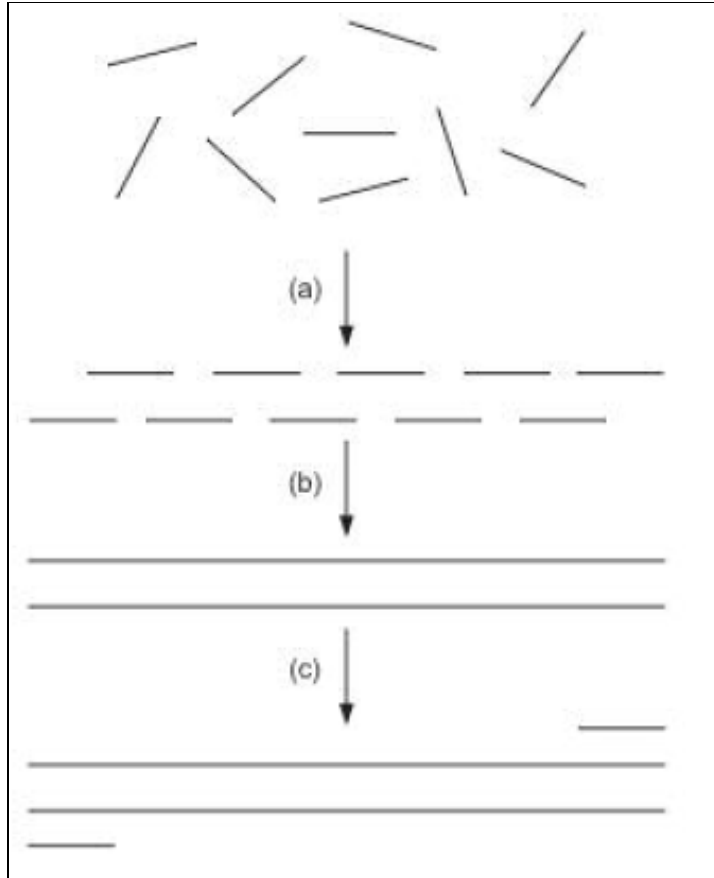


Figure 1. The assembly PCR method for constructing long DNA molecules. (a) In the first PCR step a pool of oligodeoxynucleotides anneal and are (b) elongated to produce a full-length DNA molecule. In addition to the full-length product, a host of shorter molecules also results. (c) In the second PCR step the desired full-length molecule is selectively amplified from the mixture using primers specific for the desired product.

In the first step of assembly PCR, multiple oligodeoxynucleotides that contain overlapping regions anneal, and the DNA polymerase extends the primers and fills in the regions between the primers. A range of products of different lengths results from the different possible annealing combinations that involve less than all the oligodeoxynucleotides. In the second PCR step, a pair of primers that is specific for the full-length oligodeoxynucleotide is introduced and the full-length product is selectively amplified from the mixture.

SYSTEM REQUIREMENTS

Assembly PCR Oligo Maker is a Java Applet version 1.1.

It is compatible and has been tested with a variety of browsers (Netscape, IE, Mozilla Firefox, etc.) on different platforms (Microsoft Windows, Linux, and Mac) and should not require any Java plug-ins. However, if you have difficulty running the program, you should download the latest Java Runtime Environment (JRE) available for your platform from Sun Microsystems at <http://java.sun.com>.

Mac users be aware! It is inherent to Mac OS that all menus appear at the top of the screen. The 'File' and the 'Help' menus for the Assembly PCR Oligo Maker are therefore found at the top of the screen, as shown on the screenshot below in Figure 2, instead of being located above the application window.

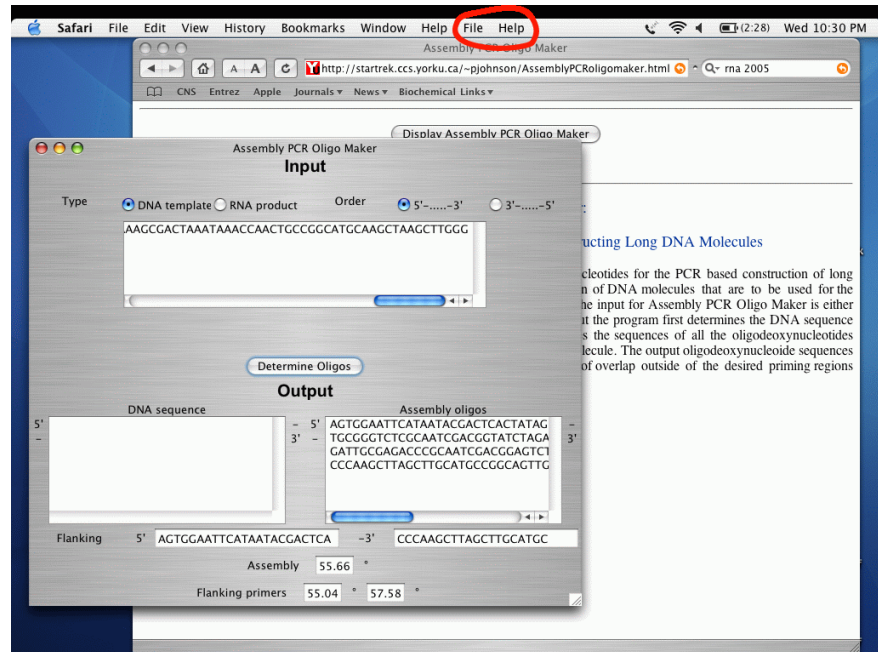


Figure 2. A Mac OS screenshot of Assembly PCR Oligo Maker depicting the location of the menu.

INTERFACE OVERVIEW

Assembly PCR Oligo Maker's interface is simple and composed of the input and the output, as shown in Figure 3. The input has only two choices: the type of DNA template or RNA product, and the order of the input 5'-...-3' or 3'-...-5'.

For the output, DNA sequence, assembly oligos, flanking primers, the assembly melting temperature, and flanking primer melting temperatures will be displayed once the green, *Determine Oligos* button is pressed.

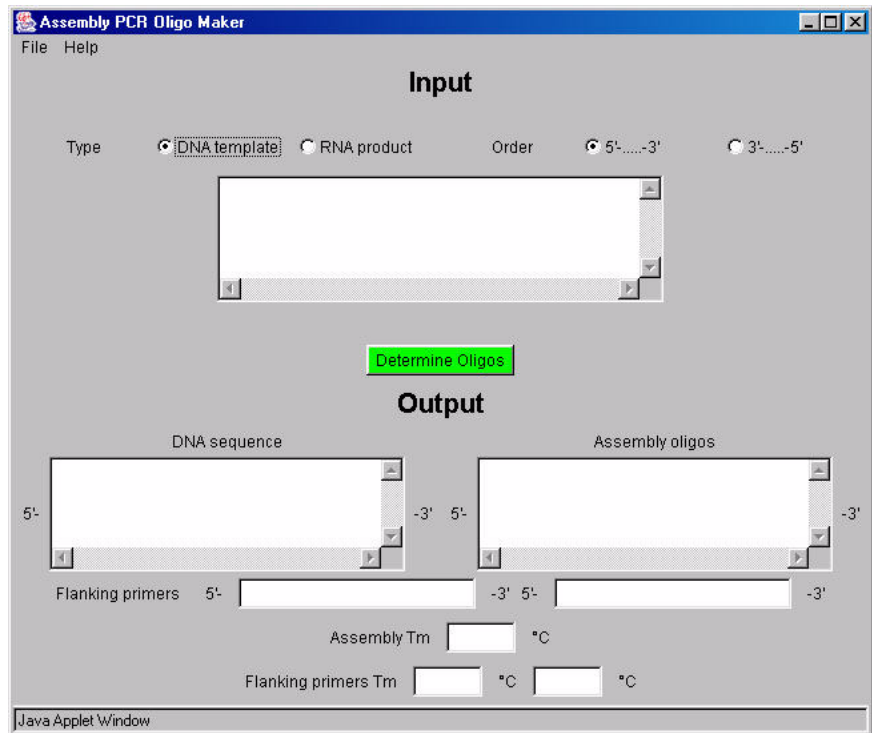


Figure 3. The Assembly PCR Oligo Maker's interface.

Figure 4 shows the File menu, which includes *Clear all fields*, *Customize*, *Show all results*, and *Exit*.

Clear all fields: Clears all textboxes, and resets all radio buttons to default.

Customize: Allows the user to customize Assembly PCR Oligo Maker.

Show all results: Displays all results in a text box, so you can copy and paste them into another application.

Exit: Closes the program, and returns to the web browser.



Figure 4. The Assembly PCR Oligo Maker's interface depicting the File menu.

Figure 5 shows the Help menu, which includes *Help* and *About*.

Help: Displays help.

About: Shows the information about the version of the program and its authors.



Figure 5. The Assembly PCR Oligo Maker's interface depicting the Help menu.

CLEARING FIELDS

This option resets all radio buttons to default values and clears all textboxes of input and output. You should use this option when you want to erase all previous input and output in order to avoid confusion.

1 Click *File > Clear all fields*.

CUSTOMIZING ASSEMBLY PCR OLIGO MAKER

Whenever you want to design oligodeoxynucleotides outside normal PCR conditions, you should use this option to input your reaction conditions. You can customize Assembly PCR Oligo Maker in the following ways:

- select the acceptable melting temperature for overlapping
- select the assembly (annealing) temperature
- select the maximum oligonucleotide length
- select the melting temperature method to be used in its calculations
- select the output in group of 3 nucleotides.

The window that allows you to customize Assembly PCR Oligo Maker is shown in Figure 6.

1 Click *File* > *Customize*.

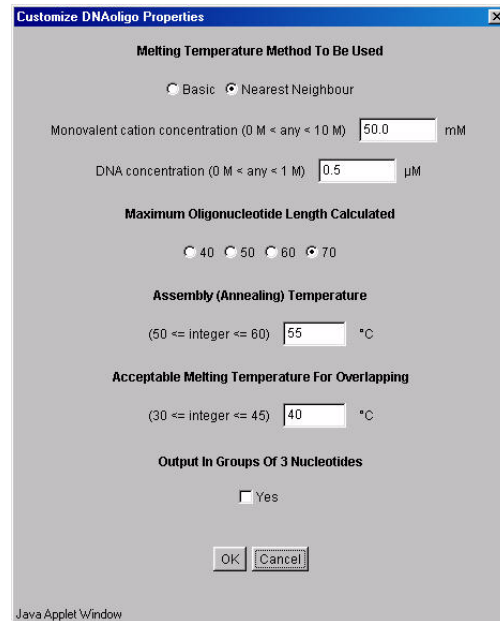


Figure 6. Assembly PCR Oligo Maker's customize dialog box.



Acceptable melting temperature for overlapping

To ensure the success of assembly PCR, it is necessary that the oligodeoxynucleotides only anneal where desired. If an oligodeoxynucleotides can anneal in multiple ways, in either an intramolecular or intermolecular fashion, the subsequent PCR reactions would produce a host of undesired products. The Assembly PCR Oligo Maker program tests the oligodeoxynucleotides calculated for both PCR reactions for regions of overlap outside of the desired location. If a stretch of nucleotides is found to overlap at a region outside of the desired site, the program calculates a T_m value for this region of overlap. If the T_m value of the overlapping region detected is greater than the defined T_m threshold, a warning appears, notifying you that an undesired overlapping region was detected. The region is also flagged in the results window. You can then make changes to the sequence of the desired PCR product or ignore this warning and go ahead and attempt the PCR reaction despite the presence of an unwanted overlap region. Alternately, upon detection of an undesired region of overlap, you can choose to calculate oligodeoxynucleotides with a smaller or greater maximum length in order to try to break up the region of unwanted overlap.

Choose the acceptable melting temperature for overlapping between the oligonucleotides, the primers, and the duplex. Allowable input is an integer between 30 and 45°C inclusive. The default value is 40°C.

- 2 Type an integer between 30 and 45 inclusive. The program will not accept values outside this range and will display an error.**

Assembly (annealing) temperature

Choose the assembly temperature during which the PCR reaction will occur. For optimal results, the PCR reaction must be performed between 50 and 60°C inclusive. The default value is 55°C.

- 2 Type in an integer between 50 and 60 inclusive. The program will not accept values outside this range and will display an error.**

Maximum oligonucleotide length calculated

Choose the length of the oligonucleotides to be calculated. The default value is 70. When you choose a smaller nucleotide length, more nucleotides will be obtained.

- 2 Choose from 40, 50, 60, or 70 nucleotides in length.**

Melting temperature method to be used

Choose this method for melting temperature calculations.

- 2 Choose either the *Basic* or the *Nearest Neighbour* method. The formulae for each method used are shown below.

Basic: $T_m(^{\circ}\text{C}) = 2(A + T) + 4(G + C)$

Nearest neighbour: $T_m(^{\circ}\text{C}) = (1000\Delta H^{\circ}/\Delta S^{\circ} + R\ln([DNA]/4)) - 273.15$

Nearest neighbour:

- 3 Type the monovalent cation concentration, a value between 0 and 10 M. The default value is 50.0 nM. Also, type in the DNA concentration, a value between 0 and 1 M. The default value is 0.5 mM.

Output in groups of 3 nucleotides

Choose whether to display the nucleotides in groups of 3's separated by a space.

- 2 Check the *Output in Groups of 3 Nucleotides* checkbox to display the nucleotides in groups of 3's. If you don't check this box, the output oligos will not be separated by a space every 3 nucleotides.

GETTING HELP

This option displays a non-searchable text version of this manual.

- 1 Click *Help > Help* again to open the help window, as shown in Figure 7.

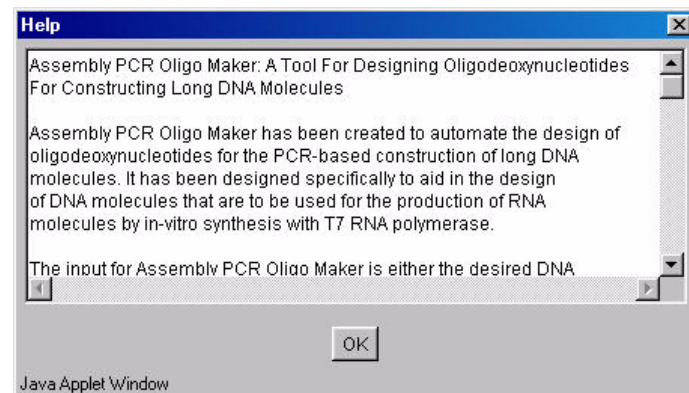


Figure 7. The Help window.

VIEWING CREDITS

This option shows the information about the version of the program and its authors.

- 1 Click **Help > About** to display the credits window, as shown in Figure 8.

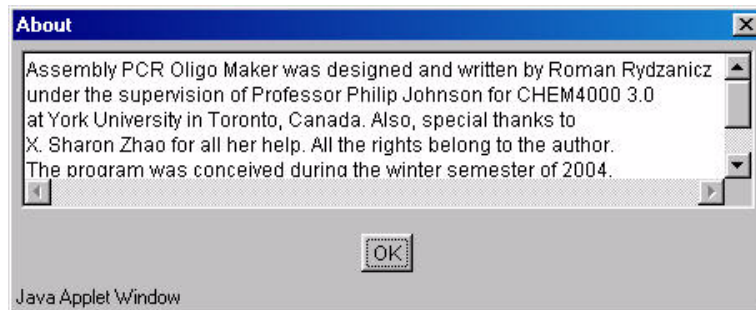


Figure 8. The About window.

MAKING OLIGOS

CALCULATING AN RNA PRODUCT

The simplest use of the program is to determine the DNA sequence needed to produce a particular RNA molecule. First, the program determines the target DNA sequence to be produced, and then it computes the necessary oligodeoxynucleotides for both steps of the two-step assembly PCR synthesis method. By default, the program only determines assembly oligodeoxynucleotide sequences for DNA targets longer than 40 nucleotides.

The program works by dividing the target DNA into an even number of segments with a user-defined maximum length.

To customize your program before determining oligos, see section Customizing PCR Oligo Maker in chapter 2.

- 1 Select *RNA product* in the input section of the program.
- 2 Select the order, *5'...-3'* or *3'...-5'*, of your RNA product.
- 3 Type or copy and paste the input into the input textbox. Allowable input for an RNA product is as follow:
 - 'c' or 'C' for cytidine
 - 'a' or 'A' for adenosine
 - 'g' or 'G' for guanosine
 - 'u' or 'U' for uridine.Nucleotides can be separated by spaces.
- 4 Click the *Determine Oligos* button.
- 5 Choose *Yes* if you want to add the Top17 sequence 5'-TATAGTGAGTCGTATTA-3' to the beginning of your DNA sequence. *No* does not add the Top17 sequence.

DNA sequence, assembly oligos, flanking primers, assembly melting temperature, and flanking melting temperature will be displayed in their appropriate boxes. Any warnings or errors will be displayed with popup boxes. Refer to the Error section to find the meaning of an error message.

CALCULATING A DNA TEMPLATE

You can also use the program to synthesize a DNA template. Assembly PCR Oligo Maker computes the necessary oligodeoxynucleotides for both steps of the two-step assembly PCR synthesis method. By default, the program only determines assembly oligodeoxynucleotide sequences for DNA targets longer than 40 nucleotides.

The program works by dividing the target DNA into an even number of segments with a user-defined maximum length.

To customize your program before determining oligos, see section Customizing PCR Oligo Maker in chapter 2.

- 1 **Select *DNA template* in the input section of the program.**
- 2 **Select the order, 5'...-3' or 3'...-5', of your DNA template.**
- 3 **Type or copy and paste the input into the input textbox. Allowable input for an DNA template is as follow:**
 - 'c' or 'C' for cytidine
 - 'a' or 'A' for adenosine
 - 'g' or 'G' for guanosine
 - 't' or 'T' for thymidine.

Nucleotides can be separated by spaces.
- 4 **Click the *Determine Oligos* button.**
- 5 **Choose *Yes* if you want to add the Top17 sequence 5'-TATAGTGAGTCGTATTA-3' to the beginning of your DNA sequence. *No* does not add the Top17 sequence.**

Assembly oligos, flanking primers, assembly melting temperature, and flanking melting temperature will be displayed in their appropriate boxes. Any warnings or errors will be displayed with popup boxes. Refer to the Error section to find the meaning of an error message.

SAVING RESULTS

You may want to save the results obtained during a calculation. All the results obtained in the output section of the program are shown in a textbox for you to copy and paste into your favourite application. Also, a visual representation of assembly oligos is shown at the bottom of the window, as shown in Figure 1.

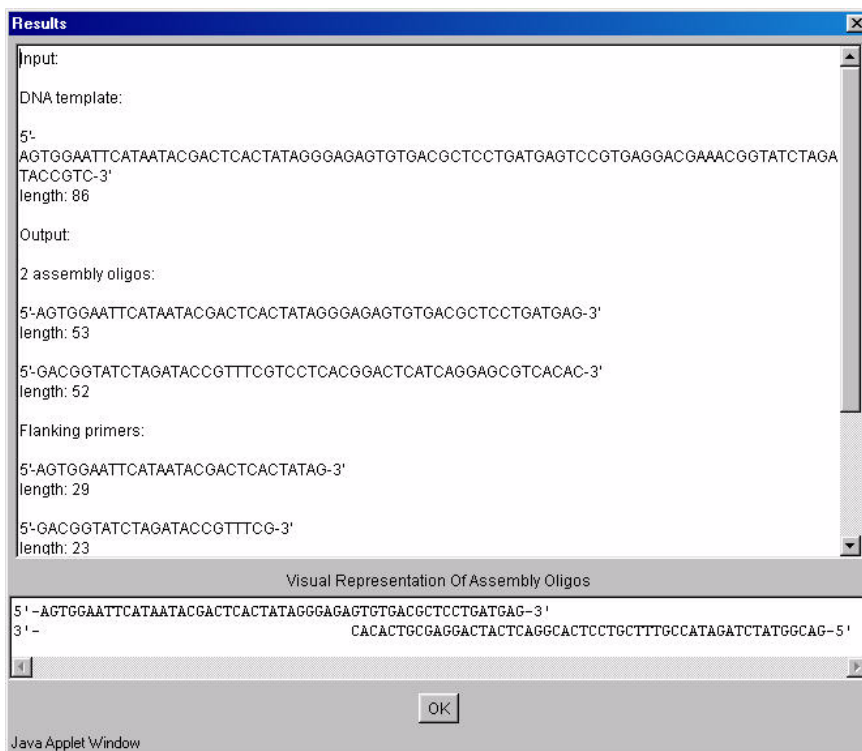


Figure 1. Assembly PCR Oligo Maker window displaying all the results in one single textbox. Also, a visual representation of assembly oligos beneath.

ERROR MESSAGES

AN OLIGO HAS A CORRESPONDING, MATCHING SEQUENCE WITHIN ITSELF

During oligo determination, if the program finds a region in one oligo that could possibly anneal to a region in the same oligo, this could be troublesome during a PCR reaction. To correct this situation, reduce the acceptable melting temperature for overlapping, or reduce the maximum oligonucleotide length calculated. Refer to the *Customizing Assembly PCR Oligo Maker* section in chapter 1 for more information. The error window is shown in Figure 1.

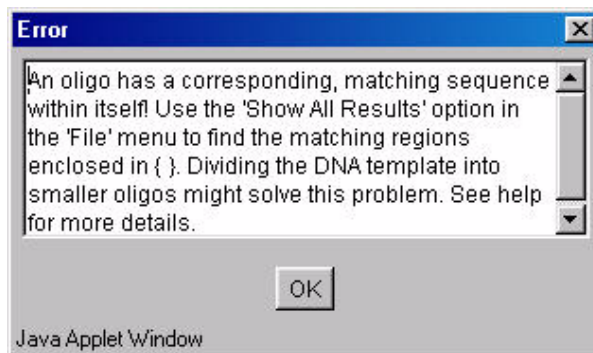


Figure 1. The Error window.

A PRIMER HAS A CORRESPONDING, MATCHING SEQUENCE WITH A DUPLEX

During oligo determination, if the program finds a region in one oligo that could possibly anneal to a region in the duplex, this could be troublesome during a PCR reaction. To correct this situation, reduce the acceptable melting temperature for overlapping or reduce the maximum oligonucleotide length calculated. Refer to the *Customizing Assembly PCR Oligo Maker* section in chapter 1 for more information. The error window is shown in Figure 2.

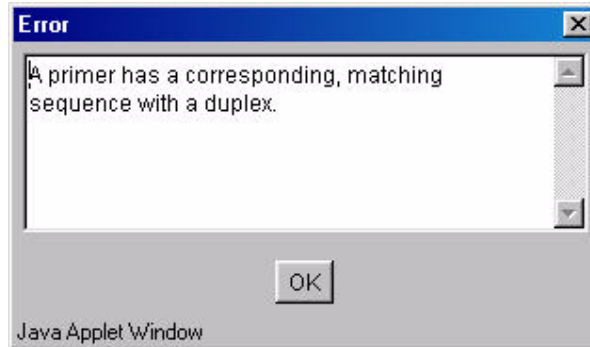


Figure 2. The Error window.

A PRIMER HAS A CORRESPONDING, MATCHING SEQUENCE WITH THE OTHER PRIMER

During oligo determination, if the program finds a region in one primer that could possibly anneal to a region in another primer, this could be troublesome during a PCR reaction. To correct this situation, reduce the acceptable melting temperature for overlapping or reduce the maximum oligonucleotide length calculated. Refer to the *Customizing Assembly PCR Oligo Maker* section in chapter 1 for more information. The error window is shown in Figure 3.

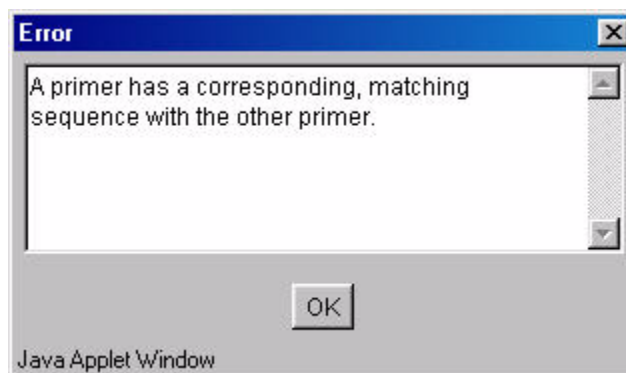


Figure 3. The Error window.

INVALID DNA TEMPLATE INVALID NUCLEOTIDE IN ()

Enter a DNA template. Valid nucleotides are 'c' or 'C' for cytidine, 'a' or 'A' for adenosine, 'g' or 'G' for guanosine, and 't' or 'T' for thymidine. Nucleotides can be separated by spaces. The error window is shown in Figure 4. The invalid nucleotide will be placed in parenthesis, as shown in Figure 5.

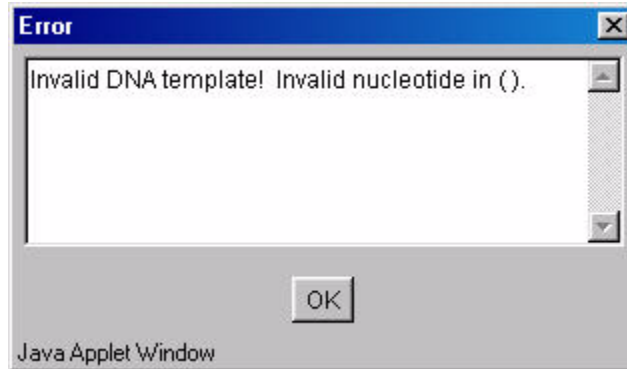


Figure 4. The Error window.

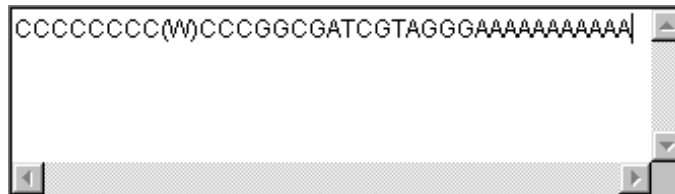


Figure 5. Figure displaying an invalid nucleotide W, which is placed between parenthesis.

INVALID RNA PRODUCT INVALID NUCLEOTIDE IN ()

Enter a valid nucleotide for an RNA product. Valid nucleotides are 'c' or 'C' for cytidine, 'a' or 'A' for adenosine, 'g' or 'G' for guanosine, and 'u' or 'U' for uridine. Nucleotides can be separated by spaces. The error window is shown in Figure 4. The invalid nucleotide will be placed in parenthesis, as shown in Figure 5.

SOME OLIGO HAS A CORRESPONDING, MATCHING SEQUENCE IN ANOTHER OLIGO

During oligo determination, if the program finds a region in one oligo that could possibly anneal to a region in another oligo, this could be troublesome during a PCR reaction. To correct this situation, reduce the acceptable melting temperature for overlapping or reduce the maximum oligonucleotide length calculated. Refer to the *Customizing Assembly PCR Oligo Maker* section in chapter 1 for more information. The error window is shown in Figure 6.

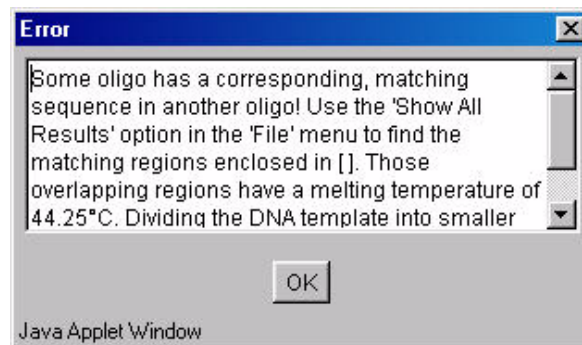


Figure 6. The Error window.

THE DNA TEMPLATE MUST BE LONGER THAN 40 NUCLEOTIDES IN ORDER TO CALCULATE OLIGOS

Enter a DNA template that is at least 40 nucleotides long. Since there is no point in determining oligos for such a short DNA template, the program's algorithm will not accept such a short input.

THE RNA PRODUCT MUST BE LONGER THAN 40 NUCLEOTIDES IN ORDER TO CALCULATE OLIGOS

Enter an RNA product that is at least 40 nucleotides long. Since there is no point determining oligos for a such short RNA product, the program's algorithm will not accept such a short input.

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