I. Introduction

This software is for use in designing, analyzing, and simulating experiments involving the polymerase chain reaction (PCR). PCR is a technique used by molecular biologists to amplify highly selected segments of DNA. If you never heard of PCR, you probably have no use for this program. If you want to know more about the process, you can look up the book, *PCR Technology*, by H.A. Erlich (1989, Stockton Press).

Very briefly, PCR will take a short stretch of DNA (usually fewer than 3000 bp) and increase its copy number about a million fold so that one can determine its size, DNA sequence, etc. The particular stretch of DNA to be amplified, called the target sequence, is identified by a pair of DNA primers, which are even shorter pieces of DNA (usually about 20 bp) that have been synthesized in large quantities. To use ***Amplify*** you must supply the sequences of the primers and the target DNA. The software then analyzes the combination of primers and target sequence you have chosen to determine what portions of the target are likely to be amplified. It also provides various bits of helpful information about the reaction products, primer binding sites, etc., that can help in planning a PCR experiment and designing primers.

II. Quick Tutorial

1. Boot up ***Amplify*** by double-clicking on the file “Primers.pri”. You should now be faced with a window showing the ***Amplify*** logo. Close it to reveal another window with a list of primers. (This is just list of primers that is currently available in my lab.) The primer sequences are in the first column and their names are in the second.

2. Find the primer named “1700” and place the cursor anywhere

in that line. Then select **Use this Primer** from the menu, or hit ⌘-1. That will open another window, and add primer 1700 to the list of primers that will be used. Then do the same for primer 2230.

3. Next, open a file of target DNA by selecting **Open Target Sequence** from the **File** menu, or by hitting ⌘-O. Find the file called “P Element.seq”. (This is the sequence of the P transposable element in *Drosophila melanogaster*.)

4. You have now specified everything needed for a PCR experiment. So pick **Run PCR** (⌘-3) from the **PCR** menu. A map showing the PCR products will then be generated as follows:

5. The arrowheads indicate points of primer binding (darker means a better match), and the bars below the sequence are potential amplified fragments (heavier bars are expected to amplify better). Explore this result by clicking on the various elements of the map. In each case, you will get an information screen with data about the item you clicked. Note that the lighter arrowhead for primer 1700 indicates a weak match that is probably not desirable in the experiment. Click on it to see how well the primer matches the target sequence.

6. To get a closer look at just a portion of the sequence, you may zoom in on any chosen section of the map. To do this, hold the command (⌘) key down and drag with the mouse to draw a rectangle around the region of interest. Then select **Zoom In** from the **PCR** menu. You can repeat the process any number of times to get even higher resolution, or else select **Zoom Out** to get back to the original scale.

III. Working With Primer Lists

To use ***Amplify*** you should keep a text file listing all the primers available to you. You can create such a list with ***Amplify***, or else use any word processing or spreadsheet program. Each line of the list should start with the sequence of the primer

(*Always in the 5´ to 3´ direction*) followed by a tab and then the name of the primer. The name should be short so that it will fit on the map. You can also append additional information about the primer on the same line after another tab.

Amplify can accept any text file as the primer list. However, if you wish to have a double-clickable file, you must create it with ***Amplify***'s **Save As…** command. In addition, the name of the file should end with ".pri" so that ***Amplify*** will know that it is not a target sequence file.

You can edit your primer list in ***Amplify***'s Primer List window just as you would edit in any Macintosh text window. In addition, you can use the **Edit** menu to interchange between upper and lower case letters in order to identify any particular part of the sequence. You can also reverse the polarity of any sequence: select the portion you want to reverse, then pick **Reverse Sequence** from the **Sequence** menu. This action takes the complementary strand and displays it in the 5´→ 3´ direction.

Only the primers listed in the Primers In Use window will be involved in the amplification. You can move primers from the main list to the In Use list by copying and

pasting or by picking **Use This Primer** (⌘-1) with the cursor on the primer you want to select. To remove a primer from the In Use list, you can use the **Remove This Primer** menu item in the same way, or else simply erase it as you would any text.

IV. Working With The Target Sequence

Amplify can read DNA sequence information from any text file. If you create a text file with ***Amplify***'s **Save As…** command, it will have the ***Amplify*** icon and be double-clickable to open the ***Amplify*** program.

The target sequence text can have an optional header section before the actual sequence starts. This header can consist of anything you like provided it ends with two periods (“..”). Anything following the “..” is taken to be part of the sequence. If you wish to use a header that includes “..” as part of the text, you can use the **Preferences** command to change the header delimiter to any two-character string.

Whenever you use the target sequence, ***Amplify*** attempts to put the DNA into a standard format. It removes any characters other than A, T, C and G, plus the corresponding lower-case letters and arranges the sequence in 80-character lines. This reformatting is done only to the sequence part of the text, and not to the header. You can change the number of characters per line to something other than 80 with the **Preferences** command.

You can edit the target sequence window as you would any Macintosh text. You can also use the upper- and lower-case commands as well as the **Reverse Sequence** command as described previously in part III.

You can search the sequence using the **Find Pattern** (⌘-F) command. This command is better for searching sequences than the usual find command available in most text editors,

including the one in the **Edit** menu of ***Amplify***. With the **Find pattern** command you can search in both orientations if you wish, and line breaks are ignored in the search. In addition, you can specify a maximum number of allowable mismatches in the search pattern. Once you have found the pattern, you can find the next occurrence with the **Find again** (⌘-G) command. Use the **Select Sequence…** command to select a stretch of sequence according to its coordinates, and the **Get sequence info** (⌘-I) command to see size, base composition, etc., of any selected portion of your sequence.

V. The Run PCR command

The **Run PCR** (⌘-3) command is what does the real work. Before you use this command, you must have loaded a target sequence and put at least one primer into the Primers In Use window. The **Run PCR** command performs the following series of actions:

1. The target sequence is formatted as described above. If it has already been formatted and no editing has been done since then, this step is skipped.

2. Each selected primer is checked against itself and all the others to determine if any are likely to form dimers.

3. The target sequence is searched in both directions for matches to the primers you have selected. Two criteria called “primability” and “stability” are used to estimate how likely a given match is to contribute to the amplification reaction. More about these later.

4. Each primer match that qualifies under both criteria is used to screen for possible amplified fragments. Each such fragment is evaluated to determine its probable degree of amplification depending on the quality of the matches involved, the length of the sequence, it’s GC content, etc.

5. The results of this analysis are displayed graphically to show the matches and the amplified fragments. If any potential dimer-forming pairs are identified, they are shown also.

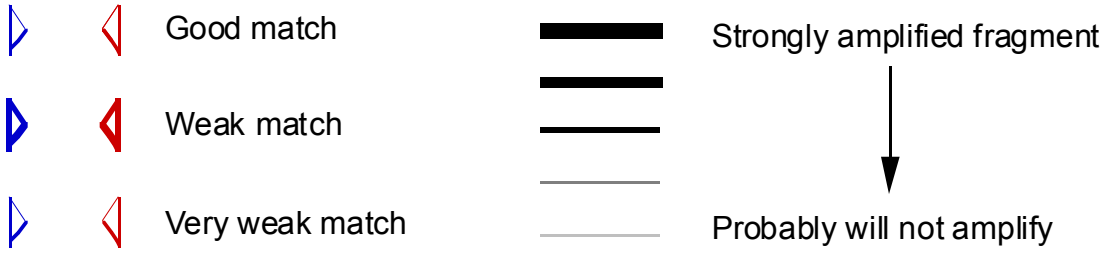
VI. Working with the amplification map

Matches and Fragments

The target sequence is represented as a horizontal line with tick marks every 100 bp. Any matches between the primers and the target sequence are shown as arrowheads. Rightward-directed matches are above the line and leftward ones below.

Segments of the target sequence that are candidates for amplification are shown as bars below the target sequence. They are drawn starting with the “best” fragments on top, *i.e.,* those expected to have the greatest abundance following the PCR.

The primer matches and amplified fragments are drawn so that darker fills and heavier lines correspond to better matches or amplification. This scheme is intended only as a rough guide to the quality of the matches and strength of the amplifications. Be sure to click on anything of interest to get more information.



Primer Dimers

If any potential dimer-forming pairs are found, they are listed below the rightward matches and above the amplified fragments. There is no theory to predict with much confidence whether dimers will actually form, but the ones identified by ***Amplify*** should include most of the likely candidates. The algorithm was designed to be conservative, so that some potential dimers will be shown even though they will not cause trouble in a real experiment. Be sure to click on the dimer warnings to see how the match is formed. For example, clicking on one dimer warning might bring up the following text:

```
5´  2611 ATCAACATCGACGTTTCCAC.....       3´
                    |||
3´       .....GTGGCTTTGACGCCTGAAGA 2465  5´
```

This indicates that primers 2611 and 2465 can pair at their 3´ ends, possibly allowing strand extension to form a dimer. However, there are only three base pair matches, and one of them is A-T. Therefore, this dimer might not be a problem. One the other hand one like this:

```
5´  2361 CCTTTGCCCAGTCGTACGAC.....       3´
                    ||||
3´       .....GCTGCGTTCCTCATCGGCTG 2360  5´
```

is potentially much more serious. And this one:

```
5´  2361 CCTTTGCCCAGTCGTACGAC.....       3´
                 |||||||||
3´       .....CAGCATGCTGACCCGTTTCC 2361  5´
```

will almost certainly kill the reaction.

Amplify also includes the commands **Check This Primer** and **Check all Primers** to help you in designing new primers. If you place your cursor on any primer in your main list and choose **Check This Primer**, it will search for potential dimer-forming combinations between the selected primer and all others in the list. If you select **Check All Primers**, the program will make a table of all potential dimer-forming pairs from your list.

You can change the parameters to make the dimer search more or less sensitive by altering the values in the dialog box invoked with the **Change Dimer Parameters…** command. The default settings are from empirical rules based on results from my own lab. You can set the minimum length of the overlap required and the number of mismatches allowed computed as a function of the number of matches.

Changing the Resolution

In some cases you will want to increase the resolution of the map for a certain area. For example, it might happen that two primer matches are so close together that their arrowheads overlap and their names are difficult to read. One thing you can do is click on them. That will put information about both matches in the Info window. Another approach is to use the **Zoom In** feature of *Amplify*. To do this, you must hold down the command (⌘) key so that your cursor becomes a “+” sign. Now drag with the mouse to make a rectangle on the screen. The left and right sides of the rectangle will determine which section of the sequence will be magnified in the close-up view. (The top and bottom of the rectangle are not

important.) Now select **Zoom In** from the **PCR** menu. You can repeat this process to get as much resolution as you need to distinguish the overlapping primer matches. To return to the original scale, select **Zoom Out**.

Saving and Exporting Graphics

You can save the map as a PICT file or copy it to the clipboard for pasting into another application. However, you must choose **Select All** (⌘-A) with the graphics window open before you perform the **Copy** of **Save As…** operation.

Alternatively, if you want to copy just a portion of the map and don't object to a jagged picture, you can do the following: First hold down the command (⌘) key and drag with the mouse to draw a rectangle around the portion you want to copy. Then select **Copy Pixels** from the **Edit** menu. Note that this method copies a pixel map rather than an object-oriented image.

VII. Primer Matches

Amplify uses two measures of the quality of a primer match. Both depend on how many G-C and A-T hydrogen-bonding pairs can form, but they weight the pairs in different ways. *Primability* gives most of the weight to the pairs near the primer's 3´ end. Thus it is a measure of how easily the DNA polymerase will be able to extend the sequence at that end. *Stability* is a measure of how tightly the primer and target are bound irrespective of where the matching pairs lie. For stability, G-C pairs are counted more heavily than A-T pairs and runs of matches are given more weight than singlets.

Consider the match below, which is shown as it would appear in the Info window.

Primer: 2607

Primability of Match = 100%

Stability of Match = 75%

The primability is 100% because all 20 nucleotides match. The stability is only 75% because not all of the pairs are G-C. Here is another example:

Primer: 2402

Primability of Match = 73%
Stability of Match = 42%

```
                5´                3´
                 CAACATCGACGTTTCCACATC
                   |  |||| |||| ||| |
TGGTGGACAAAGAGCTACGTTCAATTCCGACGACATTTCTACA
GCTATTTGTCTCCACACCGCAGGCCCT
                 |                |
                684              704
```

In this case there are several mismatches bringing the primability down to 73%. The most important one is the T-G mismatch in position 19. The stability is also reduced.

When ***Amplify*** scans the target sequence, it keeps only matches in which both the primability and stability are above a given cutoff point. In my experience, setting the primability cutoff point at 70% and the stability cutoff at 40% retains almost all matches that work for PCR, and it eliminates the majority of the matches that don't work. These values are used by ***Amplify*** as the default cutoff points. They tend to be on the conservative side, meaning that they are more likely to let a non-working match be shown than to eliminate a working match. If you wish to change the settings, you can do so by selecting the **Set Match Parameters…** item in the **PCR** menu. You can also experiment with the relative weights given to A-T and G-C pairs, the progression of weights from 3´ to 5´ used by the primability measure, and the relative weights given to runs of matches depending on the length of the run for the stability measure. However, remember that if you change any of these settings they will not take effect until you invoke the **Run PCR** command.

Window Settings

All the windows of ***Amplify*** can be size-adjusted and moved around the screen to suit your tastes and hardware. You can also change such things as the tab settings with the **Editor Setup…** command in the **Edit** menu. Use the **Save Settings** command to save your window arrangements and settings for the next time you run ***Amplify***.

Default Settings

You can change the default settings for primer matches, dimer checking, formatting of the target sequence, etc. by editing the STR# resource (ID = 5001) with a resource editor such as ResEdit. The first string in the list names the parameters whose defaults are in the second string. The third string names the fourth, and so on.

Limits

The current version of ***Amplify*** has the following upper limits:

length of target sequence.....30,000 bp
number of primers in main list.....1000
length of primer sequence .....100 bp
number of primer matches in each direction.....30
number of amplified fragments.....100
number of primer dimers displayed.....10
number of primers used for an amplification.....20

On-Line help

A condensed version of this manual is available from within ***Amplify*** if you need it. Just select a topic from the **Help** menu.

About *Amplify*

Amplify was written in Lightspeed Pascal using the programming modules from FaceWare. Support for its development was provided by Apple's Rota project. Beta tester Greg Gloor made many helpful comments and suggestions. There is no charge for ***Amplify***, but I retain the copyright. You may distribute it freely as long is it is unmodified and the documentation file is included.

I hope to add more features to ***Amplify*** when I get time. These include computation of T_m for primers, restriction site mapping, gel simulation, sequence searching with gaps allowed, inverse PCR, and perhaps more accurate evaluation of primer matches and dimer formers.

If you have any ideas or comments, you can send them to me by e-mail: WREngels@macc.wisc.edu. If you don't use e-mail, send them to William Engels, Genetics Department, University of Wisconsin, 445 Henry Mall, Madison, WI 53706.

What's new in version 1.2? I have not had time to add any new features since version 1.0 which was released about a year ago. However, there are a couple of bug fixes and slight changes in the manual. (But I haven't given up the idea of adding the new features listed above!)