
IGGPIPE INSTALLATION

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This describes how to install all elements needed to run the IGGPIPE software.

1. If not done already, download IGGPIPE files

1. Browse to <https://github.com/BradyLab/IGGPIPE>
2. At bottom of right column on screen, click "Download ZIP" and choose a place to put it on your computer.
3. Unzip the zip file on your computer.
4. Rename the unzipped folder from "IGGPIPE-master" to just "IGGPIPE".
5. If you are a GitHub user and wish to use the IGGPIPE GitHub repository directly, instead of the above steps, you can fork the IGGPIPE repository and work on your own copy. The *master* branch is where the releases reside, each tagged with a version number. You can create a branch whose contents are the same as a tagged version with the command:

```
git checkout -b my_v1.0_branch v1.0"
```

You can then do the installation process using those files.

We will try to monitor for pull requests if you make useful changes.

2. Requirements for installing IGGPIPE

1. **64-bit Mac OSX, Linux, or Windows/Cygwin System**

This description focuses on installation on a 64-bit Mac OSX system. Installation has also been tested on 64-bit Linux and 64-bit Windows, and we describe installation differences, but command line examples here are for Mac OSX, and Linux and Windows users are expected to be savvy enough to translate these to their system. For Windows systems, the Cygwin Unix-like tools must be installed. IGGPIPE will not work on 32-bit Windows systems.

2. The computer must have enough memory and speed

Before starting the installation, consider whether the computer on which the software is to be installed has enough memory for the problem at hand. We have been running IGGPIPE using 16GB of RAM, and we recommend no less than that amount. For larger genomes or more genomes, you will possibly need more. The computer we used has a 2.4 GHz processor, which has been adequate. You should monitor memory usage while the pipeline is running, and if it becomes excessive for your computer, you know you will need more memory to run those genomes.

3. Work from the command line

Most work here is done from the command line, by opening the Terminal application. You should be familiar with some basic command line commands such as *cd*, *ls*, *cp*, *rm*, *mv*, *sudo*, *less*, *head* and *tail*, *man*, and *mkdir*. If you do not know how to use the command line or don't know these basic commands, take time to learn a bit. A suggested tutorial (on the long side, but spend as little or as much time as you need to feel you can get started here) from UC Davis is at:

http://korflab.ucdavis.edu/Unix_and_Perl/current.html

4. Work in the IGGPIPE main directory unless otherwise instructed

While working from the command line to install IGGPIPE, most of the time you will be in the IGGPIPE main directory, unless instructed otherwise. If you unzipped the IGGPIPE zip file in your Documents folder, you might change into the IGGPIPE directory with this command:

```
cd ~/Documents/IGGPIPE
```

5. Know how to use a plain text editor and have one available

You must have a plain text editor you know how to use. If nothing else, the simple editor *nano*, which comes with Mac, Linux, and Cygwin, will work. On a Mac you can also use the *TextEdit* program (use Plain Text format). For Mac, the free open-source TextWrangler program is strongly recommended, and its more capable version called BBEdit is totally worth the \$50 price for anyone who regularly edits text files. TextWrangler is available from the Apple App Store (Applications, App Store) or from:

<http://www.barebones.com/products/textwrangler>

On Windows, *do not use Notepad*, as it will cause problems with DOS vs Unix line ends. If you don't want to use *nano*, look into Notepad++, a free text editor.

6. The following programs/applications will be installed as part of this installation process if they aren't already:

- a. Xcode (V7.0.1 works) (Apple App Store; *make* and *g++*)
- b. Jellyfish V2 or later (V2.2.3 works) (<http://www.genome.umd.edu/jellyfish.html>)
- c. Perl V5 or later (V5.16.0 works) (<https://www.perl.org/get.html>)
- d. R V3 or later (V3.2.1 works) (<https://www.r-project.org>)
- e. Primer3 (V2.3.6 works) (<http://sourceforge.net/projects/primer3>)

- f. NCBI e-PCR (V2.3.12-1 works) (<http://www.ncbi.nlm.nih.gov/tools/epcr>)
- g. MUSCLE (V3.8.31 works) (<http://www.drive5.com/muscle/downloads.htm>)
- 7. **Two of the above will require compilation and build on your computer using *make*:**
 - a. Jellyfish
 - b. e-PCR
- 8. **One C++ program provided with IGGPIPE will be compiled and built:**
 - a. findMers

3. Install IGGPIPE - Quick Start for the Experienced

Follow brief instructions here if you are experienced with installations and want to try to do a Quick Start installation with minimal reading. If you have trouble, check the more detailed instructions below for help.

1. Mac OSX: install Xcode and do 'xcode-select --install'; Windows: install Cygwin including Perl, make, and Bash
2. Copy allPathParameters.template file to new file allPathParameters.ours, then edit the new file and set WD to IGGPIPE directory
3. Install Jellyfish V2 or later (V2.2.3 works), configuring with ./configure, compiling with *make*, and installing with *sudo make install*
4. Install a V5 or later Perl if not already installed
5. Install R V3.2.1 or later. Windows installation may require reading more installation details below.
6. Install Primer3 V2.3.6 or later and assign CMD_PRIMER3CORE and DIR_PRIMER3CONFIG in allPathParameters.ours
7. Install e-PCR V2.3.12-1 or later and assign CMD_EPCR in allPathParameters.ours
8. Install MUSCLE V8.31 or later and assign CMD_ALIGNER in allPathParameters.ours
9. Build *findMers* using *make* in the *findMers* folder.
10. Test trashing a file using `$SHELL code/shell/trash.sh <filename>` and if it fails, set `CMD_DELETE_WHEN_CLEANING := $(CMD_DELETE)` in allPathParameters.ours
11. Copy primer3settings.default.txt to primer3settings.txt
12. On OSX in Xcode choose Open Developer Tool, FileMerge, then set it to stay in the dock.
13. Run IGGPIPE on test data using *make PARAMS=allParameters.test ALL | tee logFiles/makeLog.test.txt* then look through log file with *less logFiles/makeLog.test.txt* and compare actual

results with expected results using *source code/shell/cmpKeyFiles.sh outTestHP11 goodTest* (some files have not yet been made)

14.Run *make PARAMS=allParameters.test IndelsSNPs* and check results again with *source code/shell/cmpKeyFiles.sh outTestHP11 goodTest*

15.Run *make PARAMS=allParameters.test plotIndels* and examine *outTestHP11/Markers.indels.pdf*

16.Run *Rscript code/R/dotplot.R dotplot.template* and examine *outTestHP11/LCRs_.dotplot.png*

17.Run *Rscript code/R/annotateFile.R annotate.template* and check results again with *source code/shell/cmpKeyFiles.sh outTestHP11 goodTest*

4. Install IGGPIPE - Detailed Instructions

1. Install Xcode (Mac OSX only) or Cygwin (Windows only)

IGGPIPE makes use of a utility called *make*, and also, some of the applications used by IGGPIPE are distributed as source code that must be compiled and built into a runnable application on the user's computer, which requires a C++ compiler (g++ utility). On Linux, these utilities are already installed so you can skip this step.

Xcode (OSX)

On Mac OSX, the Apple Developer Toolkit named Xcode provides *make* and the other required utilities. Xcode is available free from the Apple App Store (Applications, App Store). If you don't have Xcode installed already, run the App Store application, search for "Xcode", and double-click the *Install* button to install it, and even if you do have it installed, make sure you are updated with the latest version. We used version 7.0.1, although later versions should work fine. *Earlier versions produce errors trying to compile Jellyfish*, so be sure you have version 7.0.1 or later.

Installation takes quite a long time, during which it appears nothing is happening.

When Xcode install is finished, you can verify that it was installed successfully by finding the Xcode application icon in Applications and running it. It may then display a box requesting your computer administrator password so it can install additional components. Then, close the Xcode application and go to the command line and enter the following command, which checks to see if the command line tools such as *make* and *g++* are installed, and if not, installs them:

```
xcode-select --install
```

To verify they are installed, you can enter this command:

```
g++
```

and you should see the error message "clang: error: no input files".

Cygwin (Windows)

On Windows, the open source Cygwin tools environment provides *make* and the other required utilities. If you don't already have Cygwin installed, it is available from:

<https://www.cygwin.com>

You should follow the instructions there for installation of Cygwin. Make sure you set the Cygwin installer so as to install:

- a. Perl
- b. Make - found under Devel (or just install all of Devel)
- c. Editors (for nano editor)
- d. bash - found under Shells

Many of these will be installed by default and the values will not need to be changed. To install a package, click the circle with arrows until it changes to a version number. This may not be a complete list of all the packages that need to be installed. Be watchful for command failures due to packages not having been installed, and if you find such a case, re-run the Cygwin installer and change the package you want from *skip* to *install*.

Although the R language is required for IGGPIPE, the version of R installed by Cygwin (available under the science category) will not be sufficient, and correct R installation instructions are given in a later step.

2. Change to IGGPIPE directory

Work from the command line from this point onwards. In OSX, the Terminal program in the Utilities folder gives you the command line, while under Windows/Cygwin, the Cygwin command line is the place to work.

Start by changing the current directory to the IGGPIPE directory where you downloaded the IGGPIPE files:

```
cd whatever/IGGPIPE
```

3. Copy allPathParameters.template file to new file allPathParameters.ours

A variety of applications must be installed. Before installing them, you should prepare a text file for editing. There is a text file in the IGGPIPE main directory that contains tool path settings for running IGGPIPE: allPathParameters.template.

The file provides settings of paths where applications have been installed, and related settings. To make your own version of this file containing your own application paths, copy the file to a new filename, replacing ".template" with ".ours":

```
cp allPathParameters.template allPathParameters.ours
```

4. Open the allPathParameters.ours file in a plain text editor

Open the new allPathParameters.ours file created above in your plain text editor for editing. If you are in a hurry, you don't need to go through the whole file, but simply need to set the parameters shown at the start of the file, up to the comment that indicates you are at the end of the quick start section.

For example, if your text editor is nano, you might use this command line to open your editor to edit the template file:

```
nano allPathParameters.ours
```

Under Windows, don't use Notepad as it will cause problems with DOS versus Unix line ends.

5. Set WD to your IGGPIPE directory in the allPathParameters.ours file

Find the WD parameter in the allPathParameters.ours file, which looks like:

```
WD := $(BRADYLAB)/Genomes/kmers/IGGPIPE
```

Change the assigned value to the path of your IGGPIPE directory (where you unzipped the IGGPIPE files). You can find the correct path to use by changing into the IGGPIPE directory (already done above) and entering this command:

```
pwd
```

Under Cygwin on Windows, this command will also work:

```
cygpath -am .
```

(note the . for current directory)

For example, maybe your WD assignment would look like this:

```
WD := /Users/johndoe/Documents/IGGPIPE
```

6. Install Jellyfish

Jellyfish is a free open-source bioinformatics application that searches FASTA sequence files for k-mers of a specified size and writes them to a file. IGGPIPE uses Jellyfish to extract unique (occurring once) k-mers from the genome sequences being used. You may already have Jellyfish installed, and if so you want to check its version number. Here is the command to see if it is installed and check the version:

```
jellyfish --version
```

Assuming you do not have it installed, or you have a version older than 2.2.3, you must do an installation. You can find the Jellyfish at:

```
http://www.genome.umd.edu/jellyfish.html
```

We chose the "latest source and binaries" link, then downloaded the .tar.gz file. Double-click this file in Finder, in the Downloads folder, and it unpacks to produce a jellyfish folder. We chose to move this folder to a directory named *src* under our user root directory:

```
cd ~
pwd
mkdir src
cp Downloads/jellyfish-2.2.3 src
```

This version of IGGPIPE was tested with Jellyfish version 2.2.3. Newer versions should work as well. *Older versions will not work, because Jellyfish changed its output file names. They used to end with "_0" but no longer do!*

Now the Jellyfish program must be configured, then compiled into an application, then installed on your computer.

Configure

To configure the Jellyfish build components on OSX, Linux, or Windows/Cygwin:

```
(change into Jellyfish directory)
./configure
```

The command worked without error on Linux and Windows, but an error occurred on OSX:

```
config.status: error: cannot find input file: `tests/compat.sh.in'
```

We ignored this error and continued on with the installation, and it worked fine.

Compile

To compile Jellyfish on OSX, Linux, or Windows/Cygwin:

```
(change into Jellyfish directory)
make
```

The *make* command worked without error on OSX and Linux, but compile errors ("impossible constraint" errors) occurred on Windows. Also, the Jellyfish installation README file said that this would not work on OSX. We found that it does not work on older OSX and Xcode versions, but it definitely does work on OSX 10.10.5 with Xcode 7.0.1.

We fixed the Windows compile errors by editing file *file_header.hpp* and adding the following lines *after* `#include <jellyfish/rectangular_binary_matrix.hpp>`:

```
namespace std {
    #include <sstream>

    template <typename T>
    std::string to_string(T value)
    {
        //create an output string stream
        std::ostringstream os ;

        //throw the value into the string stream
        os << value ;

        //convert the string stream into a string and return
        return os.str() ;
    }

    template std::string to_string<long long int>(long long int); // instantiate wi
}
```

That allowed the *make* to succeed under Windows.

The Jellyfish README says the following about compilation under OSX, despite the fact that we were able to compile successfully:

To install on Mac OS X: Jellyfish 2.0 does not compile with Apple's Xcode GCC 4.2. Instead, the easiest thing to do is to install GCC 4.8 using MacPorts (<http://www.macports.org>) using the following commands:

```
sudo port install gcc48
sudo port install gcc_select
sudo port select -set gcc mp-gcc48
```

The first command installs GCC version 4.8. The third command makes that version of GCC the default, and the second installs a package that makes the third command work. After the above, you should be able to run `./configure ; make` as normal.

Those steps might allow you to compile Jellyfish with an older version of OSX and Xcode.

Install

To install the Jellyfish program after compiling it, use this command:

```
sudo make install
```

The *sudo* command prompts for a password. Enter your computer's administrator password. On Windows/Cygwin, leave *sudo* off the above command.

The *make install* command places the Jellyfish program in the PATH variable so that the program can be run with the command *jellyfish*. (Sometimes it is necessary to log out and back in for a PATH change to take effect). Rerun the "*jellyfish --version*" command to verify that Jellyfish is installed.

The parameter `CMD_JELLYFISH` in the `allPathParameters.ours` file is already set to *jellyfish*, which is the command needed to run the Jellyfish program. You shouldn't need to change it.

Also, you shouldn't need to change the parameter `JELLYFISH_HASH_SIZE`. The value that is set for it already will usually work fine. However, if you are using a computer with lots of memory, you may want to change the value to take advantage of that. It can be especially helpful if you are working with k-mer sizes or genome sizes that produce lots more than 25 million k-mers.

7. Install Perl

Perl is a programming language used by IGGPIPE. Using it requires a Perl interpreter application on your computer. The Mac OSX system comes with a Perl interpreter already installed, and this should be sufficient. This version of IGGPIPE was tested with Perl version 5.16.0, although later versions, and earlier V5 versions, will probably be fine. You can find out if you already have Perl installed and what its version is with this command:

```
perl --version
```

If you do not have Perl installed or if the version is older than V5, you must install it, so look for it here:

<https://www.perl.org/get.html>

Explicit installation instructions are not given here. Follow the instructions provided in the downloaded installation package, then re-run the "perl --version" command to verify that it is installed. Sometimes it may be necessary to log out and log back in so that the Perl location can be added to the PATH, before the command will work.

The parameter CMD_PERL in the allPathParameters.ours file is already set to *perl*, which is the command needed to run the Perl program. You shouldn't need to change it.

8. Install R

R is a programming language used by IGGPIPE. Using it requires that the R programming environment be installed on your computer. This version of IGGPIPE was tested with R version 3.2.1, although later versions, and earlier V3 versions, will probably be fine. You can find out if you already have R installed and what its version is with this command, which invokes the command line version of the R interpreter:

```
Rscript --version
```

If you do not have R installed, or have it installed but want to update to a newer version number, look for it here:

<https://www.r-project.org>

Explicit installation instructions for R are not given here. Follow the instructions provided in the downloaded installation package, then re-run the "Rscript --version" command to verify that it is installed.

Windows presented a separate problem. If R is installed under the "Program Files" directory, which is the default for the R installer, an error occurred with the message *Error: could not find function "dir.exists"*. The only way we could find around this problem was to *reinstall R* under a different directory, such as under the *cygwin* directory, or any directory that has no SPACE character in the directory path. If you are working with Windows, install or reinstall R in such a directory, installing the Windows R binary using the regular R Windows installer. For example, we installed into directory C:/cygwin64/home/username/bin/

IGGPIPE does not use any extra R packages.

The parameter CMD_RSCRIPT in the allPathParameters.ours file is already set to *Rscript*, which is the command needed to run the Rscript program. You shouldn't need to change it, UNLESS you are running Windows. For Windows, set CMD_RSCRIPT to the full path to Rscript.exe. The correct path to use can be obtained by changing into directory containing Rscript.exe and entering the commands:

```
readlink -f primer3_core.exe
cygpath -am (output from readlink command above)
```

The *readlink* command produces a path as output, and you put that path as the argument to the *cygpath* command, and it produces a path whose output is the value to assign to CMD_RSCRIPT parameter. The *readlink* command path would probably also work.

9. Install Primer3

Primer3 is a classic bioinformatics application that generates primers from sequence data. It is used by IGGPIPE to generate primers for candidate IGG markers, so it must be installed on your computer. This version of IGGPIPE was tested with Primer3 version 2.3.6, although later versions and earlier V2 versions will probably be fine. You probably know if you already have Primer3 installed. If you don't know that you do, then you should install it. Look for it here:

<http://sourceforge.net/projects/primer3>

It comes pre-built for OSX and Windows but may need to be compiled for Linux. Make sure you download the correct version (primer3, not primer3plus). Put the downloaded directory wherever you want on your computer. The file named primer3_core (primer3_core.exe on Windows) in the root directory of the downloaded package is the executable program file. In Windows it is necessary to change the file to be executable, with this command:

```
chmod u+x primer3_core.exe
```

Now assign the parameter "CMD_PRIMER3CORE" in the allPathParameters.ours file, for example:

```
CMD_PRIMER3CORE := ~/Documents/primer3-2.3.6/primer3_core
```

For Windows, as in the previous step involving Rscript, use *readlink* and *cygpath* to get the paths needed, first changing into the directory where primer3_core.exe is located, then:

```
readlink -f e-PCR.exe
cygpath -am (output from readlink command above)
```

A set of files containing thermodynamic settings is provided with the Primer3 installation, in a subdirectory of the main Primer3 directory named *primer3_config*. The parameter "DIR_PRIMER3CONFIG" in the allPathParameters.ours file, must be set to the full path to this directory. For example:

```
DIR_PRIMER3CONFIG := /Users/tedtoal/src/primer3-2.3.6/primer3_config
```

In Windows, the correct path to use can be obtained by changing into the primer3_config directory and entering the command:

```
cygpath -am
```

10. Install e-PCR

e-PCR is an "electronic PCR" application from NCBI that uses primers and sequence data to do an *in silico* PCR amplification. It is used by IGGPIPE to test primers of candidate IGG markers to see if they generate unique amplicons of the expected length, so it must be installed on your computer. This version of IGGPIPE was tested with e-PCR version 2.3.12-1 (-V option displays version 2.3.12, but downloaded file was 2.3.12-1), although later versions will probably be fine.

To install e-PCR, look for it here:

<http://www.ncbi.nlm.nih.gov/tools/epcr>

The download link uses FTP protocol. Log in as user GUEST with no password. Look for the latest .zip version or tar.gz, copy the file or folder to your computer, and unzip it. Put the unzipped directory wherever you want on your computer.

In some cases, a binary distribution might be available, so once downloaded, you should be able to run e-PCR without further ado. At the time we downloaded version 2.3.12-1, it was only available as source code and it was necessary to run *make* to compile and build the program.

Refer to the file *BUILD.html* in the e-PCR source directory for instructions on compiling the source.

Under Windows, the program was able to be built with this command:

```
make LF64LDFLAGS= LF64CCFLAGS=-DNATIVE_LARGEFILES links depend all OPTIMIZE=6
```

Under Mac OSX, there were compile failures that required editing of the source code in order for the *make* operation to complete successfully. Perhaps these problems will have been fixed in the version you download (or perhaps a binary version will be available at the time you download). Test by trying to build e-PCR. For Mac OSX, the source was compiled by changing into the directory where the files were unzipped and entering the following command:

```
cd e-PCR-2.3.12-1
make LF64LDFLAGS= LF64CCFLAGS=-DNATIVE_LARGEFILES COMMON_CC_FLAGS=-w
```

If the *make* completes without error, there will be a file named "e-PCR" in the directory, and if you run it, it will display a page full of usage info:

```
e-PCR      (Run e-PCR to see if it works)
```

If you get errors from the *make* under OSX like we did, here are the changes we made that allowed the *make* to succeed:

- a. Edit file mmap.cpp and remove `"/"` from the start of the line that reads `"/#include <sstream>"`
- b. Edit file minilcs.hpp and insert the following two lines after the line that reads `"#include <cstring>":`

```
#include <cstdlib>
#include <sstream>
```

Now try the *make* command again, followed by running "e-PCR":

```
make LF64LDFLAGS= LF64CCFLAGS=-DNATIVE_LARGEFILES COMMON_CC_FLAGS=-w
e-PCR      (Run e-PCR)
```

The *make* should succeed and e-PCR should display its usage information, meaning you are good to go.

Now assign the parameter "CMD_EPCR" in the allPathParameters.ours file, for example:

```
CMD_EPCR := ~/Documents/e-PCR-2.3.12-1/e-PCR
```

For Windows, as in the previous step, use *readlink* and *cygpath* to get the paths needed, first changing into the directory where e-PCR.exe is located, then:

```
readlink -f e-PCR.exe
cygpath -am (output from readlink command above)
```

11. Install MUSCLE

MUSCLE is an open-domain multiple sequence aligner. It is used by IGGPIPE only if you choose to search markers or LCRs for Indels and SNPs by using the *make IndelsSNPs* command, so if you don't do that you can skip this step, although you may as well install it. This version of IGGPIPE was tested with MUSCLE version v.8.31, although later versions will probably be fine. To install MUSCLE, look for it here:

<http://www.drive5.com/muscle/downloads.htm>

The executable images are already built, so choose the correct download for your system and download the file, putting it wherever you want on your computer, such as a bin folder.

Now assign the parameter "CMD_ALIGNER" in the allPathParameters.ours file, for example:

```
CMD_ALIGNER := ~/bin/muscle3.8.31_i86darwin64
```

For Windows, as in the previous step, use *readlink* and *cygpath* to get the paths needed, first changing into the directory where muscle3.8.31_i86win32.exe is located, then:

```
readlink -f muscle3.8.31_i86win32.exe
cygpath -am (output from readlink command above)
```

12. Build findMers

findMers is a C++ program that is part of IGGPIPE. It takes as input a file full of k-mers and a genome FASTA file, and produces as output a file of the k-mers with their genomic position included as additional data columns in the file. It can also locate all contigs in the genome FASTA file and output a file that lists the starting position and length of each contig. IGGPIPE uses both of these functions of findMers to generate a list of common unique k-mers to be analyzed for LCRs (locally conserved regions). The findMers program must be compiled and built using *make*. Its source files are located in the code/cpp/findMers directory. Change into that directory and enter the command *make*:

```
cd code/cpp/findMers
make
findMers
cd ../../..
```

The *make* should compile the C++ files in the findMers folder. It should complete without error, and there will be a file named "findMers" in the directory, and when that file is run with the *findMers* command shown above following *make*, it will display a page of usage information. The path to "findMers" is already set correctly in the allPathParameters.ours file.

13. Test trashing and choose deletion method

IGGPIPE uses *make* to run data through its pipeline. A command can be given to cause *make* to delete files that it has generated by running the pipeline. There are two different ways it can delete files: it can actually delete them, or it can move them to a trash folder where they can be found and undeleted if necessary. A script file (code/shell/trash.sh) is provided to move files to the Mac OSX

trash folder, but for Linux or Windows, you must either modify that script file so that it will work with your operating system, or choose the other method that simply deletes files.

You must choose which of these methods you want. Since the trash folder method is more useful and flexible, it is the default method, but again, on Linux or Windows you will need to change it or modify `trash.sh` to work properly.

You select the method by setting the `allPathParameters.ours` parameter `CMD_DELETE_WHEN_CLEANING` to either `$(CMD_DELETE)` or `$(CMD_TRASH)`. You should make sure it is set the way you want. Also, you should test the shell script that moves files to the trash, to make sure it works. To do this, use these commands:

```
cp help.txt junk.txt
$SHELL code/shell/trash.sh junk.txt
```

Now look in the trash can to see if file "junk.txt" is there. If this doesn't work, you should set the `$(CMD_DELETE)` method as the delete method:

```
CMD_DELETE_WHEN_CLEANING := $(CMD_DELETE)
```

14. Copy `primer3settings.default.txt`

Primer3 uses a settings file to control many of the settings it uses to generate primers. Several sample settings files come with Primer3, in its root directory. One of these, **`primer3web_v4_0_0_default_settings.txt`**, was copied and modified for use with IGGPIPE. The file is named **`primer3settings.default.txt`**, in the main IGGPIPE directory. The following required changes were made to it:

- a. `P3_FILE_ID` was set to a descriptive settings title.
- b. `PRIMER_EXPLAIN_FLAG` was changed from 1 to 0.
- c. `PRIMER_PRODUCT_SIZE_RANGE` was set to a simplified 36-300 (primers are designed with most intervening DNA sequence removed)
- d. `PRIMER_NUM_RETURN` was changed from 5 to 1.
- e. `PRIMER_GC_CLAMP` was changed from 0 to 1 (optional but recommended).

You need to copy the default settings file to a new file that can be edited by you, should you want to change Primer3 settings for your needs while keeping a pristine copy in the original `primer3settings.default.txt` file. Copy it to this file name:

```
cp primer3settings.default.txt primer3settings.txt
```

This file copy is all you need to do, IGGPIPE will work with this version, and this is the required version for running the test of IGGPIPE.

The RUN instructions for IGGPIPE indicate that `primer3settings.txt` should be edited if you want to change primer settings for your needs. However, whenever you want to run the test of IGGPIPE as shown below, you should re-do the above copy to use the pristine file for testing.

15. Enable Access to FileMerge (optional and Mac only)

Parameter settings files (`allParameters.*` and `allPathParameters.*`) and Primer3 settings files (`primer3settings.txt`) can be edited by the user. You might at some time wish to see what changes were made to a file by comparing it to another similar file. The *diff* command can be used on the command line to do this. Another program, available on Mac OSX, is *FileMerge*, a great file comparison and merging tool that comes with Xcode. It is initially hidden within Xcode, but you can put it in your dock to make it more easily accessible.

To run FileMerge, start Xcode, then on the menu choose Xcode, Open Developer Tool, FileMerge. When it opens up, find its icon on the dock and set it to stay put in the dock, then you can close Xcode and in the future get to it directly from the dock.

When you run FileMerge, it prompts for two or three or four file names. To see an example of use, enter the first two file names, "left" and "right", setting "left" to `allParameters.template` and "right" to `allParameters.test`, then click "Compare". You will see a comparison of the two files, with the differences clearly shown. If you wanted to incorporate changes from one of these files into the other, you can do this easily by using the up/down arrow keys to go through the differences one by one, and use the left/right arrow keys to select whether you want the left or right side file text in the output, and you can also click in the box on the bottom that shows the merged text and edit it; when finished you can save the merged text to a new file or overwrite one of the two compared files, using File, Save Merge. Since we don't want to merge these files, exit FileMerge without saving anything.

16. Run IGGPIPE using the test parameters in `allParameters.test` and check for success

Everything is now ready to run the IGGPIPE pipeline. Data for testing it is provided in the `testFASTA` folder. This consists of two FASTA files that are truncated versions of the *S. lycopersicum* (tomato) and *S. pennellii* genomes, with only two chromosomes (1 and 2) and only about 14 Mbp for each one. The parameter file `allParameters.test` has parameters set for using these FASTA files and doing the test. It is more-or-less a copy of the `allParameters.template` file, modified for testing IGGPIPE.

To test IGGPIPE, from the command line in the IGGPIPE main directory, enter this command:

```
make PARAMS=allParameters.test ALL | tee logFiles/makeLog.test.txt
```

If all goes well, the pipeline will run quickly, and after four or five minutes, it should finish with the message **ALL files are up to date**.

The *tee* command routes the piped log output from *make* to the console and to the file `logFiles/makeLog.test.txt`. You can examine this file after the run to see what specifically happened at each step, for example with this command:

```
less logFiles/makeLog.test.txt
```

Note that the output includes timestamps telling how long each step took to run.

If the pipeline fails, an error message of some kind is displayed, and *make* stops. (There is a problem with Windows, where sometimes *make* does not stop on an error, but keeps going. We have not found a way around this. If this happens to you, you will need to go back through the output to look for errors.) If an error occurs, proceed to the next step, troubleshooting.

If no error occurs, there should be several files in the output folder "outTestHP11", including files starting with these prefixes and suffixes (shown in the order that they are produced by the pipeline):

- a. LCRs_*.tsv
- b. BadKmers_*.tsv
- c. IndelGroupsOverlapping_*.tsv
- d. IndelGroupsNonoverlapping_*.tsv
- e. NonvalidatedMarkers_*.tsv
- f. MarkerErrors_*.tsv
- g. MarkersOverlapping_*.tsv
- h. MarkersNonoverlapping_*.tsv
- i. MarkerCounts_*.pdf
- j. MarkerDensity_*.png

The MarkersOverlapping_ and MarkersNonoverlapping_ files are the final output files containing the markers.

The .pdf and .png files should be examined to see how they depict marker counts and densities.

The tables at the end of the RUN document describe the columns in these tab-separated data files.

To make sure the pipeline ran correctly, compare the MarkersOverlapping_ file to the expected result, which is in subdirectory goodTest:

```
cmp outTestHP11/MarkersOverlapping_*.tsv goodTest/MarkersOverlapping_*.tsv
```

This command should not produce any output, indicating the two files are identical. If it produces output indicating non-identity of the files, you have a problem, so proceed to the next step, troubleshooting.

17. Troubleshooting

A common problem is with file paths. Pay close attention to error messages at the end before *make* stops. Recheck file paths if messages indicate a file could not be found. Note that with Windows, which uses "\" rather than "/" to separate directories in file paths, we found that we could use "/" in all the paths in the allParameters.ours file and allParameters.test file and it worked fine; we did not have to use "\" anywhere.

In Windows, if you need to know the path that you should place into a CMD_ variable as the full path to a .exe file, use *readlink -f (exe filename)*.

Windows gave the most problems, and the most common problem with Windows was in text file line endings, which under Windows can be either "DOS" or "Unix" line endings. IGGPIPE produces files with Unix line endings exclusively, but it generally tolerates input files with either type of line ending. Most tools and programs you might use to examine the files will also tolerate either type of line ending, but occasionally, a program requires DOS line endings. Be aware of this situation during troubleshooting, and consider whether the observed problem might be one with line endings. You can determine whether a file has DOS line endings with the command:

```
cat -v filename | head
```

If the file has DOS line endings, you will see the character ^M at the end of each line. Otherwise, it has Unix line endings. To convert a file with DOS line endings to one with Unix line endings:

```
tr -d '\r' < filenameDOS > filenameUnix
```

To convert a file with Unix line endings to one with DOS line endings:

```
awk 'sub("$", "\r")' filenameUnix > filenameDOS
```

Another problem can be program versions. If you use an older or newer version of a program than what we used, the pipeline might fail, depending on what the changes are, or it might produce different output. Look carefully at version numbers and check to see if the output differs for any program that has a different version number than what we used.

If IGGPIPE produces a different marker output file than expected, as indicated by output being produced by the *cmp* command shown in the preceding step, you should do difference testing on other output files. Each of the files whose prefixes and suffixes are listed in the previous step have a "good" version of the file containing the expected results, in folder *goodTest*. Each of those files can be compared to the output IGGPIPE produced when you ran it using a *cmp* command to see which ones are good. No output means the files match. A shell script named *cmpKeyFiles.sh* is provided that runs *cmp* on each of these files. To use it with the *allParameters.test* output files:

```
source code/shell/cmpKeyFiles.sh outTestHP11 goodTest
```

It will show only a single line of output for each file, saying it compared the file, if the files match. If they don't match, you will get a lot of output from the mismatches. A single file can be compared with this command, for example to compare the *LCRs_* file:

```
cmp outTestHP11/LCRs_*.tsv goodTest/LCRs_*.tsv
```

If the final output file does not match, but one or more output files do match (starting with the first file listed in the previous step), then you can tell which step produced an incorrect result based on which file in the list is the first one that is incorrect. The following *make* steps produce the following output files (*italicized output files are those available in the goodTest subdirectory for comparison to your files*):

<i>make</i> command or other command	Produces output file(s)
a. <i>make</i> PARAMS=myFilename getSeqInfo	GenomeData/*.idlens
b. <i>make</i> PARAMS=myFilename getContigFile	GenomeData/*.contigs
c. <i>make</i> PARAMS=myFilename getKmers	Kmers/Kmers_*.kmers

<i>make</i> command or other command	Produces output file(s)
d. make PARAMS=myFilename kmerStats	Kmers/Kmers_*.stats
e. make PARAMS=myFilename kmersToText	Kmers/Kmers_*.kmers.txt
f. make PARAMS=myFilename getGenomicPosIsect	Kmers/Kmers_*.isect
g. make PARAMS=myFilename mergeKmers	Kmers/Kmers_*.merge
h. make PARAMS=myFilename sortCommonUniqueKmers	Kmers/common.unique.kmers
i. make PARAMS=myFilename findLCRs	<i>LCRs_*.tsv, BadKmers_*.tsv</i>
j. make PARAMS=myFilename findIndelGroups	<i>IndelGroupsOverlapping_*.tsv,</i> <i>IndelGroupsNonoverlapping_*.tsv</i>
k. make PARAMS=myFilename getDNAseqsForPrimers	<i>IndelGroupsOverlapping_*.dnaseqs</i>
l. make PARAMS=myFilename findPrimers	<i>NonvalidatedMarkers_*.tsv</i>
m. make PARAMS=myFilename ePCRtesting	<i>MarkerErrors_*.tsv</i>
n. make PARAMS=myFilename removeBadMarkers	<i>MarkersOverlapping_*.tsv,</i> <i>MarkersNonoverlapping_*.tsv</i>
o. make PARAMS=myFilename plotMarkers	<i>MarkerCounts_*.pdf, MarkerDensity_*.png</i>
p. make PARAMS=myFilename getDNAseqsForIndelsSNPs	<i>*.withseqs.tsv</i>
q. make PARAMS=myFilename IndelsSNPs	<i>*.indels.tsv, *.snps.tsv</i>
r. make PARAMS=myFilename plotIndels	<i>*.indels.pdf</i>
s. Rscript code/R/dotplot.R dotplot.template	<i>LCRs_*.dotplot.png</i>
t. Rscript code/R/annotateFile.R annotate.template	<i>MarkersAnnotated_*.tsv</i>
u. Rscript code/R/annotateFile.R annotate/ HP11_isInNearColumn.markers	<i>MarkersAnnotated_WithInNearFeatures_*.indels.tsv</i>
v. Rscript code/R/annotateFile.R annotate/ HP11_to_gff3.markers	<i>MarkersAnnotated_GFF3_*.gff3</i>

(Note that some of the files listed above are produced by steps to be described below).

Different results between the goodTest directory files and your outTestHP11 directory files might be due to running a software package of a different version than what we used for testing. For example, a different version of e-PCR might cause a mismatch starting at file *MarkerErrors_*.tsv*. Another possibility to be aware of is that the files may in fact be identical except one might have Unix line ends and the other might have DOS line ends.

18. Run *make IndelsSNPs* to align markers and find Indels and SNPs

Pipeline software is also provided to read a file of LCRs, non-overlapping Indel Groups, or non-overlapping Markers, extract the DNA sequences from the genomes in each LCR or Marker region

and align them, then locate all Indels and SNPs in the aligned sequences and write their positions to files. This part of the pipeline is NOT run when the *make ... ALL* target is built. Run it as follows:

```
make PARAMS=allParameters.XY getDNaseqsForIndelsSNPs      (replacing with your allParameters name)
make PARAMS=allParameters.XY IndelsSNPs                  (replacing with your allParameters name)
```

This produces three files in your output folder whose names end with "withseqs.tsv", "indels.tsv" and ".snps.tsv", the latter two containing tables of all Indels and SNPs found in the marker regions.

Check that the output files exist with:

```
ls outTestHP11/NonoverlappingMarkers*.indels.tsv
ls outTestHP11/NonoverlappingMarkers*.snps.tsv
```

This should list the files *outTestHP11/*

MarkersNonoverlapping_K11k2L100D10_2000A100_2000d10_100N2F0X20V3000W8M3G1.indels.tsv
and *outTestHP11/*

MarkersNonoverlapping_K11k2L100D10_2000A100_2000d10_100N2F0X20V3000W8M3G1.snps.tsv

You can examine them with Excel or a text editor to see the Indel and SNP data they contain.

19. Run *make plotIndels* to plot Indel information

Another R program that is NOT run as part of the pipeline when the *make ... ALL* target is built, but which can be run using *make ... plotIndels*, reads the Indels file produced by *make ... IndelsSNPs* and plots information from it in a pdf file. The program is called *plotIndels.R*. Run it as follows:

```
make PARAMS=allParameters.test plotIndels
```

Check that the output file exists with:

```
ls outTestHP11/Markers*.indels.pdf
```

This should list the file *outTestHP11/*

MarkersNonoverlapping_K11k2L100D10_2000A100_2000d10_100N2F0X20V3000W8M3G1.indels.pdf

You might want to open it and look at the plots.

20. Run *dotplot.R* to make a dot plot

The *LCRs_* file contains a list of common unique k-mers assigned to locally conserved regions (LCRs), and it can be used to make a dotplot depicting alignment of the two genomes. The R program *dotplot.R* is provided to do this. It is driven by a parameter file, a sample of which has been provided, *dotplot.template*, that is set for using the test data produced by running IGGPIPE with *allParameters.test*. Run *dotplot.R* as follows:

```
Rscript code/R/dotplot.R dotplot.template
```

Check that the output file exists with:

```
ls outTestHP11/LCRs_*.dotplot.png
```

This should list the file *outTestHP11/LCRs_K11k2L100D10_2000.dotplot.png*, an image file. You may want to examine it (e.g. in the OSX Preview app) to see the dot plot.

There are other sample parameter files in subdirectory *dotplot*, although the parameter file is fairly straightforward and you probably don't need other examples to work from.

21. Run `annotateFile.R` to make new files containing annotated marker data in different formats

A common need is to add additional annotation information the table of markers. For example, you might be working with an introgression line population and wish to annotate each marker with the names of the lines whose introgressions that marker lies within, along with the marker position relative to the introgression. Or, you might want to annotate each marker with the ID of the nearest gene and its distance away. You may also want to change file format, from .tsv (tab-separated) to .gff3 or .gtf for adding the markers to a browser track. All this can be done with the R program `annotateFile.R` that is provided with IGGPIPE. It is driven by a parameter file, a sample of which has been provided, `annotate.template`, that is set for using the test data produced by running IGGPIPE with `allParameters.test`, along with additional annotation test data in folder `code/R/test_GFFfuncsAndMergeData`. Run `annotateFile.R` as follows:

```
Rscript code/R/annotateFile.R annotate.template
```

Check that the output file exists with:

```
ls outTestHP11/MarkersAnnotated*
```

This should list file

MarkersAnnotated_K11k2L100D10_2000A100_2000d10_100N2F0X20V3000W8M3G1.tsv

in the `outTestHP11` folder. You can examine this file with a text editor

or Excel to see the new column that was added compared to the input file

MarkersOverlapping_K11k2L100D10_2000A100_2000d10_100N2F0X20V3000W8M3G1.tsv.

There are other sample parameter files in subdirectory *annotate* which produce other types of files or do other types of file data manipulation. The parameters can be challenging to set properly, especially when merging data from a separate file, so these sample files can be helpful. Also, when .gff3 files are used, they must conform well to the expected GFF3 format else an error is likely to occur.

22. Edit `primer3settings.txt` (optional)

After finishing installation, and prior to any run of IGGPIPE, you may want to edit `primer3settings.txt` file and make any changes that are important for your needs. For example, you might change the parameters that determine the acceptable *range of primer Tm values*. If you have several different setting values you use, you will probably want to keep a directory of different `primer3settings.txt` files and copy the needed one prior to each run of IGGPIPE.

The Primer3 user manual (http://primer3.sourceforge.net/primer3_manual.htm) describes all the parameters.

An explanation of the sequence data IGGPIPE gives Primer3 in order to generate primers will be helpful, particularly in understanding the setting of the parameter `PRIMER_PRODUCT_SIZE_RANGE`. Since IGGPIPE is making primers to be used in different genomes with different sequences and sequence lengths between the two primer sites, it cannot use the typical method of giving Primer3 the entire sequence between the two primer sites. Instead, IGGPIPE gives Primer3 the concatenation of two short sequences,

one around each of the two k-mers that define and anchor the candidate IGG marker. Each sequence is equal to K plus twice `EXTENSION_LEN` in length. Both K (the k-mer length) and `EXTENSION_LEN` (the number of bases to add on each side of the k-mer) are defined in `allParameters.template`. Thus, the sequence that Primer3 uses for designing the primers is equal to $2K + 4 * \text{EXTENSION_LEN}$ in length. IGGPIPE also gives Primer3 a value for its parameter `SEQUENCE_PRIMER_PAIR_OK_REGION_LIST`. This tells Primer3 to design one primer in the left half of the sequence and one primer in the right half. Thus, the primer product size will appear to Primer3 to be much smaller than the actual amplicon size will be, which is why `PRIMER_PRODUCT_SIZE_RANGE` can be set to a smaller value than the amplicon sizes.

Although Primer3 is a stable program and unlikely to change a lot, if new versions of Primer3 add parameters, you might want to incorporate them into `primer3settings.txt`. You will see new parameters if you compare `primer3settings.txt` to Primer3's file `primer3web_v4_0_0_default_settings.txt` (for example by using *diff* or *FileMerge*).

That completes the installation of IGGPIPE.

5. To run IGGPIPE to generate markers

- Find file `RUN.pdf` or `RUN.html` in the IGGPIPE folder on your computer and open either one and follow the instructions.

5.1. For problems and help:

- Post an issue on GitHub under BradyLab/IGGPIPE repository
- Contact me, Ted Toal, twtoal@ucdavis.edu [<mailto:twtoal@ucdavis.edu>]