PrimerPipeline

Instruction Manual v1.0

Hannah Macdonald & Greg Macdonald

14/07/2015
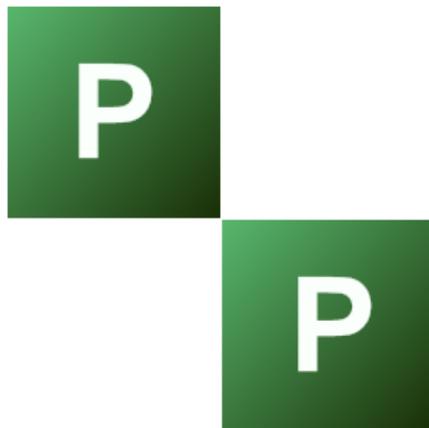
Contents

Table of Figures

1.0 Getting started

1.1 Operating System: Windows 7 or later

No installation required. All you need to do is download the **PrimerPipeline.zip** folder (available via the website here) and *Extract all* the contents together to a folder anywhere on your computer. It is not recommended to do this in the root of your C drive, or in Program files, as this will require you to run **PrimerPipeline** as an administrator in order for it to write files.

If required, make a shortcut to **PrimerPipeline.exe**.

Depending on your computers security settings, the first time you run **PrimerPipeline** you might have to click *Run Anyway*, because it won't be recognised.

1.2 Operating System: Windows XP

If you are using Windows XP, you can still use **PrimerPipeline** as above, but in addition you have to ensure you have 'Microsoft .NET Framework 4.0' installed.

To find out whether you have it already, go to:

Start > Control Panel > Add/Remove Programs

And look for 'Microsoft .NET Framework 4.0'. If you do not have it, you can download it from Microsoft here.

1.3 Contents of PrimerPipeline.zip

Contains the following 5 items:

- primer3_config (Folder containing setting files used by Primer3)
- primer3_core.exe (This is Primer3, **PrimerPipeline** runs this program in the background during the Primer3 stage of the process)
- **PrimerPipeline.exe** (This is the **PrimerPipeline** program itself)
- **PrimerPipeline**_Instruction_manul_v1.0.pdf (The document you're reading)
- example.fasta (An example fasta file of sequence data)

The first three files all need to be in the same folder for **PrimerPipeline** to work. The manual and the example file can be moved or removed if required.

> **Tip:** The example.fasta file can be used for a practise run. The file contains 50,000 sequences, and under default settings **PrimerPipeline** finds 59 microsatellites, and designed primers around 8 of them correctly. This takes roughly 30 seconds (depending on your computer specifications).

2.0 Using PrimerPipeline

2.1 Input File

> **Tip:** We recommend that the input file has already gone through Quality Control (to ensure ambiguous bases and errors are removed, which program you use to do this depends on what NGS platform you used, e.g. Musket removes common Illumina errors, available here). Ideally, also perform a *de novo* assembly (for example, with SOAPdenovo, available here). This assures that your sequences are good quality so the results can be trusted, and preforming a *de novo* assembly assemblies your sequences into larger contigs to aid microsatellite mining; the larger the sequence length, the higher the chance that primers will be able to be designed around microsatellite's (SSR's) found.

Format: Fasta file

Location: The file can be anywhere on your computer, but be aware that the output files from **PrimerPipeline** (see section 4) will be placed in same location as the input file.

> **Note 1: Make sure output files are not overwritten by mistake**
>
> If you repeat the pipeline with the same input file, make sure you either rename the input file so that the output files have different names (see section 4) or make a new folder to avoid accidentally overwriting previous files.

Example of fasta file format:

```
>2080906_length_274_cvg_7.0_tip_0
ATTAGGTTTCAAGAAATAGTCTTCGCACGCACACAATGATATTCTATTCTTGTCACGCAAATGCCTT
GTGGAATGCCTTTAGACCCCTGTCAAAGTTTTCTTTTTGGTTTTGCTAATACAAAATATTTGAAAAT
GCCCACAAAAATAACACTGGGGCACAGTATTAAAATATAATGGAGTCTTCGGAAGCAAGTTTTTTTT
TTTATTAAAATGATACTCCAAAGGCAGAAAACAAAGAAAATATAGCACACAAATGTTTAAAAATACA
ATTTGA

>2080908_length_274_cvg_10.0_tip_0
ATTCAATCATGCAGCAAAGTGGGAGAAGAAGCAGCCGCCTCCAGGAATACATTAGACACGCACCGTT
GCGTCTCGTGCGTGTTGCAAACTTTTCCACTCCTCTTTTCCATTCCACGTCAAAAGTATTAAATTTT
GTATTCGGAAAGTGGCAAAAATTTAAATTTAATCCCATTTATCCTTTTGTAGTTGGGCCCCCAGTGT
GCGCATTGGACGTATGGAAATGTAAAGTAGCGTCAATGTAGCATGAAAACAAGCACTTGAGCCGAAC
ACTTGC
```

See example.fasta file.

2.2 Open Input File in PrimerPipeline

- Open **PrimerPipeline.exe** by double clicking (Figure 1).
- Click the *Open files* button and find the file containing the sequences you wish to run **PrimerPipeline** on. Or drag and drop the file into the box.
- The status should now say 'Ready to process' (Figure 2).
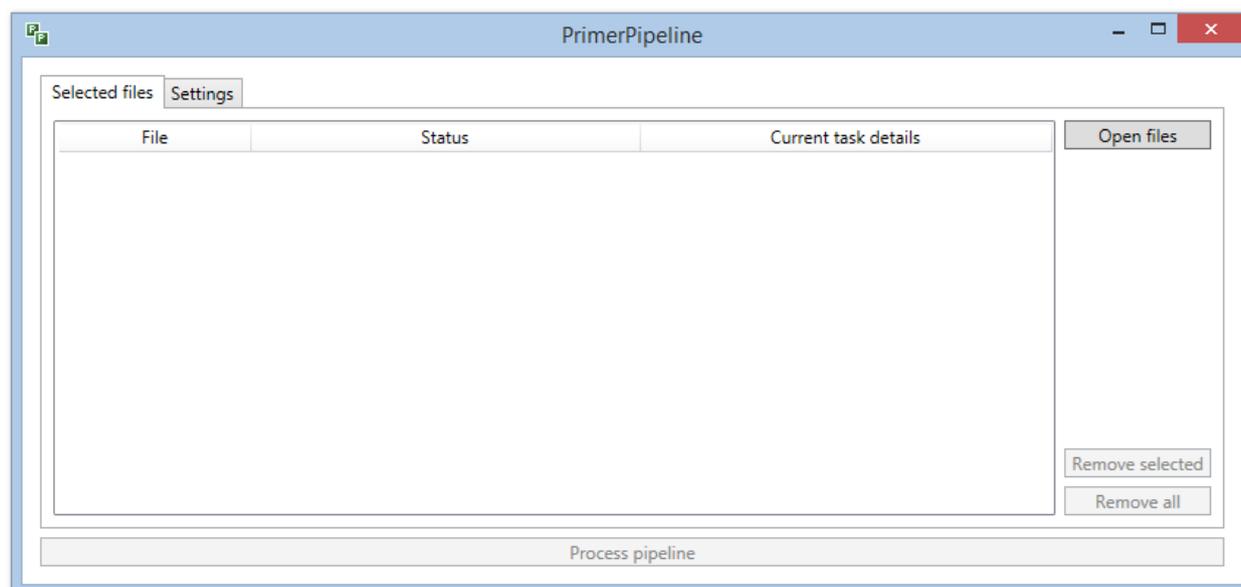
Figure 1. How **PrimerPipeline.exe** looks when opened.
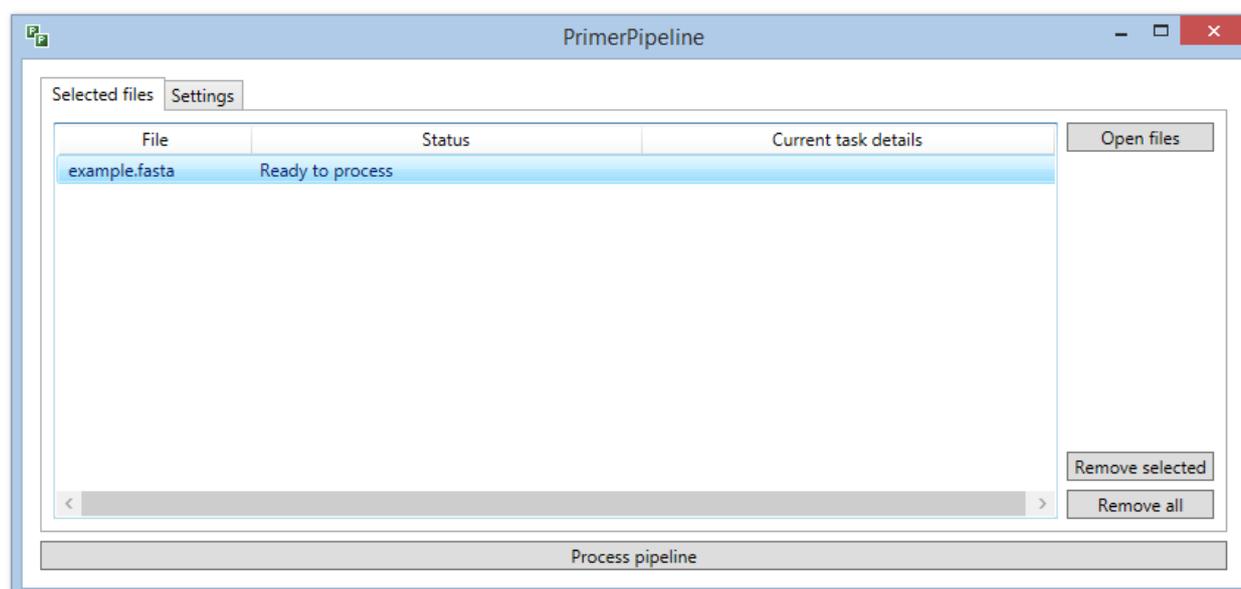
Figure 2. **PrimerPipeline** with input file loaded.

3.0 Editing Settings

Select the file you wish to run, (it will be highlighted in blue as in Figure 2) and then go to the *Settings* tab (Figure 3).

Note 2: PrimerPipeline remembers previous settings.

The first time you open **PrimerPipeline** it will load the default settings for each stage. After the first time, **PrimerPipeline** will load the settings used in the previous run.

It does this by saving a copy of the three settings files (Trim.Settings.txt, misa.ini, and primer3_v1_1_4_default_settings.txt) in the same location as '**PrimerPipeline.exe**' and replaces these files after each run). If you want to return to default settings use the *Reset to default* button in each of the three setting tabs. Also **PrimerPipeline** will return to default settings if these setting files are removed or deleted.

3.1 Sequence Trimming

- Within the *Settings* tab select *Sequence trimming* on the left hand side (Figure 3).
- To edit settings click *Edit argument*, a small window will open where you can change any of the settings (Figure 4).
- If you want to add anything to the trimming stage you can add arguments by clicking *Add argument*.
- Click *Reset to default* to return to default settings at any point. Default Settings are shown in Figure 3.
- Settings can be saved without running **PrimerPipeline** using *Save settings* and load previous settings files using *Load settings*.

Tip: **PrimerPipeline** saves settings files automatically if you run the pipeline, use *Save settings* in any of the three different settings windows (Sequence Trimming, MISA and Primer3) when you're not planning to run **PrimerPipeline** but want so save settings for later or to send to a colleague. The ability to load settings files for all sections makes it easier to: 1) repeat what a colleague has done; 2) make your runs 100% repeatable, and 3) allows you to hone your settings gradually. For example, load a settings file from a previous run and change one thing, this will allow you to see the effect of the change in your results, particularly important in the Primer3 settings stage.

Note 3: Further information on Trimming Settings

PrimerPipeline assumes Quality Control (QC) has already been performed however has some QC during this step.

1. 'Ambiguous trim. Number of bases =2, Window size = 200.'

Searches and removes sequences with more than 2 N's (unknown bases) in a row, it searches 200 base pairs (bp) at a time. You can edit the number of N's allowed and the window size. Increasing these numbers makes the trimming more relaxed, decreasing makes it more strict.

2. 'Remove stretches of Type A from 5' end. Minimum accepted repeat = 5, window size = 200.'

3. 'Remove stretches of Type A from 3' end. Minimum accepted repeat = 5, window size = 200.'

An artefact of some sequencing (usually older types) is stretches of one bp repeated (in default settings this is A) at the beginning (5') and end (3') of the sequence. You can edit the number of accepted repeats and the window length, again, increasing these numbers makes the trimming more relaxed, and decreasing makes it stricter.

You can add Arguments of Type G, C and T, to search for repeats of these bases, whether this is necessary depends on the type of sequencing used to acquire the data, and on the QC performed prior to using **PrimerPipeline**.

4. Cut off. Minimum value = 500, maximum sequence size = 50700.

The minimum and maximum sizes (in bp) of sequences going onto the next step (mining for microsatellites). This should be adjusted according to your average sequence length of your data. Only use default if a large proportion of your sequences are over 500bp, otherwise you could be cutting out a significant percentage of your data. If unsure reduce minimum to 200.
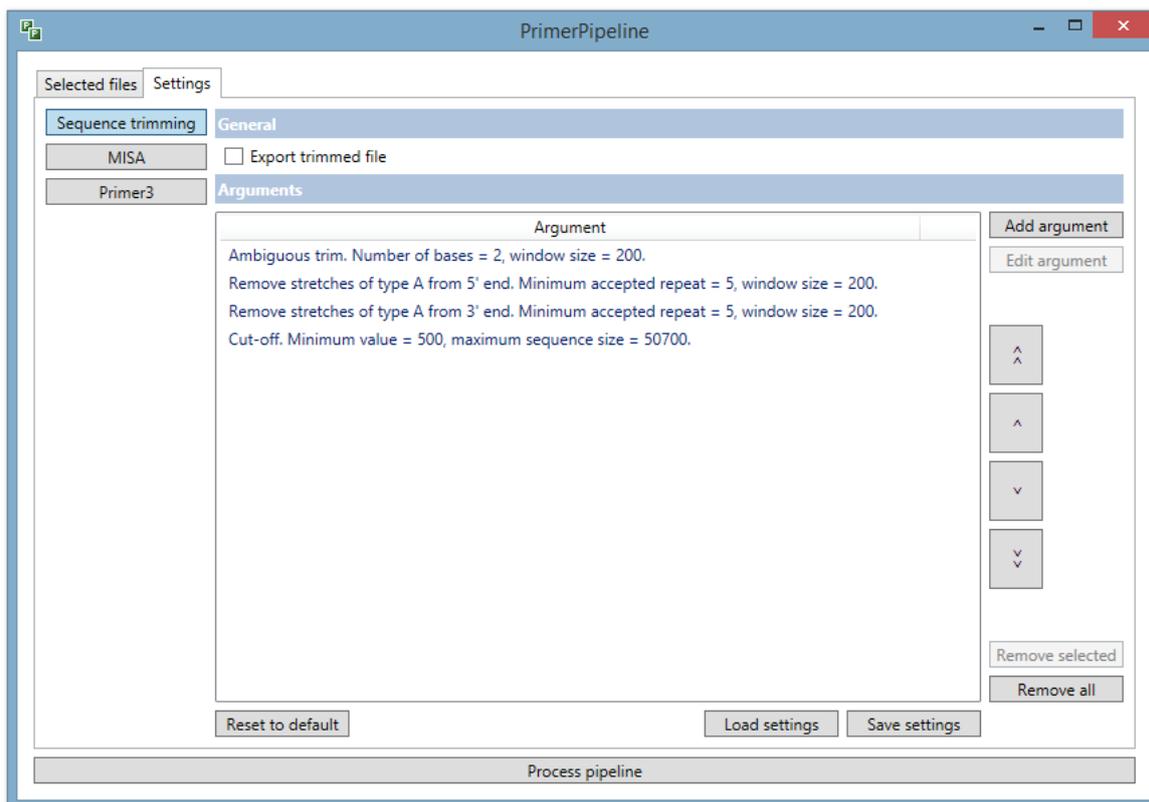
Figure 3. Settings Tab showing *Sequence trimming* selected.
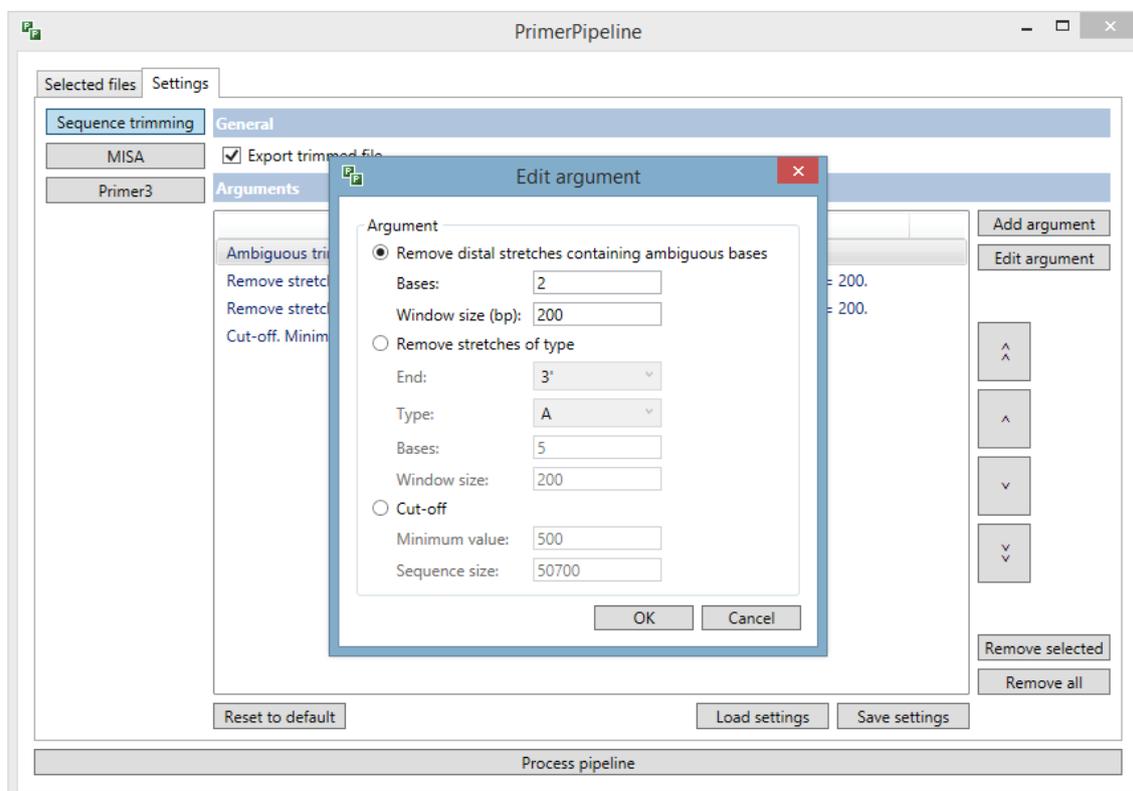
Figure 4. Sequence trimming *Settings* tab, with *Edit argument* clicked so you can edit all trimming parameters.

3.2 MISA

- Within the *Settings* tab, Select *MISA* on the left hand side (Figure 5).
- Can edit 'Interruptions' and SSR minimum repeats. Select one 'Unit size' at a time and then click *Edit definition* to change the minimum repeats.
- Press the *Reset to default* to return to default settings at any point. Default settings are shown in Figure 5.
- Again, settings can be saved using *Save settings* and settings files loaded using *Load settings*.

Note 4: Further information on MISA Settings

1. 'Interruptions: 0'

This value means the maximum difference between two SSRs allowed, i.e. the number of base pairs between SSRs. Default value 0 will allow no interruptions within a microsatellite, however if you change this setting, MISA will then find compound microsatellites e.g. Interruptions: 1 = $(AAG)_7A(AAG)_{24}$.

2. 'Unit Size – Minimum repeats'

'Unit Size' refers to the SSR/microsatellite type, e.g Unit size 2 = $(CA)_{23}$, dinucleotide.

Unit size 3 = $(TGA)_{12}$ trinucleotide ect.

The minimum repeats refers to the length of the microsatellite, for example, 'Unit size 2, minimum repeats 20' means that MISA will only search for dinucleotides that are repeated 20x or more, like $(CA)_{23}$.
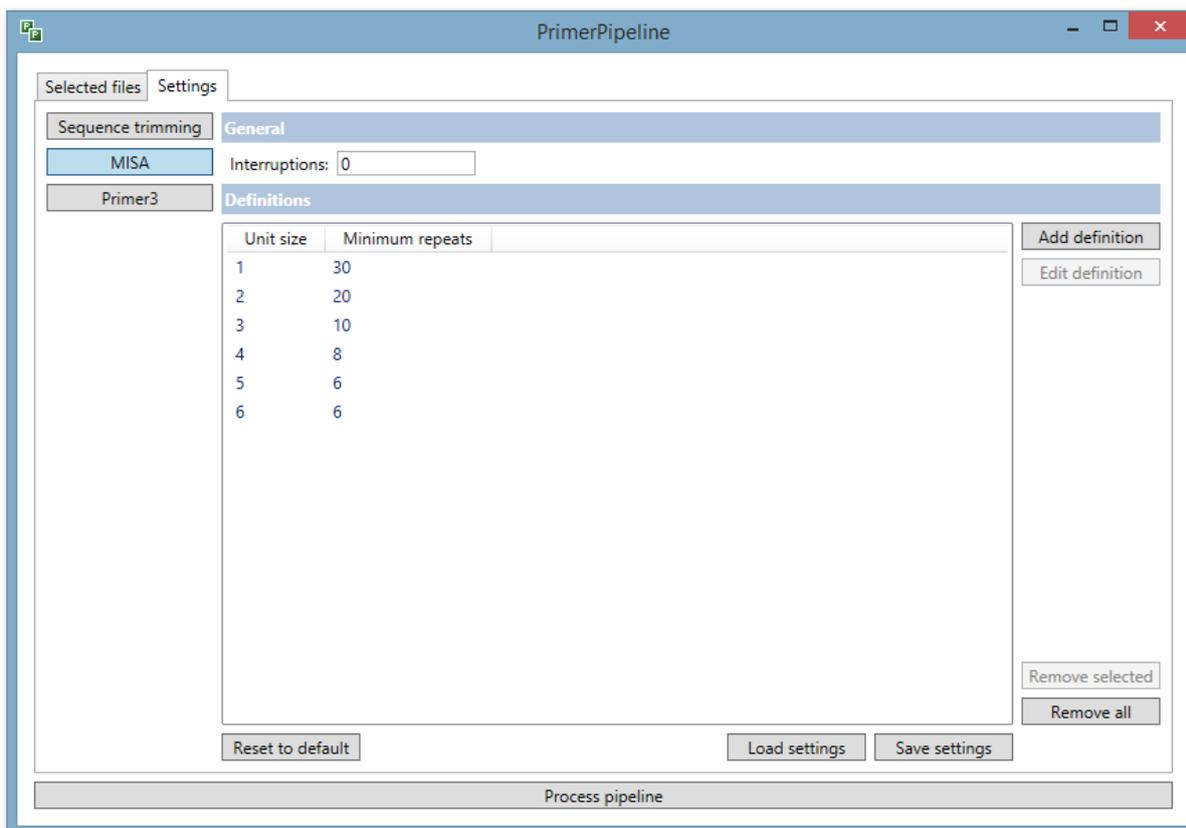
Figure 5. *Settings* tab, with *MISA* selected, allowing you to edit MISA settings.
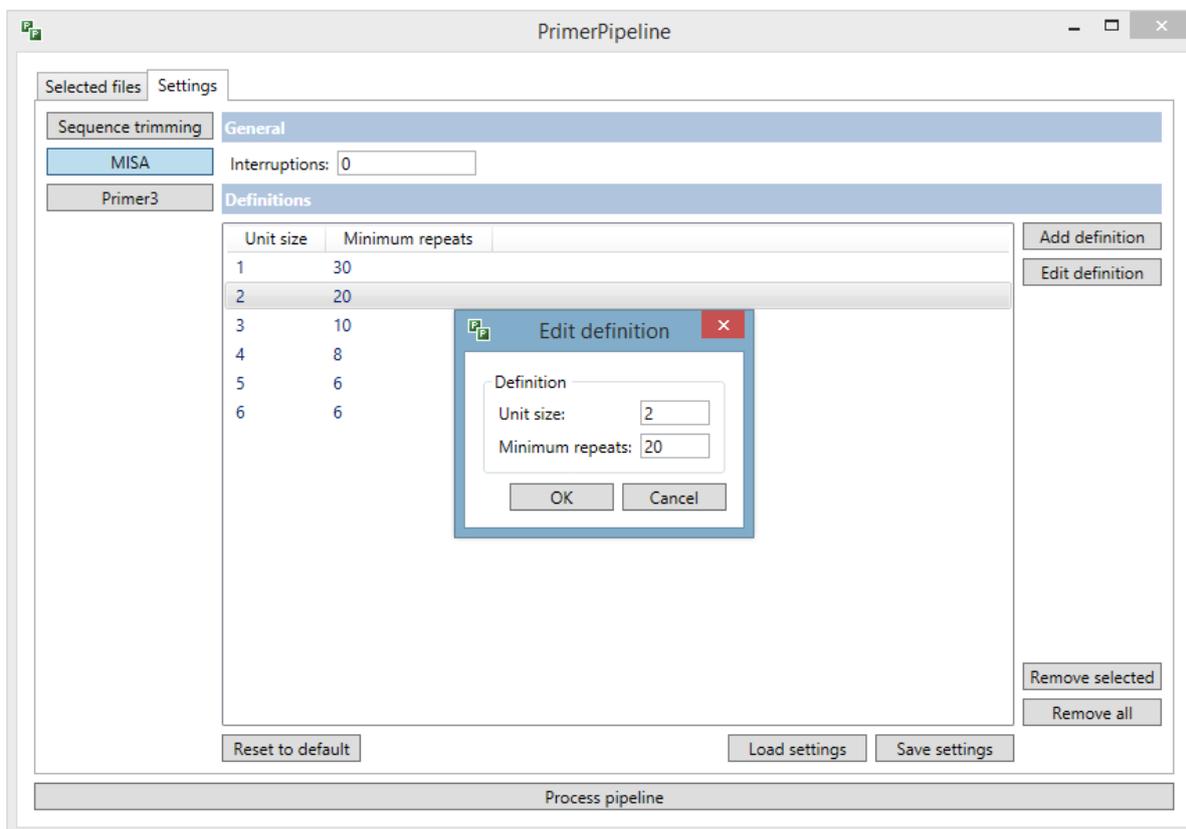
Figure 6. MISA settings tab, with *Edit definition* clicked so you can edit that unit size minimum repeats.

3.3 Primer3

- Within the *Settings* tab, Select *Primer3* on the left hand side (Figure 7)
- Can edit any of the Primer3 settings directly on this tab
- Press the *Reset to default* to return to default settings at any point, some of which are shown in Figure 7.
- If you wish to see the advanced settings Tick the box at the bottom 'Include advanced settings' and the advanced settings will be added to the list in grey (Figure 8).
- Again, settings can be saved using *Save settings* and settings files loaded using *Load settings*.
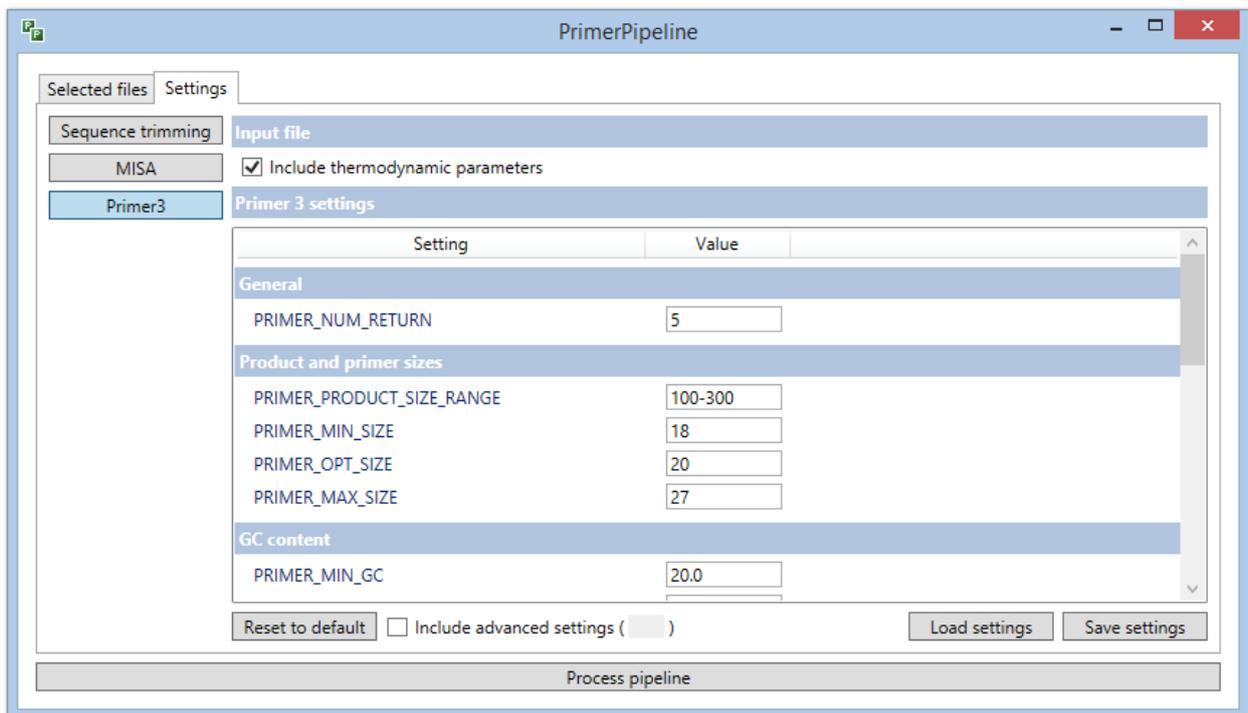
Figure 7. *Settings* tab, with *Primer3* selected, allowing you to edit Primer3 settings (without advanced settings displayed).
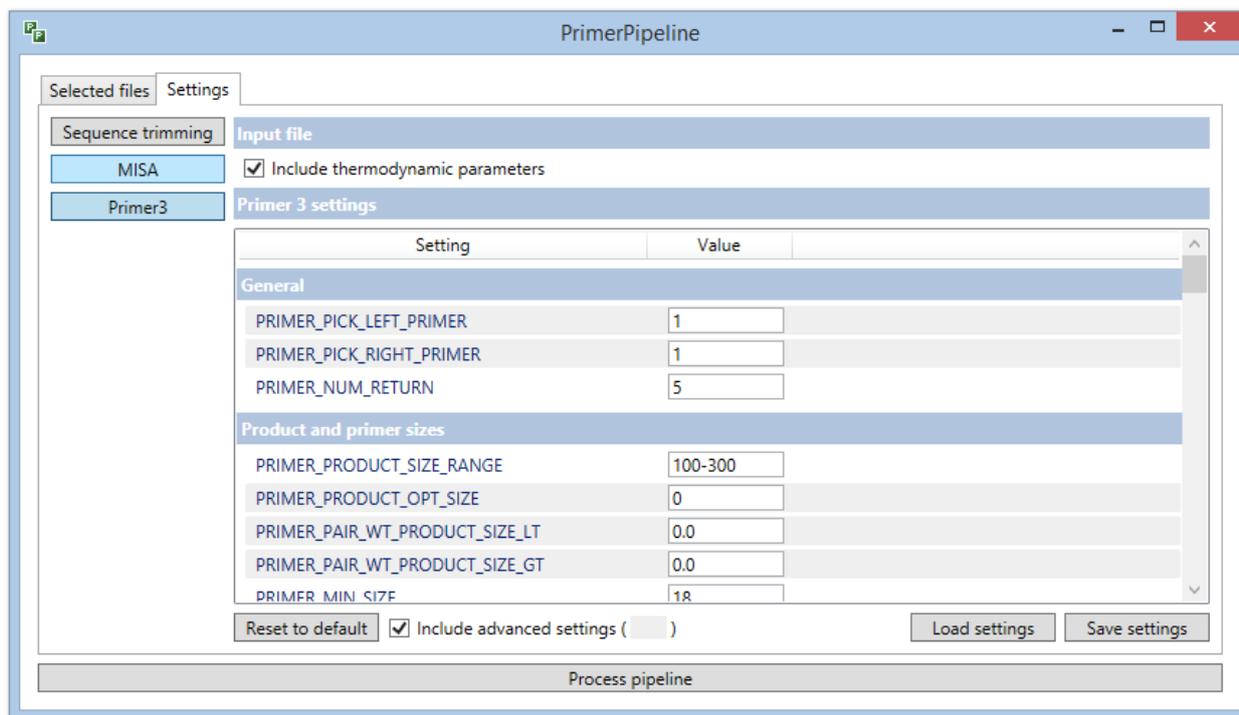
Figure 8. Settings tab, with Primer3 selected, allowing you to edit Primer3 settings (with advanced settings displayed).

Note 5: Further information on Primer3 Settings

All setting names in **PrimerPipeline** have been kept consistent with Primer3 so you can refer to Primer3's website here for more information.

There are too many settings in Primer3 to explain them all fully in this manual, however, for all settings explanations are available within **PrimerPipeline**. If you hover over the setting name a tip box will appear with a short description of the setting, however some are too long to present fully in this way, so if you read 'See Primer3 website for more details', then refer to the Primer3 website for a longer explanation.

For simple navigation of Primer3 settings, they have been gathered into seven groups:

'General'
'Product and primer sizes'
'GC content'
'Melting temperature'
'PCR conditions' (advanced only)
'Self-binding (primer-dimer and hairpins)'
'PolyX and other'

'Include thermodynamic parameters' tick box at the top. This tick box specifies the path to the directory that contains all the parameter files used by the thermodynamic approach ('PRIMER_THERMODYNAMIC_PARAMETERS_PATH' in Primer3). If this is unticked, Primer3 will not be able to locate the files it needs.

'Include advanced settings' tick box at the bottom. In order to make PrimerPipeline even more user-friendly, many of the Primer3 settings that will not be needed by the average user have been placed in the advanced settings. However, if you wish to change more settings, click 'advanced' at the bottom for the full functionality of Primer3.

Note. In the interest of user friendliness, and to focus on primer design on a large scale, specifically for microsatellites some Primer3 settings have been hidden from the user. This includes the following: the option to pick 'hybridization probe (internal oligo)' and associated settings; 'Junction List' overlap settings; 'Mispriming library' settings and 'Sequence quality' settings.

Tip: There is a lot of advice online about designing primers which you can read; however here are some of my suggestions of settings to change to design better quality primers:

1. 'PRIMER_MIN_SIZE', 'PRIMER_OPT_SIZE' and 'PRIMER_MAX_SIZE'

   I advise primers to be between 18-30 bases, optimum 20, this will make sure the primers are specific and increase amplification success.

2. 'PRIMER_MIN_GC', 'PRIMER_OPT_GC_PERCENT' and 'PRIMER_MAX_GC'

   I recommend that the primer GC% be between 40-60%, with the optimum 50%. This is due to fact that there are three hydrogen bonds between G and C, whereas there is only two between A and T. This means the bonds between G and C are stronger, and a good percentage of C's and G's across your primer will ensure the primers will bind affectively and increase amplification success.

3. 'PRIMER_GC_CLAMP'

   For the same reason as above, it is good to have a GC clamp at the end of your primer, meaning the primer ends in a G or C (3' end). Default is 0, meaning not all primers will have one, you can change this to 1 if you want a GC clamp.

4. 'PRIMER_PAIR_MAX_DIFF_TM'

   This is the maximum acceptable difference between the melting temperatures of the left and right primers. Default is 100, however for PCR it is better that the annealing temperature of your primers to be similar. I advise you to change this to 2.

5. 'PRIMER_MAX_POLY_X'

   This is the maximum allowable length of a mononucleotide repeat within a primer, for example AAAAAA. I advise a maximum repeat of 3. This reduces the chance of slippage occurring during annealing step of the PCR reaction.

Other things you could consider changing are the annealing temperatures ('PRIMER_MIN_TM', 'PRIMER_OPT_TM' and 'PRIMER_MAX_TM') and product size range ('PRIMER_PRODUCT_SIZE_RANGE').

In the end, how picky you can be depends on how many primers you have to choose from. We advise running the Primer3 stage of the process more than once to get the optimum number and quality of primer pairs.

4.0 Output Files from PrimerPipeline

During each run, **PrimerPipeline** creates nine files. These files will be placed in the same location as the original input file.

Files are named after the original input file, for example, when using the input file name example.fasta the files will be named as follows:

4.1 Trimming

1. example_TrimSettings.txt (The trimming settings file so that for every run of **PrimerPipeline** there is a record of what settings were used (a duplicate TrimSettings.txt settings file is created in the same folder as **PrimerPipeline.exe**))

4.2 MISA

2. example_misa.ini (MISA settings file. PrimerPipeline will read and write your MISA settings to this file, (a duplicate misa.ini settings file is created in the same folder as **PrimerPipeline.exe**))
3. example.misa (The output file from MISA, containing details of the microsatellites found)
4. example.misa_statistics (The MISA statistics file containing details of the MISA step, including how many files were examined)
5. example.p3in (Input file for Primer3)

Note 6: Running Primer3 only

Drag the Primer3 input file, example.p3in, into **PrimerPipeline** if you want to re-run the Primer3 stage only, **PrimerPipeline** will then ask for the example.misa file, direct it to this file, and then you can adjust primer3 settings and click *Process Pipeline* to re-run Primer3 and results stage. Remember Note 1.

4.3 Primer3

6. example.p3out (Primer3 output file, containing details of all Primers designed)
7. example.Primer3_ErrorLog.txt (Primer3 error log file)
8. example.Primer3_Settings.txt (Primer3 Settings file, (a duplicate Primer3_Settings.txt settings file is created in the same folder as **PrimerPipeline.exe**))

4.4 Results

9. example.FinalResults.csv (This is the Results file that can be opened in **PrimerPipeline** and also excel)

5.0 Running the Pipeline

- When happy with the settings for all three stages, return to the *Selected Files* tab (Figure 2)
- Select your file so that *Process pipeline* button becomes enabled (blue), then click *Process pipeline* button to start.
- The pipeline will now run until results are ready.
- In the 'Status' column you will always be able to check the progress of the pipeline, it will state which step (of 3) it is on and the percentage complete.

Note 7: Move to next task/ cancel process

At any point during the process you can 'Move to next task' or 'Cancel process'. To do this right click on the running process and the options will pop up like in Figure 9.

If you click 'Move to next task' during 'Step 1/3 – Searching for microsatellites' **PrimerPipeline** will continue with 'Step 2/3 – Running Primer3' will the microsatellites already found.

If you click 'Move to next task' during 'Step 2/3 – Running Primer3' **PrimerPipeline** make the results file with the primers designed so far.

This may be useful if you are short of time, or if your input file is very large.
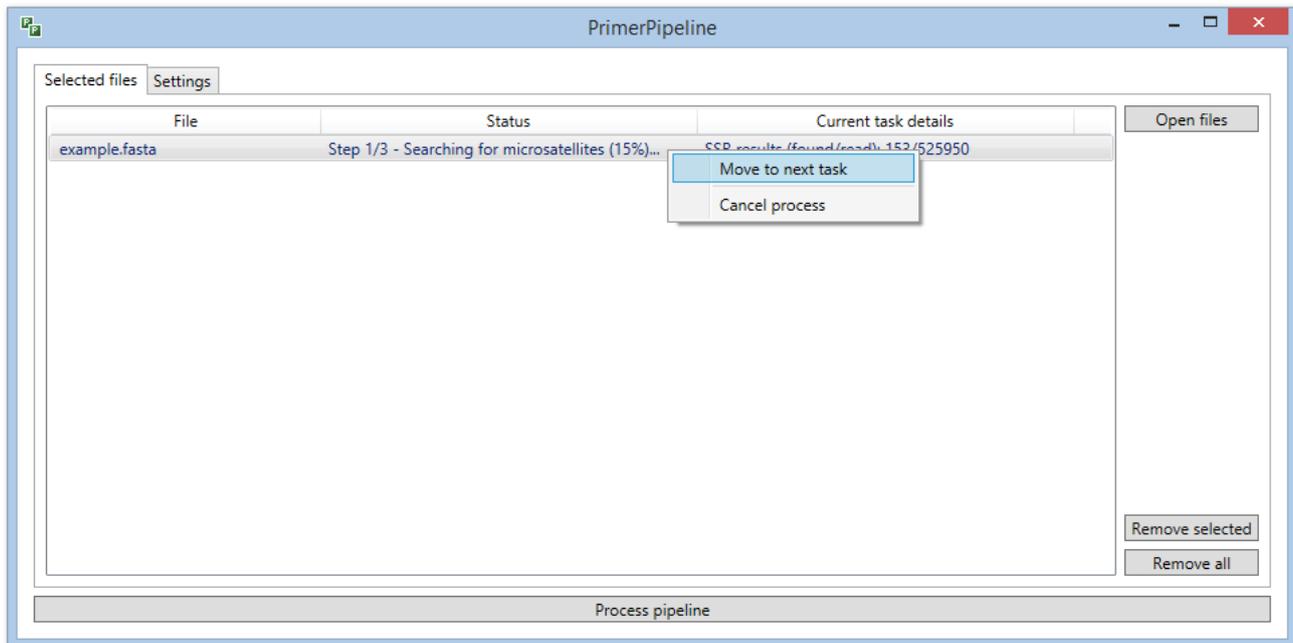
Figure 9. While **PrimerPipeline** is running you can 'Move to next task' or 'Cancel process' at any time.

5.1 Step 1.

This step incorporates Sequence trimming and MISA (finding microsatellites), and when complete makes a Primer3 input file for the next step.

- Within the 'Current task details' column the 'SSR results (found/read)' are shown and updated every 50 sequences, therefore is constantly updating (Figure 10).
- Within the 'Status' column the percentage complete compares the amount of sequences read to the total number of sequences in the input file (Figure 10).

Note 8: Current task details for Step 1

In the 'Current task details' column 'Read' = the number of sequences read by **PrimerPipeline** so far (Read for the Trimming stage, not necessarily searched for SSR's, this depends on your trimming settings); 'found' = the number of microsatellites found so far.

Tip: If your sequences are listed in size order, smallest to largest sequence, (like outputs from SOAPdenovo are) then it will take proportionately longer per sequence to complete, therefore , during 'Searching for microsatellites' and 'Running Primer3' the percentage complete will increase quickly at the start and slow down towards the end.
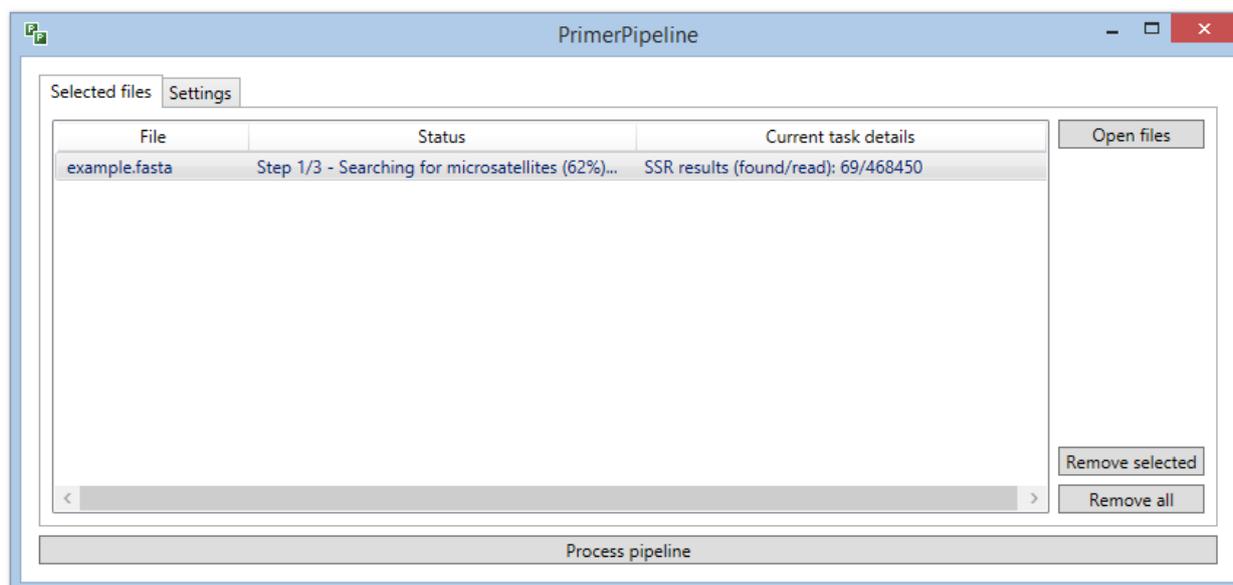
Figure 10. How **PrimerPipeline** looks whilst running step 1/3 – Searching for microsatellites.

5.2 Step 2.

Running Primer3 (Figure 11 and 12)

- During the Primer3 step the 'Current task details' and percentage complete in 'Status' updates every 30 seconds. This was a decision based on a compromise to keep the user informed during the process but not to significantly increase running time. So for the first 30 seconds of Primer3 running, the 'Current task details' will say '(updates every 30 seconds)' as in Figure 11.
- After 30 seconds the 'Current task details' will show 'Results found (valid/total)' (Figure 12).
- In the 'Status' column the percentage is calculated by comparing the total amount of sequences in the Primer3 input file to the amount of sequences so far in the Primer3 output file.

> **Note 9: Current task details for Step 2**
>
> In the 'Current task details' column 'total' is the number of sequences (that contain an SSR) that have been read so far and, 'valid', are the number of results that Primer3 has been able to design primers for.

5.3 Step 3.

Creating Results file.

- This is a very quick step, therefore is usually not shown on the display.
- When **PrimerPipeline** has finished (Figure 13) you can right-click on the file and go to 'view results' which will take you to the *Results* window (Figure 14).

5.4 Time Taken

Steps 1 and 2 are the longest steps; with time taken depending on your computer specifications, your input file and the settings that are chosen (e.g. number of sequences being read, the number of sequences that go through to the MISA stage, number of microsatellites found, and the number of primers that can be designed). Therefore it is difficult to predict the total time taken for each process, (however if in a hurry, please see Note 7).
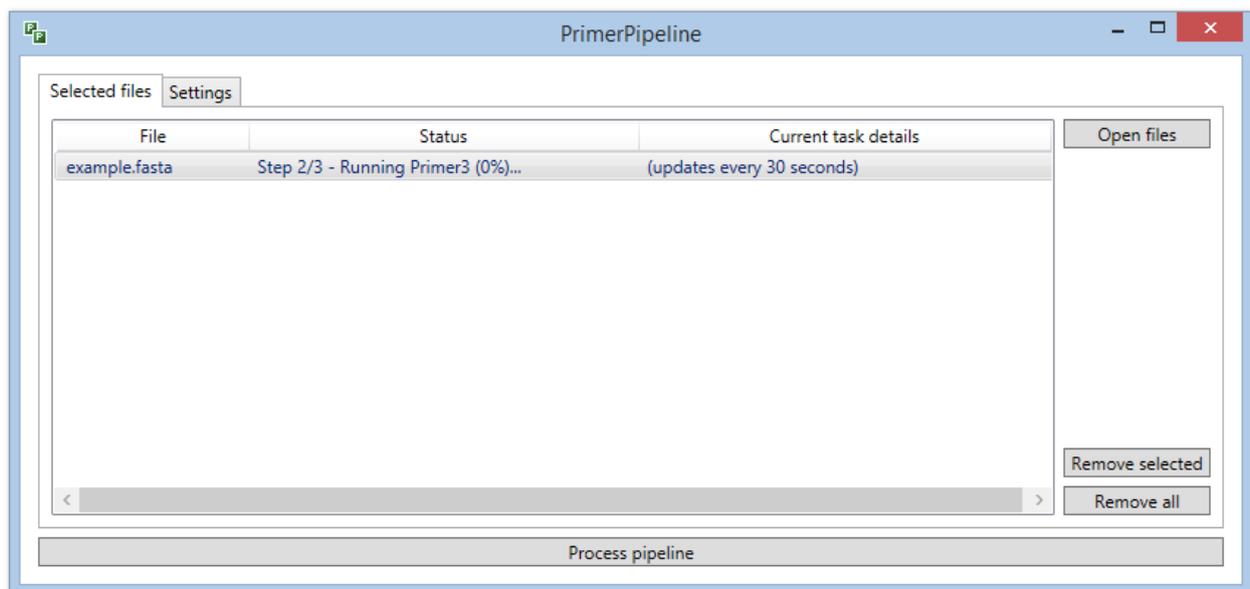
Figure 11. How **PrimerPipeline** looks for the first 30 seconds of step 2/3 – Running Primer3.
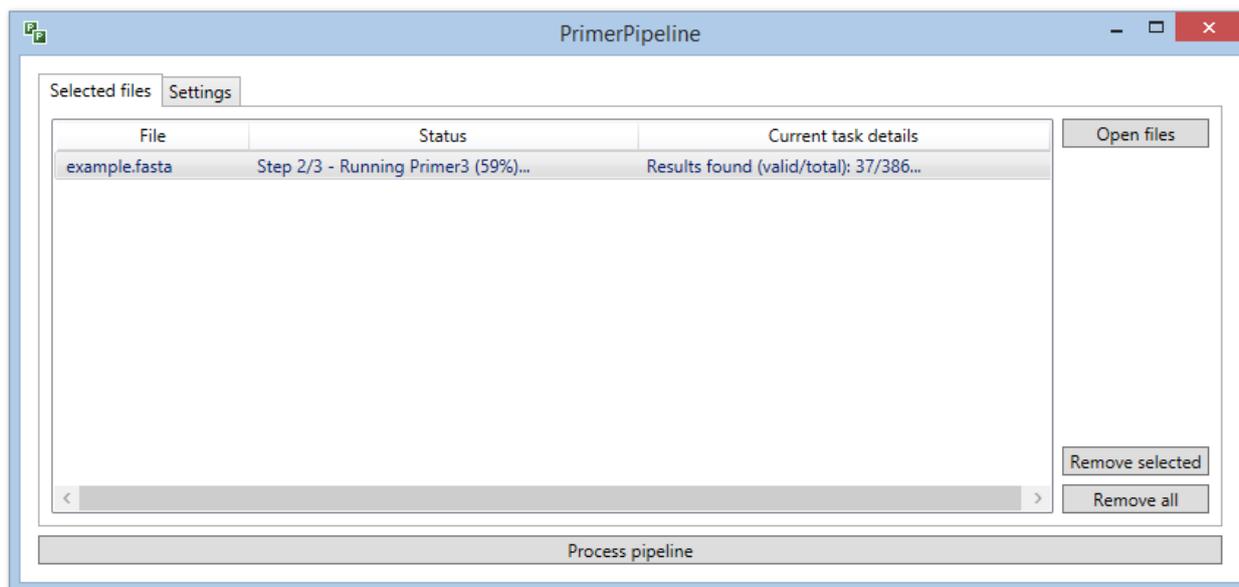
Figure 12. How **PrimerPipeline** looks when running step 2/3 – Running Primer3.
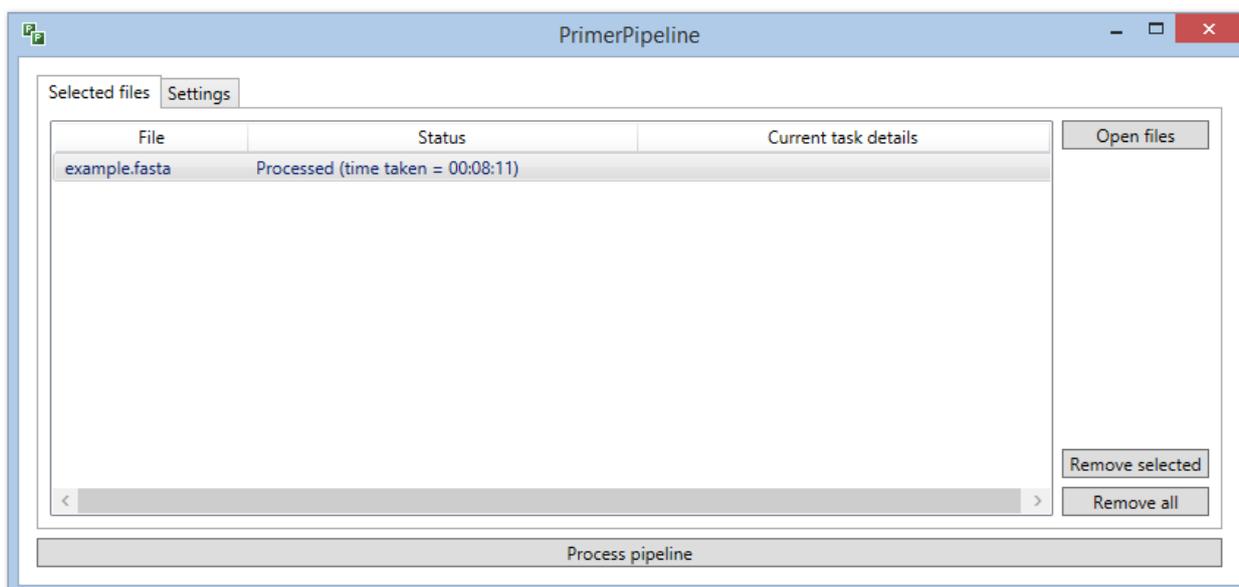
Figure 13. How **PrimerPipeline** looks when the pipeline has finished.
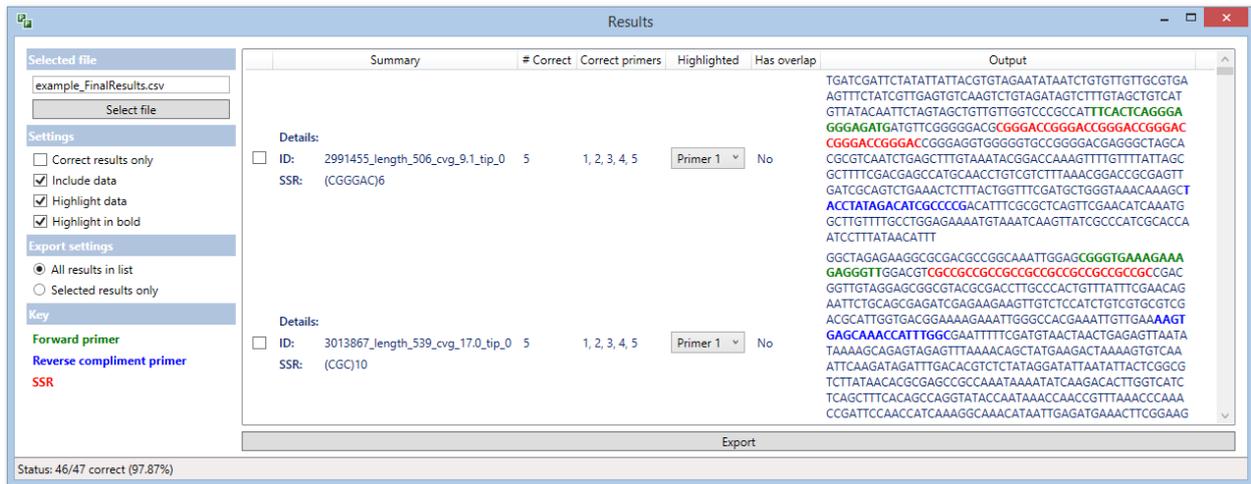
Figure 14. **PrimerPipeline** Results Window.
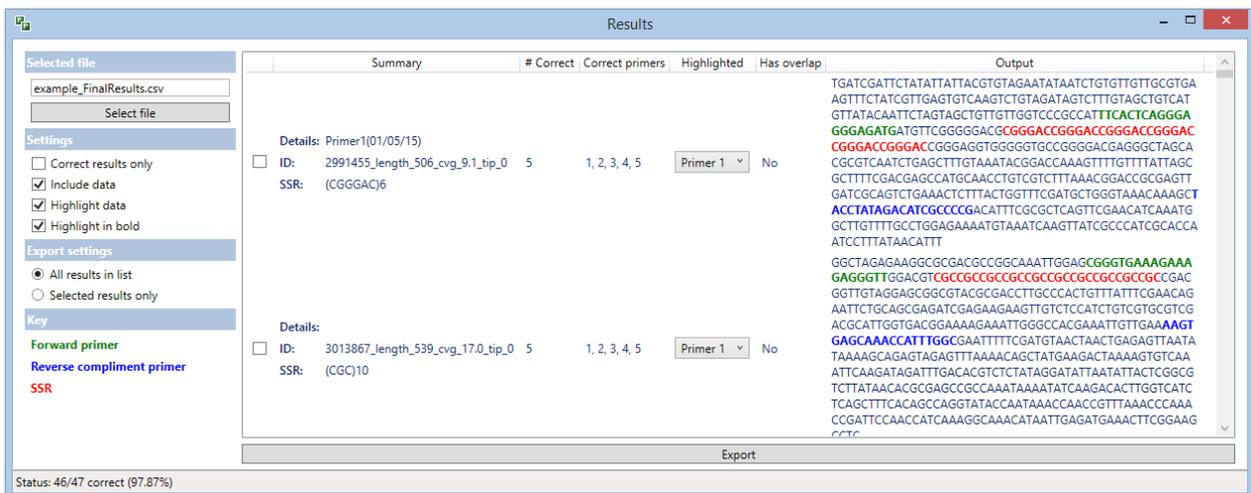
Figure 15. **PrimerPipeline** Results Window displaying information added in the 'Details' column of the FinalResults.csv.

6.0 Viewing results

When the pipeline has finished, the status will say 'Processed' (Figure 13). Right-click on the file, and click *View results*, this will open the 'Results' window (Figure 14).

> **Viewing.** You can view the results with or without the Data (Data means the sequence in the output column) by selecting or deselecting *Include data*, and you can chose whether to have the data highlighted or not, again by selecting or deselecting *Highlight data* and *Highlight in bold*.

Tip:	We recommend viewing the results with *Include Data, Highlight data* and *Highlight in bold* all selected because seeing the sequence with primers and SSR clearly defined allows you to check the flanking regions and pick the primers you will test in an easy, well informed way.

> **Ensuring correct results**. The status at the bottom of the 'Results' window (Figure 14) shows the results from an automatic check. The program has checked that the microsatellite and primer positions are in the correct order and do not overlap with each other. So you know that no errors in Primer3 have been made. If you see that a few primers are not correct, you can select *Correct results only* in the 'Settings', and click the *Export* button at the bottom (Figure 14), **PrimerPipeline** will ask you to 'Save as' to export all the correct sequences into a new .csv file.

> **Exporting.** If you wish to make a new .csv file with selected sequences only, click the boxes to the Left of the 'Summary' column, of the chosen sequences and then in *Export Settings*, click *Selected results only* before clicking the *Export* button at the bottom of the page.

> **Copying.** You can copy and paste sequences with formatting (with the forward and reverse primers and SSR highlighted) into Word, powerpoint, ect, if you wish.

> **Using the results file in Excel.** It is useful to open example.FinalResults.csv file in excel to edit, it is very flexible and allows you to plan your next steps in the microsatellite development. For example:
> 1) To order the sequences by SSR type or length.
> 2) To add a more user-friendly name for the primer pairs that you will order (a 'Details' column in the FinalResults.csv file is felt empty for this purpose, see Figure 15).
> 3) If you're only interested in certain SSRs, you can make a new .csv file with only dinucleotide microsatellites or with a certain size range so you can open a smaller, more manageable number of sequences in **PrimerPipeline** 'Results' window.

➢ **Reopening Results files in PrimerPipeline.** To reopen a file in **PrimerPipeline** save as .csv and drag into **PrimerPipeline**, it will recognise the file as a results file and immediately say 'Processed' in the 'Status' column, allowing you to right click on the file and click on *View Results*.

6.1 Results Window: Each column explained

1. Summary

Containing:

Details: Is the empty 'Details' column in the .csv file, allowing you to give the primers a new name or add any other extra information. If you write it in the column in excel and save as '.csv' it can be displayed by **PrimerPipeline**. If you haven't added your own names, this will be empty like in Figure 14, if you add a detail; it will look like Figure 15.

ID: Is the ID of the sequence from the original input file uploaded.

SSR: The SSR type and number of repeats e.g. (TG)24

2. # Correct: The number of primer pairs that are correct (out of however many you chose in the Primer3 settings 'Number of primers to return', default is 5). Correct means forward primer first, SRR then reverse primer with no overlap between the three.

3. Correct Primers: States which primer pairs (called 1 to 5) are correct.

4. Highlighted: This column has a drop down menu to enable you to change which primer pair is shown overlaid on the sequence, for each microsatellite. All show Primer pair 1 as default because Primer3 sorts primer pairs by quality, 1 being the highest quality. However if you are looking for a different product size for multiplexing then you may wish to use one of the other Primer pairs.

5. Has Overlap: Tells you if there are any overlap between the microsatellite and each primer. If you select *Correct only* under 'Settings', or if the status says 100% correct, then this will be 'No'. If there are overlap it will say 'Yes', avoid these primers if you can.

6. Output: This column shows the sequence in normal text, and if *Highlight data* and *Highlight in bold* are both selected the microsatellite (SSR) will be overlaid in **red**, and forward primer in **green** and the reverse compliment primer in **blue** (see key on Figure12). This allows you to easily view and check your primers. This column is only there if *Include data* is highlighted in 'Settings'.

7.0 Tips

1. Not finding enough microsatellites?

Consider relaxing the settings. To see where you need to change your settings first check the 'example.misa_statistics' to see how many sequences were examined and compare this number to the total number of sequences in the original input file. If there is a large difference, it means that the *Trimming* step is possibly too strict, and isn't letting enough sequences through to the MISA stage. Consider decreasing you minimum sequence and increasing your maximum sequence.

If the trimming settings are fine, in the MISA settings consider decreasing the minimum repeat so that smaller microsatellites can be found or allowing interruptions so that compound microsatellites can be found.

2. Not finding enough Primers?

If you are finding plenty of microsatellites but are not getting enough primers, it may be due to the position of the microsatellite in the sequence. If the microsatellite is at the very beginning or very end of the sequence there is no room for Primer3 to design a primers around it. Check this by looking at the 'example.misa' file and looking at the start and end column. This shows where the microsatellites are located in the sequence.

There are a lot of settings during the Primer 3 stage which you can adjust, it is always a compromise between quality and quantity of primers. Look into more detail at the Primer3 settings and consider making them more relaxed. To read more about Primer3 settings please visit Primer3's website here (see Note 2 to run Primer3 only, without having to re-run Trimming and microsatellite mining steps).

8.0 Acknowledgments

We'd like to thank the creators of MISA and Primer3 for allowing us incorporate their amazing programs into ours.

I'd also like to thank all my colleagues in my lab for help and guidance, and a special thank you to my supervisors at Cardiff University for not being too hard on me for completely failing in my first attempt at bioinformatics, when I spend 5 months of my PhD trying to optimise primers that were incorrect. It turned out there was an error in the Linux pipeline I was using which meant that Primer3 designed primers randomly in the sequence, not around the SSR. We created **PrimerPipeline** to help others avoid such pitfalls when first embarking in bioinformatics.

9.0 References

9.1 MISA

For original MISA and trimmer pearl scripts:

Program name: misa.pl
Author: Thomas Thiel
Release date: 14/12/01 (version 1.0)
Website: http://pgrc.ipk-gatersleben.de/misa/

9.2 Primer3

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M and Rozen SG. Primer3--new capabilities and interfaces. Nucleic Acids Res. 2012 Aug 1;40(15):e115.

The paper is available at http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3424584/
Source code available at http://sourceforge.net/projects/primer3/
Website: http://primer3.ut.ee/