TASSEL 3 Genotyping by Sequencing (GBS) pipeline documentation

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Introduction

This document describes the GBS pipeline available in the TASSEL 3 standalone for species with a reference genome. If your species does not have a reference genome, we suggest that you try the UNEAK pipeline, which is available as part of the TASSEL 3 standalone. Documentation for the UNEAK pipeline is available here: http://www.maizegenetics.net/images/stories/bioinformatics/TASSEL/uneak_pipeline_documentation.pdf

The reference genome-based GBS analysis pipeline described below is an extension to the Java program TASSEL. On Linux, Unix, or Mac operating systems (or Windows machines with perl installed), GBS commands are run as

TASSEL plugins via the command line by calling a perl script (which in turn launches Java) with the following syntax:

```
run_pipeline.pl -fork1 -PluginName --plugin-option(s) -endPlugin -runfork1
```

On a Windows machine without perl installed use `run_pipeline.bat` instead. Each step of the pipeline is specified with a "-fork" command and a number, since TASSEL can run several processes at once, split and recombine their results, and use the output of one "fork" as the input to the next.. The fork option is followed by the name of the plugin, and any plugin-specific options. "-endPlugin" signals the end of plugin-specific options, and "-runfork1" then runs the specified plugin. In all of our examples here for the GBS pipeline, we run only a single fork at a time (always "-fork1").

All of the GBS plugins will print out their available options/arguments if you call them without any:

```
run_pipeline.pl -fork1 -PluginName -endPlugin -runfork1
```

Please see http://www.maizegenetics.net/tassel/docs/TasselPipelineCLI.pdf for general instructions on how to install the TASSEL 3.0 Standalone Build on your computer. These GBS-specific instructions assume that you have unzipped the standalone into the directory (folder):

```
/programs
```

and then renamed the directory:

```
/programs/tassel3.0_standalone
```

to:

```
/programs/tassel
```

If not, you will have to edit the example commands appropriately (*e.g.*, replace "`tassel`" with "`tassel3.0_standalone`").

If you have more memory available on your machine than 1.5GB, then you can increase the amount of memory available to TASSEL by opening `run_pipeline.pl` (or `run_pipeline.bat` if running on Windows) in a text editor and modifying "`-Xmx1536m`" to (for example) "`-Xmx6g`" (the `-Xmx` option controls the maximum amount of memory available to the java pipeline). Note that the first step of the pipeline, FastqToTagCountsPlugin, required at least 6G of memory in order to run (under the original default value of its -s parameter of 200,000,000 good, barcoded reads). Therefore, **in order to run the TASSEL GBS pipeline, you need a computer with at least 8G of RAM**. Because recent fastq files often contain more than 200,000,000 good, barcoded reads, we have recently raised the default value of the -s parameter to 300,000,000 -- hence you might need more than 8G of RAM to run the pipeline using this default setting (16G should suffice).

If you are launching the pipeline via the perl script, you can also allocate memory directly in the command line, for example:

```
run_pipeline.pl -Xmx6g -fork1 -PluginName --plugin-option -endPlugin
-runfork1
```

Many of the GBS commands produce a large amount of console output ("stdout" = "standard output"). Although we won't describe this output in detail here, some of it is very informative in tracing bugs or finding problems with your input files or command syntax. You will likely find it helpful to either copy and paste it to a text log file or, better, to redirect stdout to both the console and a log file. In Linux, this can be done by appending "` | tee GBSlogfile20110915.txt`" on the end of your pipeline command (rename the log file as you see fit).

How to cite the TASSEL-GBS pipeline

Glaubitz JC, Casstevens TM, Lu F, Harriman J, Elshire RJ, Sun Q, Buckler ES. TASSEL-GBS: A high capacity genotyping by sequencing analysis pipeline. PLoS ONE (in press) [manuscript].

TASSEL Google Group

The TASSEL Google Group (https://groups.google.com/group/tassel) provides a forum where TASSEL users in general can pose questions to other TASSEL users or the TASSEL developers, browse the answers to previous questions, or request new features. It also provides these functions for users of the TASSEL GBS pipeline. It is always a good first resource if you get stuck: someone else may have already encountered the same problem.

TASSEL Source Code is Available on SourceForge

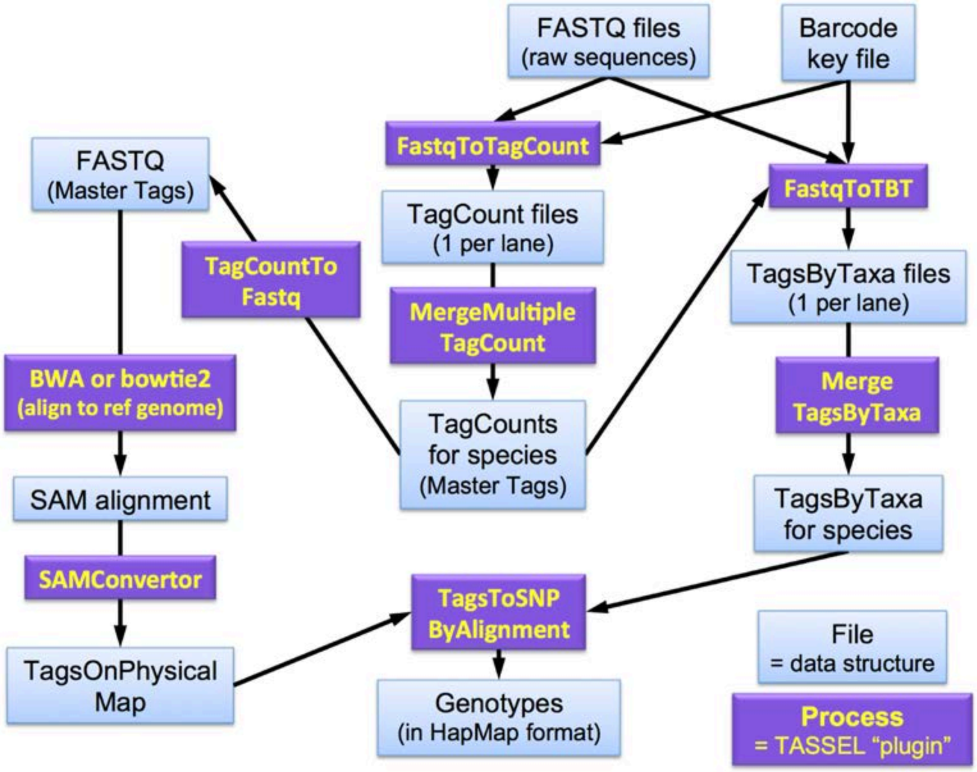
The most detailed, accurate, and up-to-date documentation of the Tassel GBS Pipeline is the code itself. So, if you are able to understand Java, you can get the source code here:

Tassel3: http://sourceforge.net/p/tassel/svn/2538/tree/
Tassel4 & 5: http://sourceforge.net/p/tassel/code/ci/master/tree/

If you both understand Java and are brave, you might even modify/customize the code to better suit your own purposes.

Discovery Pipeline Overview

The flow chart below shows how the steps of a possible GBS “Discovery Pipeline” analysis link together (variations on this approach are possible). Light blue boxes represent files (or data structures) produced at each step of the analysis, and purple boxes represent the processes (Tassel3 plugins) that produced them:



Most of the intermediate files are in a binary format. You can convert some of the file types (TagCounts, TagsByTaxa, TagsOnPhysicalMap) to human-readable, tab-delimited text format using the BinaryToTextPlugin. These text files will often be too large to open in a text editor or Excel. However, in Linux (or with Cygwin on a PC) you can use the "head" and "tail" commands to extract a section from the middle of a file. For example the command:

```
head -2000000 myLargeFile.txt | tail -10000 > myLargeFileMid10KLines.txt
```

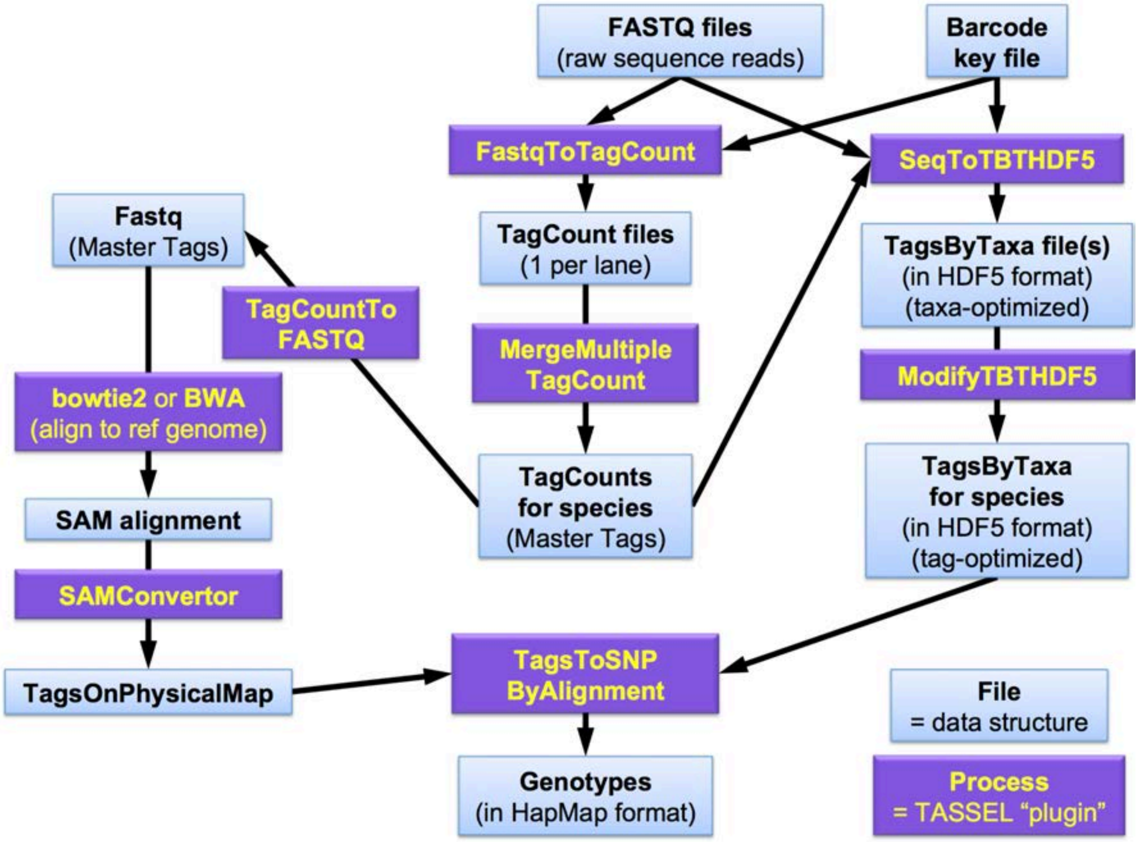
will extract lines 1,990,001 to 2,000,000. Excel can usually open a file with 10,000 lines in it quite quickly.

Once you have genotypes, subsequent possible steps include:

- **MergeDuplicateSNPsPlugin**: To merge duplicate SNPs called from overlapping tags on opposite strands.
- **GBSHapMapFiltersPlugin**: To filter SNPs based upon (1) amount of missing data, (2) minor allele frequency, (3) deviation of observed from expected heterozygosity (F_{IS} = 1-H_O/H_E), or (4) amount of linkage disequilibrium (LD) with nearby markers (if you are working with a population with extensive linkage disequilibrium such as a biparental linkage mapping population). The LD filter works only for highly homozygous samples (*e.g.*, RILs).
- **BiParentalErrorCorrectionPlugin**: If the samples that you are analyzing include one or (preferably) more biparental families, you can use this plugin to remove SNPs with high error rates (*i.e.*, SNPs that are not actually segregating 1:1 in one or more of the families but appear to be weakly polymorphic in those families because of high error rates) and SNPs that are not in LD with their neighboring SNPs (in families where they *are* actually segregating). The error detection part of this plugin works only for families with expected segregation ratios of 1:1 (*e.g.*, F2 or RILs derived from F2). The LD filter part works only for highly homozygous families (*e.g.*, RILs).
- **MergeIdenticalTaxaPlugin**: To merge the genotypes of taxa with identical short names (up to the first colon of their full name) but run on different lanes or in the same lane but with different barcodes.
- **FastImputationBitFixedWindowPlugin**: If a large proportion of your samples ("taxa") are inbred lines or have very low heterozygosity, you can use this plugin to impute missing data. This plugin will not be documented here. Imputation is a tricky problem that is the subject of a lot of ongoing research. We recommend that you use the FastImputationBitFixedWindowPlugin only if you are able to read the Java code and understand the underlying assumptions.

If you are working with very large numbers of samples (taxa) in your discovery build (*e.g.*, >10,000 samples), and the master TagsByTaxa (TBT) file produced by the MergeTagsByTaxa file is too large, you can use the alternate route in the flowchart below (top of next page) to produce a master TBT file in HDF5 format. The only differences in the flowchart below (compared to the one above) is that the SeqToTBTHDF5 and ModifyTBTHDF5 plugins are used in place of the FastqToTBT and MergeTagsByTaxaFiles plugins.

The only drawback with using a TBT HDF5 (instead of a TBTByte) is that it is currently not possible to convert a TBT HDF5 to text via the BinaryToTextPlugin. However, you can use the program HDF5View (available from http://www.hdfgroup.org/hdf-java-html/hdfview/) to manually inspect a TBT HDF5 file.



Discovery versus Production Pipelines

The above flow charts and subsequent steps describe a typical **Discovery Pipeline**. "Discovery Builds" are typically done at the species level with all available GBS sequence data for that species, from multiple lanes. Filtering for good GBS SNPs is usually best done at the species level.

Once you have run a large-scale, species-wide Discovery Pipeline, it is possible to use the knowledge of useful variants that you gained from that (stored in the TagsOnPhysicalMap file by using the -mUpd flag during SNP calling) to quickly call known SNPs in newly sequenced samples, without re-analyzing all of the samples sequenced to date. We refer to this as the **Production Pipeline**, which is carried out by the single step RawReadsToHapMapPlugin. The RawReadsToHapMapPlugin (Production Pipeline) can be used to match GBS tags found in a single lane of sequence data to those already present in the TagsOnPhysicalMap (TOPM) file created and populated with variants during the most recent Discovery Build. It calls SNPs based on the known useful variants stored in the TOPM, producing a HapMap format genotype file just for that single lane of sequence data. More details are provided in the documentation for the RawReadsToHapMapPlugin, below.

Recommended directory (folder) structure for a GBS analysis

A dot (.) represents the working directory (folder) for your analysis, which will be your current working directory (*e.g.*, `/home/myUserName/myGBSstudyName`)

The example commands below for each plugin don't create the directories (and will fail if the directories don't already exist), so at the start of the analysis, create the following directories inside your working directory:

```
./fastq OR ./qseq   (original raw data files, one file per flowcell lane)
```

```
./tagCounts          (for output from FastqToTagCountPlugin)
./mergedTagCounts    (for output from MergeMultipleTagCountPlugin)
./topm               (for output from SAMConverterPlugin)
./tbt                (for output from FastqToTBTPlugin)
./mergedTBT          (for output from MergeTagsByTaxaFilesPlugin)
./hapmap
./hapmap/raw         (for output from TagsToSNPByAlignmentPlugin)
./hapmap/mergedSNPs (for output from MergeDuplicateSNPsPlugin)
./hapmap/filt        (for output from GBSHapMapFiltersPlugin)
./hapmap/bpec        (for output from BiParentalErrorCorrectionPlugin)
```

FastqToTagCountPlugin

Summary:

Derives a tagCount list for each FASTQ file in the input directory (and all subdirectories thereof). Keeps only good reads having a barcode and a cut site and no N's in the useful part of the sequence. Trims off the barcodes and truncates sequences that (1) have a second cut site, or (2) read into the common adapter.

Input:

- Barcode key file (see example in Appendix 1)
- Directory (folder) containing FASTQ files

Output:

- Directory (folder) containing a corresponding tagCount (.cnt) file for every FASTQ file in the input directory

Arguments:

FastqToTagCountPlugin	
-i	Input directory containing FASTQ text (_fastq.txt) or gzipped FASTQ (_fastq.gz) text files. NOTE: Directory will be searched recursively, and should be written without a slash after its name.
-k	Key file listing barcodes for each sample and plate layout. See Appendix 1.
-e	Enzyme used to create the GBS library (*Ape*KI, *PstI* or several others).
-s	Maximum number of good, barcoded reads per lane. Default: 300,000,000.
-c	Minimum number of times a tag must be present to be output. Default: 1
-o	Output directory to contain ouput .cnt (tag count) files, one per input FASTQ file. Defaults to input directory (the default is not recommended - it is best to use a separate directory).

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -FastqToTagCountPlugin -i fastq -k
myGBSProject_key.txt -e ApeKI -o tagCounts -endPlugin -runfork1
```

Gory Details:

This is the initial step of a GBS “Discovery Pipeline” analysis. It reads a **user-supplied key file** (**-k option**) in tab-delimited text format which indicates, for each lane of interest from a flowcell, which barcodes are assigned to which sample. An example key file is provided in Appendix 1. Note that you can combine lanes from multiple flowcells into a single key file and GBS analysis. In fact, to take full advantage of the features of our pipeline, we encourage you to lump all samples using the same restriction enzyme from multiple lanes/flowcells together into a single, species-level analysis.

After reading the key file the FastqToTagCountsPlugin then ***recursively*** searches the specified **input directory** (**-i option**) and all of its subdirectories for FASTQ files matching one of the flowcell/lane combinations in the key file and with one of the following acceptable file naming conventions:

FLOWCELL_LANE_fastq.txt	(example: **42A87AAXX_2_fastq.txt**)
FLOWCELL_LANE_fastq.txt.gz	(example: **42A87AAXX_2_fastq.txt.gz)**
FLOWCELL_LANE_sequence.txt	(example: **42A87AAXX_2_sequence.txt**)
FLOWCELL_LANE_sequence.txt.gz	(example: **42A87AAXX_2_sequence.txt.gz**)
FLOWCELL_s_LANE_fastq.txt	(example: **42A87AAXX_s_2_fastq.txt**)
FLOWCELL_s_LANE_fastq.txt.gz	(example: **42A87AAXX_s_2_fastq.txt.gz**)
code_FLOWCELL_s_LANE_fastq.txt	(example: **10225395_42A87AAXX_s_2_fastq.txt**)
code_FLOWCELL_s_LANE_fastq.txt.gz	(example: **10225395_42A87AAXX_s_2_fastq.txt.gz)**

Note that both compressed (.gz) and uncompressed (.txt) files can be read - we recommend using compressed files to save disk storage space. The "code" part of the latter two file name examples is a numerical tracking code that our sequencing center used to generate. Our GBS pipeline doesn't actually use this numerical code, so you can substitute any text or numbers. The same thing goes for the "_s_" part: you can substitute any text or numbers (but not underscores) for the "s". The underscores are essential for correct parsing of the parts of each FASTQ file name (only FLOWCELL and LANE are actually used by our pipeline).

For each FASTQ file that has samples in the key file with a matching flowcell and lane, the FastqToTagCountPlugin finds all reads that begin with one of the expected barcodes immediately followed by the expected cut site remnant (CAGC or CTGC for *Ape*KI) and trims them to 64 bases (including the cut site remnant but after removing the barcode). Reads containing N within the first 64 bases after the barcode are rejected. If a read contains either a full cut site (from incomplete digestion or chimera formation) or the beginning of the common adapter (from restriction fragments less than 64bp) within the first 64 bases it is truncated appropriately and padded to 64 bases with polyA (where "polyA" = "AAAA…"). The actual length of all reads (64 bases or less, if truncated) is recorded. Once all of the reads in an input FASTQ file have been loaded into memory (or when the maximum number of good, barcoded reads specified by the **-s** option have been read from the file), the plugin then sorts all of the reads and collapses identical reads (over the first 64 bases after the barcode) into a single tag. It then writes out this list of tags into an output tagCount file.

Hence, the output of FastqToTagCountPlugin is a single tagCount file in the **specified output directory** (**-o option**) for every matching FASTQ file in the input directory. The tagCount files are named after their corresponding FASTQ file, with "_fastq.txt.gz" (or "_fastq.txt", etc.) replaced by ".cnt". The tagCount files are binary, and can only be read by our pipeline (you can use the BinaryToTextPlugin to convert them into human readable text if you wish). They contain the 64 base sequence of each observed tag (padded with polyA if truncated), the actual length of the tag (either 64 bases or less if it is padded with polyA), and the number of times that tag was observed in the corresponding FASTQ file. The tags are sorted by their sequence.

In the future (in Tassel4, eventually) we will modify the pipeline to allow the user to retain tags of any desired (but fixed) length, not just 64 bases. But until we make the changes required for that, keep in mind that the sequencing error rate tends to rise dramatically after 64 bases, so the extra sequence may not be worth the bother. For most purposes, 64 base tags should suffice.

The restriction **enzyme** used to create the GBS library is indicated via mandatory option **-e**. Currently, our pipeline only accepts the enzymes (or pairs of enzymes) in the table below combined with the indicated common adapter sequence. Also provided in the table are the initial cut site remnant(s) expected to occur in each read immediately after the barcode, and the full cut sites that are diagnostic of either incomplete restriction digestion or chimera formation. Reads that contain either a full cut site or the beginning of the common adapter sequence are truncated appropriately. The first few bases of the common adapter (not shown in the table) are defined by the restriction enzyme "sticky end". The "Y-adapter" employed by Poland *et al.* (2012) as the common (non-barcoded) adapter ensures unidirectional cloning of doubly digested restriction fragments. Pairs of restriction enzymes are specified in the -e option separated by a hyphen (for example "`-e PstI-MspI`").

Enzyme or Enzyme Pair	Initial Cut Site Remnant(s)	Full Cut Site(s)	Common Adapter
*Ape*KI	CAGC or CTGC	GCAGC or GCTGC	Elshire et al. 2011
*Apo*I	AATTC or AATTT	AAATTT, AAATTC, GAATTC or GAATTT	Elshire et al. 2011
*Bam*HI	GATCC	GGATTC	Elshire et al. 2011
*Eco*T22I	TGCAT	ATGCAT	Elshire et al. 2011
*Hin*P1I	CGC	GCGC	Elshire et al. 2011
*Hpa*II	CGG	CCGG	Elshire et al. 2011
*Mse*I	TAA	TTAA	Elshire et al. 2011
*Msp*I	CCG	CCGG	Elshire et al. 2011
*Nde*I	TATG	CATATG	Elshire et al. 2011
*Pas*I	CAGGG or CTGGG	CCCAGGG or CCCTGGG	Elshire et al. 2011
*Pst*I	TGCAG	CTGCAG	Elshire et al. 2011
*Sau*3AI	GATC	GATC	Elshire et al. 2011
*Sbf*I	TGCAGG	CCTGCAGG	Elshire et al. 2011
*Asi*SI-*Msp*I	ATCGC	CCGG or GCGATCGC	Poland et al. 2012
*Bss*HII-*Msp*I	CGCGC	CCGG or GCGCGC	Poland et al. 2012
*Fse*I-*Msp*I	CCGGCC	CCGG or GGCCGGCC	Poland et al. 2012
*Pae*R7I-*Hha*I	TCGAG	GCGC or CTCGAG	Poland et al. 2012
*Pst*I-*Ape*KI	TGCAG	GCAGC, GCTGC, or CTGCAG	Poland et al. 2012
*Pst*I-*Eco*T22I	TGCAG or TGCAT	CTGCAG or ATGCAT	Elshire et al. 2011
*Pst*I-*Msp*I	TGCAG	CCGG or CTGCAG	Poland et al. 2012
*Pst*I-*Taq*I	TGCAG	TGCA or CTGCAG	Poland et al. 2012
*Sal*I-*Msp*I	TCGAC	CCGG or GTCGAC	Poland et al. 2012
*Sbf*I-*Msp*I	TGCAGG	CCGG or CCTGCAGG	Poland et al. 2012

If you would like us to add additional enzymes or enzyme combinations to the pipeline, please post your request on the TASSEL Google Group (groups.google.com/group/tassel). We are also considering adding a feature that allows the user to specify all the information needed for any combination of restriction enzymes and common adapters (but that has not been implemented yet - a possible problem with this is it will likely lead to a lot of user errors).

The **-s option** (***maximum number of good reads per lane***) is used to set an upper limit on memory usage. Our initial default value of the -s option was 200 million, which required 6G of available RAM, allowing the pipeline to be run on a computer with a total of 8GB of RAM. However, as Illumina next gen sequencing technology has improved, FASTQ files with more than 200 million good, barcoded reads have become more commonplace. Therefore, we have now increased the default value of -s to 300 million, which might require a computer with more than 8GB of RAM (a 16GB machine should suffice).

If the console output of the FastqToTagCountsPlugin indicates that *exactly* 300 million good, barcoded reads (or whatever you set -s to) were found in one or more of the input files, then you should increase the -s parameter, provided that your computer has enough memory. In this case, it is extremely likely that the FASTQ file actually contained more than 300 million good, barcoded reads. Unlike most of the steps in the GBS pipeline, the FastqToTagCounts step will not overwrite pre-existing output tagCount (.cnt) files, so in order to rerun with a higher value for the -s option, you will first have to delete (or move) the previously output tagCount (.cnt) files.

If we are combining the results of multiple lanes in our analysis, we usually keep the **-c option** (***minimum number of times a tag must be present in a FASTQ file to be output***) at its default value of 1. In that case, the minimum number of times that a tag must be seen *across the entire experiment* to be retained can instead be controlled by the -c option at the next step, MergeMultipleTagCountPlugin. Tags that occur only once in a given flowcell lane (input FASTQ file) might occur multiple times in other lanes, so they might be real (*i.e.*, not from sequencing error). In contrast, if your analysis consists of only a single lane's worth of data (*i.e.*, a single input FASTQ file), then you should consider setting the -c option in the FastqToTagCount step to value higher than its default of 1, depending on the lowest allele frequency of interest and expected level of coverage. The trade-off is as follows: the lower is the value of the -c option, the more sequencing errors you will include in your analysis; the higher is the value of the -c option, the more rare alleles will be missed. Keep in mind that tags that contain one or more sequencing errors can still be useful to score SNPs at the non-error positions, and to increase the depth of coverage at these non-error positions. The balancing act is to keep the amount of sequencing error down to a reasonable level via the -c option, and then to remove most of the remaining error-prone SNPs at subsequent steps in the pipeline (GBSHapmapFiltersPlugin and/or the BiParentalErrorCorrectionPlugin; see below for more details on these plugins).

If your sequence data **predates** CASAVA 1.8 (a version of the Illumina software for generating the sequence files released in 2011) and you thus have it in both QSEQ and FASTQ format, we recommend using the QSEQ files if possible because they contain *all* reads, not just the ones passing Illumina's quality filters. (CASAVA 1.8 only provides FASTQ files but these contain all reads.) We have found that perfectly good reads - exactly matching a 64 base tag that we have seen many times - can fail to pass Illumina's filters. To analyze QSEQ input files instead of FASTQ, use the QseqToTagCountPlugin, which uses exactly the same arguments as the FastqToTagCountPlugin.

MergeMultipleTagCountPlugin

Summary:

Merges each tagCount file in the input directory into a single "master" tagCount list. Only keeps tags with a total count (after merger) greater than or equal to that specified by the **-c option** (***minimum number of times a tag must be present to be output***). It has two output formats: (1) a binary output format (.cnt) that is used by the FastqToTBTPlugin to construct tags by taxa (TBT) files, and (2) a fastq text format (.fq) that is used as an input to BWA or bowtie2 to align tags to the reference genome. For clarity, the latter functionality (conversion of a master tagCount list into fastq format) has been made into its own plugin (see TagCountToFastqPlugin below).

Input:

- Input directory (folder) containing tagCount (.cnt) files

Output:

- Merged tagCount file (it is best to send this to a separate directory from the input directory)

Arguments:

MergeMultipleTagCountPlugin	
-i	Input directory containing tagCount (.cnt) files.
-o	Output file name (should be in a separate directory from the input).
-c	Minimum number of times a tag must be present to be output. Default: 1
-t	Specifies that reads should be output in FASTQ text format (for use as input to either BWA or bowtie2 for alignment to the reference genome).

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -MergeMultipleTagCountPlugin -i
tagCounts -o mergedTagCounts/myMasterGBSTags.cnt -c 5 -endPlugin -runfork1
```

Gory Details:

The MergeMultipleTagCountPlugin step merges multiple tagCount files produced by the FastqToTagCountPlugin step (from multiple lanes and/or flowcells) into a single "master" tagCount file. (For a description of the tagCount file format, see FastqToTagCountPlugin.) All binary tagCount (.cnt) files in the specified input directory (argument **-i**) are merged into a single, output tagCount file.

To remove rare or singleton tags that possibly result from sequencing errors, we use the **-c option** (***minimum number of times a tag must be present to be output***). A **-c** option setting between 5 and 20 is typical, but when deciding on an appropriate cutoff, you should consider the number of individuals in your analysis, the expected coverage (currently about 0.3-0.5x for maize with *Ape*KI at 384 plex), the expected segregation ratio, minimum minor allele frequency of interest, etc. The trade-off is as follows: the lower is the value of the **-c** option, the more sequencing errors you will include in your analysis; the higher is the value of the **-c** option, the more rare alleles will be missed. Keep in mind that tags that contain one or more sequencing errors can still be useful to score SNPs at the non-error positions, and to increase the depth of coverage at these non-error positions. The balancing act is to keep the amount of sequencing error down to a reasonable level via the **-c** option, and then to remove most of the remaining error-prone SNPs at subsequent steps in the pipeline (GBSHapmapFiltersPlugin and/or the BiParentalErrorCorrectionPlugin; see below for more details on these plugins).

The merged tagCount output file is used as a master tag list for two subsequent steps: alignment to the reference genome (via BWA or bowtie2) and/or the FastqToTBTPlugin step. The output of the MergeMultipleTagCountPlugin is, by default, in binary tagCount (.cnt) format, which serves as the input format for the FastqToTBTPlugin step.

Alignment to the reference genome is performed with external software: BWA and bowtie2 are currently supported by our pipeline. To obtain a master tagCount file in FASTQ format for use as input to BWA or bowtie2, invoke the **-t** option. Omitting this option produces a binary tag count file (the default). However, output binary master tag count (.cnt) file can be directly converted to a FASTQ file for input to BWA or bowtie2 using the TagCountToFastqPlugin described immediately below.

Note that, instead of using the binary master tag list tagCount (.cnt) file as the input Master Tags List specified in the -t option of the FastqToTBTPlugin, you may alternatively use the TagsOnPhysicalMap (TOPM) file produced by the SAMConverterPlugin. More details are provided in the FastqToTBTPlugin section.

TagCountToFastqPlugin

Summary:

Converts a master tagCount file containing all the tags of interest for your species/experiment (*i.e.*, all of the tags with a minimum count greater than the **-c** parameter used in the MergeMultipleTagCountPlugin) from binary (.cnt) format into a FASTQ format file (.fq) that can then be used as input to one of the aligners BWA or bowtie2.

Input:

- A binary tag count (.cnt) file containing all tags of interest (= master tag list).

Output:

- The master tag list in FASTQ format (.fq). Can be used as input to BWA or bowtie2.

Arguments:

TagCountToFastqPlugin	
-i	Input binary tag count (.cnt) file
-o	Output FASTQ file to use as input for BWA or bowtie2.
-c	Minimum count of reads for a tag to be output (default: 1)

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -TagCountToFastqPlugin -i
myMasterGBSTags.cnt -o myMasterGBSTags.fq -c 5 -endPlugin -runfork1
```

Gory Details:
If you have already run MergeMultipleTagCountPlugin with the -t option and with your desired minimum tag count (-c), then there is no need to run this TagCountToFastqPlugin. However, if you did not use the -t option when you ran the MergeMultipleTagCountPlugin, or if you have only a single lane of GBS sequence data that you are working with, and a single corresponding tagCount (.cnt) file, then you can use this TagCountToFastqPlugin to convert from binary, tagCount (.cnt) format to FASTQ (.fq) format. The FASTQ output file will contain all the tags present in the input tagCount (.cnt) file, and can be used as an input to BWA or bowtie2 to try to align all of the tags against the index reference genome. The quality string for each tag is given the arbitrary value of "ffffff…" (all f), corresponding to an arbitrary, and very high, Phred score of 69 at all positions.

Indexing with BWA

Summary:
Creates a series of support files needed to operate BWA. You must have BWA installed on your computer: http://bio-bwa.sourceforge.net. For more details, consult the BWA manual:
http://bio-bwa.sourceforge.net/bwa.shtml

Input:

- A FASTA file containing one record for each chromosome or contig in the genome. In order for the SAMConverterPlugin (see below) to work correctly, the header of each record should contain only a single integer corresponding to that chromosome or contig's number. The suffix "chr" prior to the chromosome number is permissible, but no others. For example, the headers ">1", ">2", ">3", etc. are acceptable, as are ">chr1", ">chr2", ">chr3", etc.

Output:

- A series of support files with the same name as the FASTA file and different suffixes (.sa, .rsa, .rpac, .rbwt, .pac, .bwt, .ann, .amb).

Key Arguments:

bwa index	
-a	Indexing algorithm: "is" or "bwtsw".

Example command:

```
bwa index -a bwtsw referenceSequence/rice.fa
```

Gory Details:
In order to quickly align short reads, BWA needs to go through an initial, time-consuming, indexing step that generates a series of lookup tables from the input genome sequence. There are two alternative algorithms available. The default, "is", does not work with genomes larger than 2GB, but has no lower limit on genome size, and is the fastest option. The alternative, "bwtsw", is slower and cannot be used for genomes under 20MB, but it is capable of indexing human, bovine, maize, or other large genomes.

The Tassel3 GBS pipeline can use tag alignments produced either by BWA or by the default (-M) mode of bowtie2. In our experience, bowtie2 is more sensitive than BWA, and produces results that are more similar to BLAST. The tradeoff of the greater sensitivity of bowtie2 is that misalignment of tags will occur more often (e.g., tags from paralogous loci or from inserted sequences not present in the reference).

Alignment with BWA

Summary:

Aligns the master set of GBS tags to the reference genome. This input master tag list is stored in the fastq (.fq) file produced by TagCountToFastqPlugin (or the MergeMultipleTagCountPlugin with the -t option). You must have BWA installed on your computer: http://bio-bwa.sourceforge.net.

Input:

- Tag count file in FASTQ format (.fq) produced by the TagCountToFastqPlugin (or the MergeMultipleTagCountPlugin with the -t option).

Output:

- Alignment file in SAI (binary) format.

Key Arguments:

bwa aln	
-t	Number of CPU cores on which to run the program. Speeds up execution on multi-core computers.

Example command:

```
bwa aln -t 4 referenceSequence/rice.fa mergedTagCounts/myMasterTags.cnt.fq >
mergedTagCounts/myAlignedMasterTags.sai
```

Gory Details:

The angle bracket (greater than sign, ">") here indicates that the output from this program should be stored in the given filename. Otherwise, BWA prints the output to the console.

As of this writing, our version of BWA (0.5.6), has several options dealing with insertions and deletions that we have not modified (we use the default settings). For guidance on these or other options, go to http://bio-bwa.sourceforge.net/bwa.shtml or type "man bwa" on UNIX systems.

Exporting BWA Alignments in SAM Format

Summary:

Converts the BWA-specific binary alignment (.sai) file into a text-based SAM (.sam) file.

Input:

- SAI format alignment (.sai) file produced by the Linux program BWA

Output:

- SAM alignment file that can be read by the SAMConverterPlugin of our GBS pipeline, as well as by MAQ and other bioinformatics software.

Key Arguments:

bwa samse	We use the defaults. Consult http://bio-bwa.sourceforge.net/bwa.shtml for possible options.

Example command:

```
bwa samse referenceSequence/rice.fa
mergedTagCounts/myAlignedMasterTags.saimergedTagCounts/myMasterTags.cnt.fq
> mergedTagCounts/myAlignedMasterTags.sam
```

Gory Details:

The last two letters of “samse” stand for “single-ended”. Paired-end read alignment is possible, but not used in GBS (which produces single end reads).

BWA outputs only one record (one line of text) for each read regardless of how many places it aligns to the reference. The user cannot specify which alignment is chosen to “represent” a read with multiple mappings. However, the coordinates of the alternative mappings can be found at the end of the record, prefixed with “XA:”.

Indexing with bowtie2

Summary:

Creates a series of support files needed to operate bowtie2. You must have bowtie2 installed on your computer. For more details, consult the bowtie2 manual (http://computing.bio.cam.ac.uk/local/doc/bowtie2.html).

Input:

- A FASTA file containing one record for each chromosome or contig in the genome. In order for the SAMConverterPlugin (see below) to work correctly, the header of each record should contain only a single integer corresponding to that chromosome or contig’s number. The suffix “chr” prior to the chromosome number is permissible, but no others. For example, the headers “>1”, “>2”, “>3”, etc. are acceptable, as are “>chr1”, “>chr2”, “>chr3”, etc.

Output:

- A series of support files with the same name as the output base name but with different suffixes.

Key Arguments:

bowtie2-build	
input fasta files	a comma separated list of input fasta files (see above for header information)
output base name	output file base name (e.g. ZmB73_RefGen_v2.fa)

Example command:

```
bowtie2-build
chr1.fasta,chr2.fasta,chr3.fasta,chr4.fasta,chr5.fasta,chr6.fasta,chr7.fasta
,chr8.fasta,chr9.fasta,chr10.fasta,chrPt.fasta,chrMt.fasta,chrUNKNOWN.fasta
ZmB73_RefGen_v2.fa
```

Gory Details:

This command builds a set of indices from the reference genome. These indices are subsequently used by the “bowtie2” command for fast alignment of tags. For more details see the bowtie2 manual.

The Tassel3 GBS pipeline can use tag alignments produced either by BWA or by the default (-M) mode of bowtie2. In our experience, bowtie2 is more sensitive than BWA, and produces results that are more similar to BLAST. The tradeoff of the greater sensitivity of bowtie2 is that misalignment of tags will occur more often (e.g., tags from paralogous loci or from inserted sequences not present in the reference).

Alignment with bowtie2

Summary:

Aligns the master set of GBS tags to the reference genome. This input master tag list is stored in the fastq (.fq) file produced by TagCountToFastqPlugin (or the MergeMultipleTagCountPlugin with the -t option).

Input:

- Tag count file in FASTQ format (.fq) produced by the TagCountToFastqPlugin (or the MergeMultipleTagCountPlugin with the -t option).

Output:

- SAM alignment file that can be read by the SAMConverterPlugin of our GBS pipeline.

Key Arguments:

bowtie2	
-M X	The "-M mode" is the default mode of bowtie2, where it "*searches for at most X+1 distinct, valid alignments for each read. The search terminates when it can't find more distinct valid alignments, or when it finds X+1 distinct alignments, whichever happens first.* ***Only the best alignment is reported***" (ties are resolved at random). If multiple valid alignments are found, the alignment score for the second best alignment is stored in the XS:i field of the SAM output (alignment info) for that tag. In bowtie 2.1, this -M flag is deprecated, as it is the default mode. Therefore, omitting the -M flag should work in the same manner, except that search depth is now controlled by the -D and -R options (defaults of 15 and 2, respectively). Consult the bowtie2 manual for more details: http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml
-p X	The number of processors to be used. More is faster.
--very-sensitive-local	This sets the sensitivity.
-x	The basename of the reference genome index.
-U	The input fastq file.
-S	The output sam file name.

Example command:

```
bowtie2 -M 4 -p 15 --very-sensitive-local -x ../zeareference/bowtie2/ -U
AllZeaMasterTags_c10_20120607.fq -S AllZeaMasterTags_c10_20120613.sam
```

Gory Details:

This plugin performs an alignment of the GBS tags to a reference genome and reports the results in a SAM formatted file. Consult the bowtie2 manual for more details: http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml

The output SAM file will be converted to a Tags On Physical Map (TOPM) file in a subsequent step (SAMConverterPlugin). This subsequent conversion to a TOPM will only work properly if the default -M mode ("*search for multiple alignments, report the best*") of bowtie2 was used here.

SAMConverterPlugin

Summary:
Converts a SAM format alignment (.sam) file produced by one of the aligners, BWA or bowtie2, into a binary tagsOnPhysicalMap (.topm) file that can be used by the TagsToSNPByAlignmentPlugin for calling SNPs.

Input:

- SAM format alignment (.sam) file produced by BWA or by the default (-M) mode of bowtie2

Output:

- binary tagsOnPhysicalMap (.topm) file that can be used by the TagsToSNPByAlignmentPlugin for calling SNPs

Arguments:

SAMConverterPlugin	
-i	Alignment file in SAM format (.sam) produced by BWA or bowtie2.
-o	Output TagsOnPhysicalMap (TOPM) file that can be used by the TagsToSNPByAlignmentPlugin for calling SNPs (or by the SeqToTBTHDF5Plugin or FastqToTBTPlugin as a master tag list). We recommend using the extension ".topm". In addition to the tags that aligned to a single best genomic position, tags with either multiple positions or no alignment are still included in this output TOPM (in other words, all tags that were fed into BWA or bowtie2 should end up in the TOPM file produced by this plugin, regardless of whether they could be aligned to the genome or not, or of how many positions they aligned to).

Example command:
```
/programs/tassel/run_pipeline.pl -fork1 -SAMConverterPlugin
-i mergedTagCounts/myAlignedMasterTags.sam -o topm/myMasterTags.topm
-endPlugin -runfork1
```

Gory Details:
In order for this step to work correctly, **the chromosome names** (sequence headers) **in the FASTA** reference genome **file** used as input to BWA or bowtie2 **must be integers**, *e.g.*, >1, >2, or >3. We currently do not have any provision for text names such as >chrom1, >chrom2, >chrom3, etc. The **only exception** is that we do allow usage of the prefix "chr" so that >chr1, >chr2, >chr3, etc. are acceptable headers. In the case of non-numerical chromosome names (X, Y, mt, cp) or polyploid genomes, you will need to rename them as integers (and keep track of how your new names back-translate).

If you used bowtie2 instead of BWA, in order for this SAMConverterPlugin to work correctly, you must have run bowtie2 in its default (-M) mode ("*search for multiple alignments, report the best*"). With bowtie2 output, when there are multiple valid alignments for a tag and the best two have identical alignment scores, then it is not possible to know how many different genomic positions are tied for first place. Hence, when there are multiple ties for best genomic position for a tag in bowtie2, the number of positions is recorded as "99" in the output TagsOnPhysicalMap (.topm) file for that tag.

FastqToTBTPlugin

Summary:
Generates a TagsByTaxa file for each FASTQ file in the input directory (or in subfolders thereof). One TagsByTaxa file is produced per FASTQ file. Requires a master list of tags of interest, which may come either from a tagCount (.cnt) or tagsOnPhysicalMap (.topm) file. **If your input files are in QSEQ format, use**

QseqToTBTPlugin instead (same arguments). To obtain a single TagByTaxa file in HDF5 format, and thus reduce the amount of disk space required for a large analysis, use the SeqToTBTHDF5Plugin instead of this FastqToTBTPlugin

Input:

- Directory (folder) containing FASTQ files
- Barcode key file (see example in Appendix 1)
- Master tag list in the form of either a ***binary*** tagCount (.cnt) file or a tagsOnPhysicalMap (.topm) file

Output:

- Directory (folder) containing a corresponding tagsByTaxa file for every FASTQ file in the input directory

Arguments:

FastqToTBTPlugin	
-i	Input directory containing FASTQ files with raw GBS sequence reads.
-k	Barcode key file. See Appendix 1 for an example.
-e	Enzyme used to create the GBS library (e.g., ApeKI).
-o	Output directory.
-c	Minimum taxa count within a FASTQ file for a tag to be output. Default: 1
-t	Master tagCount (.cnt) file containing the tags of interest. This file must be binary (.cnt). The -t option is mutually exclusive with the -m option.
-m	TagsOnPhysicalMap (.topm) file containing the tags of interest. The -m option is mutually exclusive with the -t option.
-y	Output in TBTByte format (counts from 0-127) instead of TBTBit (0 or 1)

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -FastqToTBTPlugin -i fastq -k
myGBSProject_key.txt -e ApeKI -o tbt -y –t mergedTagCounts/myMasterTags.cnt
-endPlugin -runfork1
```

Gory Details:

Similar to FastqToTagCountPlugin, FastqToTBTPlugin parses FASTQ files containing raw GBS sequence data for good reads that contain a barcode and cut site remnant and that have no N's in the first 64 bases after the barcode, and trims them to 64 bases (not including the barcode). As in FastqToTagCountPlugin, FastqToTBTPlugin appropriately truncates reads that contain either a full cut site or the beginning of the common adapter within the first 64 bases, and pads them to 64 bases with polyA. In a given GBS analysis, the same **key file (-k option)**, containing the names of the taxa corresponding to each barcode in each lane, is used for both plugins (see Appendix 1 for an example key file).

The difference between FastqToTBTPlugin and FastqToTagCountPlugin is that FastqToTBTPlugin uses the barcode information to keep track of which taxa each tag of interest is observed in. Each good read in each FASTQ file is checked for a match against a set of tags of interest. A tagsByTaxa output file is produced for every FASTQ file in the input directory (and all of its sub-directories) with a matching flowcell and lane in the key file. Each output file is named after its corresponding input FASTQ file but with "_fastq.txt.gz" or "_fastq.txt" (etc.) replaced by ".tbt.bin" or ".tbt.byte" (depending on the format selected: tbt.bin by default, tbt.byte if the -y option is invoked, which we recommend). Each output tagsByTaxa file is in binary format (only readable by our pipeline), but can be thought of as a grid where the rows are the tags of interest (the actual length in bases of each tag - not including the polyA padding - is also recorded), the columns headers are taxa (sample) names (including flowcell, lane and well information) and the cells indicate the number of times a tag was observed in a given taxon (= read depth of each tag in each taxon).

When the **-y option** is used (**recommended**), cells have a maximum value of 127 reads per tag per taxon; by default, they have a value of only 1 or 0 (indicating presence or absence of a tag in a taxon). Storing the number of reads per tags per taxon (with the -y option) makes it possible to score heterozygotes & homozygotes quantitatively and thus attempt to distinguish true heterozygotes from apparent heterozygotes resulting from sequencing error. For example, if one allele at a SNP is observed 20 times in an individual and the other allele is observed only once, then that individual will be called homozygous at that SNP (the actual SNP calling is performed by the TagsToSNPByAlignmentPlugin). In contrast, if one allele is observed 12 times and the other 8 times, then that individual will be called heterozygous for the SNP in question.

In the output TBT, each taxon (sample) is named “SampleName:Flowcell:Lane:LibraryPrepID” (or, if the key file does not contain LibraryPrepIDs, then “SampleName:Flowcell:Lane:Well”). The “**short name**” of the taxon (sample) is the “SampleName” part (up to the first colon). The “**full name**” is “SampleName:Flowcell:Lane:LibraryPrepID” or “SampleName:Flowcell:Lane:Well”.

The set of tags of interest are those that are present in the **input master tagCount file** (using the **-t option**) ***or*** **tagsOnPhysicalMap file** (using the mutually exclusive **-m option**). We usually use the **-t** option, using the output of TagCountToFastqPlugin (or MergeMultipleTagCountPlugin) as the **-t** option input file for this FastqToTBTPlugin step.

If you use the -t option, the input tagCounts file (master tag list) must be binary (.cnt). In other words, if you use the Linux command `head` (or `less`) on this file, it should look like random gibberish, not human-readable text. If your analysis (discovery build) encompasses only a single lane of raw GBS sequence data, then the input master tagCount (.cnt) file for this FastqToTBTPlugin will have been produced by the FastqToTagCountPlugin. On the other hand, if your analysis (discovery build) encompassed more than one lane of raw GBS sequence data, then the input master tagCount (.cnt) file for this FastqToTBTPlugin will have been produced by the MergeMultipleTagCountPlugin without the -t option (i.e., a binary .cnt file, not a text fastq [.fq] file).

The restriction enzymes currently supported by our pipeline (and their corresponding common adapters) are indicated in the FastqToTagCount section above.

We generally leave the **-c option** (*minimum taxa count within a FASTQ file for a tag to be output*) at its default value of 1. Filtering of *tags* based upon the number of taxa they appear in would be better performed at the MergeTagsByTaxaFilesPlugin step, but is not currently implemented (however, filtering of *SNPs* based upon data coverage/amount of missing data can be performed with the GBSHapMapFiltersPlugin). With the default **-c option** of 1, tags that are in the master tagCount file but are not present in a given FASTQ file will not be output into the corresponding tagsByTaxa file - this is a good thing, as it saves disk space (no need to store rows containing nothing but zeros).

If your sequence data **predates** CASAVA 1.8 (a version of the Illumina software for generating the sequence files released in 2011) and you thus have it in both QSEQ and FASTQ format, we recommend using the QSEQ files if possible because they contain *all* reads, not just the ones passing Illumina’s quality filters. (CASAVA 1.8 only provides FASTQ files but these contain all reads.) We have found that perfectly good reads - exactly matching a 64 base tag that we have seen many times - can fail to pass Illumina’s filters. To analyze QSEQ input files instead of FASTQ, use the QseqToTBTPlugin, which uses exactly the same arguments as this FastqToTBTPlugin.

The multiple tagsByTaxa files produced by this FastqToTBTPlugin can be merged into a single master tagsByTaxa file in the next step, MergeTagsByTaxaFilesPlugin.

MergeTagsByTaxaFilesPlugin

Summary:
Merges all .tbt.bin and/or (preferably) .tbt.byte files present in the input directory and all of its subdirectories.

Input:

- Directory (folder) containing multiple tagsByTaxa (.tbt.byte or .tbt.bin) files (produced by FastqToTBTPlugin). For the best genotyping results (proper calling of heterozygotes), we recommend using .tbt.byte files as input (produced by the FastqToTBTPlugin using the -y option)

Output:

- Merged tagsByTaxa file (it is best to send this to a separate directory from the input directory)

Arguments:

MergeTagsByTaxaFilesPlugin	
-i	Input directory containing multiple tagsByTaxa files (preferably tbt.byte files).
-o	Output file name (should be in a separate directory from the input). Use extension matching the type of input file (".tbt.byte" or ".tbt.bin").
-s	Maximum number of tags the TBT can hold while merging (default: 200,000,000). Reduce this only if you run out of memory (omit the commas).
-x	Merges tag counts of taxa with identical short names. Not performed by default.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -MergeTagsByTaxaFilesPlugin -i tbt
-o mergedTBT/myStudy.tbt.byte -endPlugin -runfork1
```

Gory Details:

This step merges the separate tagsByTaxa files produced by the FastqToTBTPlugin (and/or QseqToTBTPlugin) into a single, experiment-wide tagsByTaxa (TBT) file for all of the flow cell lanes in your experiment.

The **-s option** controls the maximum number of tags that can be stored in the TBT tag list during the merger process. It defaults to 200,000,000. This is much larger than is needed for most purposes. If you try to run MergeTagsByTaxaFilesPlugin but run out of memory, invoke this option with a number smaller than 200,000,000. Use the largest possible number that your memory capacity can handle. This should be *at least* twice the number of tags in the master tagCounts (or master tagsOnPhysicalMap) file that you used to generate the individual tagsByTaxa files (in the FastqToTBTPlugin).

The **-x option** (off by default) can be invoked to merge the tag counts of taxa with identical short names (*i.e.*, with the same "SampleName" part of their full name, where the full name is either "SampleName:Flowcell:Lane:LibraryPrepID" or "SampleName:Flowcell:Lane:Well"). These taxa have the same SampleName (or DNASampleName) in the key file but were run on different flow cells, lanes or in the same lane but with different barcodes. However, we recommend that you do not invoke the -x option and thus leave in any duplicated taxa for now, as they can be used in a later step (GBSHapMapFiltersPlugin, BiparentalErrorCorrectionPlugin, or MergeIdenticalTaxaPlugin) to check error rates and to verify that there have been no sample mix-ups among the replicates.

SeqToTBTHDF5Plugin

Summary:

This plugin processes all of the raw GBS sequence files (FASTQ or QSEQ format) in the input directory (and all of its subdirectories) and generates a "Tags by Taxa" (TBT) data file in HDF5 format. This plugin and the ModifyTBTHDF5Plugin are newer additions to the pipeline that can be used in place of the FastqToTBTPlugin and the MergeMultipleTagsByTaxaFilesPlugin (which do not produce HDF5 formatted output). Only reads that match one of the tags in the input master tag list will be recorded in the output TBT HDF5. The input master tag list can be in the form of either a binary tag count (.cnt) file or a TagsOnPhysicalMap (.topm) file.

Input:

- Directory (folder) containing FASTQ or QSEQ raw GBS sequence files
- Barcode key file (see example in Appendix 1)
- Master tag list in the form of either a binary tagCount (.cnt) file or a tagsOnPhysicalMap (.topm) file

Output:

- A single TagsByTaxa (TBT) file in HDF5 format (*TBT.h5) recording how often each GBS tag in the master tag list was observed in each taxon (sample) present in the input FASTQ or QSEQ files. A "taxon" in the output TagsByTaxa file represents an individual DNA sample from a particular flowcell lane that has been distinguished by a particular barcode.

Arguments:

SeqToTBTHDF5Plugin	
-i	Input directory containing FASTQ or QSEQ raw GBS sequence files
-k	Barcode key file. See Appendix 1 for an example.
-e	Enzyme used to create the GBS library (see FastqToTagCountPlugin for list of available enzymes).
-o	Output TagsByTaxa (TBT) file in HDF5 format. Use the extension ".h5".
-s	Max good reads per lane. (Optional. Default is 500,000,000).
-L	Output log file
-t	Master tagCount (.cnt) file containing the tags of interest. This file must be binary (.cnt). The -t option is mutually exclusive with the -m option.
-m	TagsOnPhysicalMap (.topm) file containing the tags of interest. The -m option is mutually exclusive with the -t option.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -SeqToTBTHDF5Plugin -i fastq
-k key/AllZea_key.txt -e ApeKI -o tbt/AllZeaTBT.h5 -s 900000000 -L
tbt/AllZeaTBT.log -t mergedTagCounts/AllZeaMasterTags.cnt -endPlugin
-runfork1
```

Gory Details:

The purpose of the SeqToTBTHDF5Plugin is to produce a TagsByTaxa (TBT) file in HDF5 format recording how many times each GBS tag of interest was observed in each taxon (sample). It is more recent alternative to the FastqToTBT and MergeMultipleTagsByTaxaFiles plugins, which (together) also produce a single, master TBT file, but that TBT file is not in HDF5 format.

The tags of interest are limited to those that appear in either the master tag count file or the TOPM derived from that master tag list. If you use the **-t option**, then the tags of interest are those present in the supplied master tag counts (.cnt) file produced by the MergeMultipleTagsByTaxaPlugin (or, if your experiment encompasses only one lane of data, by the FastqToTagCountPlugin) which must be a binary file (see the FastqToTBTPlugin for more explanation of this). Alternatively, if you use the **-m option**, the master list of tags of interest can be obtained from a binary TagsOnPhysicalMap (.topm) file.

The information in the key file (flowcell, lane, barcode) is used to assign GBS reads matching one of the tags of interest to a given sample (taxon). Each taxon is named "SampleName:Flowcell:Lane:LibraryPrepID" in the output TBT (or, if the key file does not contain LibraryPrepIDs, then "SampleName:Flowcell:Lane:Well"). If the same GBS library (combination of a DNA sample, barcode, and well in GBS library prep plate) was sequenced multiple times (on different lanes or flowcells) then the TBT HDF5 will contain multiple, replicate entries -- these can be merged in the next step, ModifyTBTHDF5Plugin, provided that the key file contains LibraryPrepIDs.

The change to the TBT HDF5 format was driven by our need to efficiently process hundreds of flow cell lanes and tens of thousands of samples. HDF5 is a mature format designed to efficiently work with large data sets (*e.g.*, weather data, astronomical data, etc.). The only drawback of the TBT HDF5 format compared to our previous TBT.byte format (produced by the FastqToTBT and MergeMultipleTagsByTaxaFiles plugins) is that the TBT HDF5 file cannot be converted to a human-readable text format (*i.e.*, the BinaryToTextPlugin does not work for TBT HDF5). However, you can use the program HDF5View (available from http://www.hdfgroup.org/hdf-java-html/hdfview/) to manually inspect a TBT HDF5 file.

Similarly to the tbt.byte format, the HDF5 TBT format holds counts from between 0-127 recording the number of times each tag of interest was observed in each taxon (= read depths per tag per taxon). Counts larger than 127 are recorded as 127.

ModifyTBTHDF5Plugin

Summary:
This plugin makes changes to a TBT HDF5 file. There are three things that it can do, but it can only do one of them at a time:

1. Merge two TBT HDF5 files into one, or
2. Merge taxa with identical LibraryPrepIDs, or
3. Transpose the TBT HDF5 into an orientation that is more efficiently used for SNP calling (by allowing faster access of all the counts across taxa for a particular tag).

Input:

- TBT HDF5 (*TBT.h5) file (one or multiple depending on action)

Output:

- Modified TBT HDF5 (*TBT.h5) file

Arguments:

ModifyTBTHDF5Plugin	
-o	Target TBT HDF5 (*TBT.h5) file to be modified
One of either:	(depending on the modification you wish to make)
-i	TBT HDF5 (*TBT.h5) file containing additional taxa to be added to the target TBT HDF5 file
-c	Merge taxa with same LibraryPrepID in the target TBT HDF5 file
-p	Pivot (transpose) the target TBT HDF5 file into a tag-optimized orientation

Example commands:

Merging two TBT HDF5 files:

```
/programs/tassel/run_pipeline.pl -fork1 -ModifyTBTHDF5Plugin -o
mergedTBT/mergedTBT.h5 -i tbt/part2TBT.h5 -endPlugin -runfork1
```

Merging taxa with the same LibraryPrepID:

```
/programs/tassel/run_pipeline.pl -fork1 -ModifyTBTHDF5Plugin -o
mergedTBT/mergedTBT.h5 -c -endPlugin -runfork1
```

Pivot (transpose) a TBT HDF5 file:

```
/programs/tassel/run_pipeline.pl -fork1 -ModifyTBTHDF5Plugin -o
mergedTBT/mergedTBT.h5 -p pivotedTBT/pivotedTBT.h5 -endPlugin -runfork1
```

Gory Details:

The gory details for this plugin are organized by the three different functions this plugin can perform:

1) Merging two TBT HDF5 files into one TBT HDF5 file (**-i option**):

If you are working on a large project, to reduce the total amount of time it takes to create the "master" TBT HDF5 file, you might chose to run the SeqToTBTHDF5Plugin on multiple computers or processors and thus create multiple TBT HDF5 files. This will result in multiple TBT HDF5 files which must be combined into one master file. To use the ModifyTBTHDF5Plugin to merge two TBT HDF5 files, use the **-o option** to specify an existing **target TBT HDF5 file** and the **-i option** to specify an existing **input TBT HDF5** to be added to the target. In practice, it is best to make a copy of what is to be the initial target TBT HDF5. This allows the original file to be kept. This plugin is then run repeatedly until all TBT HDF5 files generated in the SeqToTBTHDF5 step are merged into the target. For example, assume that the SeqToTBTHDF5Plugin was run in three stages (with each stage working with a different set of input FASTQ files), and that the output TBT HDF5 files were in a folder named "tbt" and were named

`part1TBT.h5,`
`part2TBT.h5`, and
`part3TBT.h5`.

To merge these three TBT HDF5 files, first, make a copy of `part1TBT.h5` named `mergedTBT/mergedTBT.h5`:

```
cp tbt/part1TBT.h5 mergedTBT/mergedTBT.h5
```

Then, run this ModifyTBTHDF5Plugin with the arguments:

```
-o mergedTBT/mergedTBT.h5 -i tbt/part2TBT.h5
```

Then, run it again with the arguments:

```
-o mergedTBT/mergedTBT.h5 -i tbt/part3TBT.h5
```

The TBT HDF5 file `mergedTBT/mergedTBT.h5` will then be a merger of all three parts.

2) Merging taxa by LibraryPrepID (**-c option**):

We typically run GBS at 384-plex and, if higher depth of coverage is desired, run the resulting pooled GBS library in replicate on multiple flow cell lanes (usually on four different lanes, with each lane on a different flow cell). In addition to increasing depth of coverage, this has the added benefits of spreading out systematic sequencing errors and of allowing lane effects and sample effects to be distinguished in statistical analyses of read depth per tag. To identify the replicate runs of each library prep (where a library prep is a particular combination of sample DNA and barcode in a particular well of a library prep plate), we assign each library prep a distinct LibraryPrepID which is recorded in the barcode key file (see Appendix 1). If you have run some of your library preps in replicate in this manner, and have recorded distinct LibraryPrepIDs in the key file for each Sample/Barcode/libraryPlateWell combination, then you can use the **-c option** of the ModifyTBTHDF5Plugin to merge the tag counts of the replicate library preps. When LibraryPrepIDs are present in the key file, taxa in the TBT files are named as SampleName:Flowcell:Lane:LibraryPrepID (rather than SampleName:Flowcell:Lane:Well). Replicate library preps will have the same SampleName and LibraryPrepID but the Flowcell and/or Lane portions of their name will be different. Using ModifyTBTHDF5Plugin with the **-c option** will merge the tagCounts for each set of taxa having the same LibraryPrepID by summing the counts for each tag. The resulting, merged taxon will be named SampleName:MRG:4:LibraryPrepID, where the 4 indicates that four replicates with the same LibraryPrepID were merged. The -c option of this plugin operates on only one file (the target TBT HDF5 file specified by the -o option) and changes that file. It is best to make a copy of the original TBT HDF5 file before performing this operation.

3) Pivot (transpose) TBT HDF5 into a tag-optimized orientation (**-p option**):

The TBT HDF5 created so far are in a taxon-optimized orientation best suited for operations involving taxa (adding and merging taxa). In order for the SNP caller (TagsToSNPByAlignmentPlugin) to run efficiently, the master TBT HDF5 needs to be in a tag-optimized orientation, allowing fast retrieval of the counts across taxa for a particular tag. You can produce a new, tag-optimized TBT HDF5 by using the **-p option** of this plugin. The target TBT HDF5 file (specified by the -o option) will not be changed by this operation.

TagsToSNPByAlignmentPlugin (the Discovery SNP Caller)

Summary:
Aligns tags from the same physical location against one another, calls SNPs from each alignment, and then outputs the SNP genotypes to a HapMap format file (one file per chromosome).

Input:

- TagsByTaxa file (.tbt.byte or a tag-optimized TBT.h5) indicating the number of times each tag of interest was observed in each taxon. Use of a TBTBit (.tbt.bin) file is not recommended.
- TagsOnPhysicalMap file (.topm) containing genomic position of each tag of interest

Output:

- One HapMap format genotype file (.hmp.txt or .hmp.txt.gz) per chromosome.

Arguments:

TagsToSNPByAlignmentPlugin	
-i	Input TagsByTaxa (TBT) file. If you are using a TBT in .tbt.byte format, then use the -y option as well.
-y	Indicates that the input TBT specified by the -i option is in TBTByte (.tbt.byte) format (with counts from 0-127) rather than TBT HDF5 (*TBT.h5) format (also with counts from 0-127) or TBTBit (.tbt.bin) format (with counts of 0 or 1). Either TBTByte (.tbt.byte) or TBT HDF5 (*TBT.h5) format are recommended. If you use a TBTBit (.tbt.bin), then heterozygotes will be improperly called at higher coverage SNPs. If you don't use the -y option, then the type of TBT input file (TBT HDF5 or TBTBit) is determined from its file extension (.h5 or .tbt.bin, respectively)
-m	TagsOnPhysicalMap (.topm) file containing genomic position of tags.
-mUpd	Update the TOPM file with variants called during SNP calling.
-o	Output HapMap genotype file. Use a plus sign (+) as a wildcard character in place of the chromosome number (*e.g.,* -o hapmap/raw/myGBSGenos_chr+.hmp.txt). If you use a ".gz" suffix at the very end of the filename, the output genotype files will be gzip compressed.
-mxSites	Maximum number of sites (SNPs) output per chromosome (default: 200,000).
-mnF	Minimum value of F (inbreeding coefficient = 1-Ho/He). Not tested by default.

-p	Optional pedigree file containing full sample names & expected inbreeding coefficient (F) for each. Only taxa (samples) with expected F >= mnF used to calculate F (= 1-Ho/He) when applying the -mnF filter. See Appendix 2 for an example pedigree file. Default: use ALL taxa to calculate F.
-mnMAF	Minimum minor allele frequency (default: 0.01). SNPs that pass *either* the specified minimum minor allele frequency (mnMAF) or count (mnMAC) will be output.
-mnMAC	Minimum minor allele count (default: 10). SNPs that pass *either* the specified minimum minor allele count (mnMAC) or frequency (mnMAF) will be output.
-mnLCov	Minimum locus coverage, *i.e.*, the proportion of taxa (samples) with at least one tag present from the TagLocus covering a SNP (default: 0.1).
-errRate	Average sequencing error rate per base (used to decide between heterozygous and homozygous calls) (default: 0.01).
-ref	Path to reference genome in fasta format. Ensures that a tag from the reference genome is always included when the tags at a locus are aligned against each other to call SNPs. The reference allele for each site is then provided in the output HapMap files, under the taxon name "REFERENCE_GENOME" (first taxon). DEFAULT: Don't use reference genome.
-inclRare	Include the rare alleles (3rd or 4th states) at sites. These are ignored by default (genotypes containing rare alleles are set to missing).
-inclGaps	Include sites where the major or minor allele is a gap. These sites are excluded by default.
-callBiSNPsWGap	For SNPs where the major and minor alleles are nucleotides, but the third allele is a gap (-), include the gap alleles in the genotype calls (default: ignore the gap alleles)
-sC	Start chromosome. Must be an integer.
-eC	End chromosome. Must be an integer.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -TagsToSNPByAlignmentPlugin -i
mergedTBT/myStudy.tbt.byte -y -m topm/myMasterTags.topm -mUpd
topm/myMasterTagsWithVariants.topm -o hapmap/raw/myGBSGenos_chr+.hmp.txt
-mnF 0.8 -p myPedigreeFile.ped -mnMAF 0.02 -mnMAC 100000 -ref
MyReferenceGenome.fa -sC 1 -eC 10 -endPlugin -runfork1
```

Gory Details:

In this step, a multiple sequence alignment is created for each "TagLocus" which is defined as a set of tags that align to the exact same genomic position and strand. The genomic position of a tag is defined by that of the first base on its barcoded end (after removing the barcode). SNPs are called from each TagLocus alignment. Tags with multiple or unknown physical genomic positions are not used for SNP calling. The SNP calls from each TagLocus are written to a genotype file in HapMap format, with one HapMap file produced per chromosome. With the **-o (output file) option**, you must provide the relative path and "generic" name of the output HapMap genotype file. This filename must include the wildcard character '+' in place of the chromosome number. For example, if you use the argument:

```
    -o hapmap/raw/myGBSGenos_chr+.hmp.txt
```

then the '+' character will be replaced by each chromosome number (from -sC to -eC) in the output files. If you only want genotypes for one chromosome (*e.g.*, chromosome 9), then the **-sC (start chromosome) and -eC (end chromosome) options** should both be the same (*e.g.*, `-sC 9 -eC 9`). If you use '.gz' as the suffix at the very end of the output file name, then the output HapMap file will be gzip compressed. Note that *.hmp.txt.gz files can be directly read by the Tassel GUI (you do not have to decompress them first), or by subsequent steps in the GBS pipeline (as long as you include the '.gz' in the generic input file name).

If you are working with highly homozygous inbred lines or a selfing species, then be sure to use the **-mnF (minimum F) option** (we suggest setting mnF to 0.8 or 0.9), where 'F' means 'inbreeding coefficient', and is calculated for each SNP as:

`F = 1 - Ho/He,`

where `Ho` = observed heterozygosity, and

`He` = expected heterozygosity = `2p(1-p)`, where `p` = the frequency of the major allele.

SNPs with a calculated F less than -mnF will be removed from the output. In species like maize which contain abundant paralogs (from ancient chromosomal duplications), this can filter out numerous bad SNPs.

If you are NOT working with inbred lines or a selfing species, then invoke the -mnF option with a low cutoff such as -0.1 (use double quotes to specify a negative number: -mnF "-0.1").

If the samples in your study (discovery build) are a mixture of inbred lines and outbred material, then you can use a **pedigree file** (**-p option**) to specify which samples are inbred. In that case, when applying the -mnF cutoff, only the samples with an expected F in the pedigree file that is greater than or equal to the value specified by the -mnF (minimum F) option will be used in the calculation of F for each SNP (for comparison to the cutoff set by -mnF). For more information on the format and content of a pedigree file, see Appendix 2.

The options **-mnMAF** (minimum minor allele frequency) and **-mnMAC** (minimum minor allele count) can be used to filter out SNPs with rare minor alleles that possibly result from sequencing errors. Keep in mind that SNPs that pass *either* of these criteria will be output. If you are working with a biparental family with 1:1 segregation you might try a mnMAF of 0.2 and an impossible to reach mnMAC much larger your total number of taxa, so that it is irrelevant (in that case, only the mnMAF will matter). With unrelated individuals and no expected range of acceptable minor allele frequencies, you might want to try a mnMAF of 0.02 (and an impossible to reach mnMAC much larger than your total number of taxa).

The **-mnLCov (minimum locus coverage)** option can be used to filter out SNPs with very high amounts of missing data from the output. "Locus Coverage" is the proportion of taxa (samples) that are covered by at least one of the tags comprising the TagLocus to which a SNP belongs. If the coverage at a TagLocus is less than that specified by the -mnLCov option, then none of the SNPs in that TagLocus will be output. TagLoci with high amounts of missing data most likely result from large restriction fragments (>400 bp) that are not amplified as efficiently in the PCR steps of the GBS protocol. The default value of -mnLCov is 0.1. If you want fewer SNPs, but those with higher coverage, then increase -mnLCov.

The TagsToSNPByAlignmentPlugin ("Discovery SNP Caller") can work with three different types of TBT input files, a TBTBit file (.tbt.bin), a TBTByte file (.tbt.byte), or, more recently, a tag-optimized TBT HDF5 file (*TBT.h5). The **input TBT file** is specified by the **-i option**. We started off working with TBTBit files that simply used 1 or 0 to record the presence or absence of each tag in each taxon. We then realized that at sites (SNPs) with higher depths of coverage, sequencing errors could result in homozygotes being erroneously called as heterozygotes (for example, if a TBTBit is used, a taxon covered by 20 reads of a tag for one allele, and 1 read of a tag for the other allele that actually came from a sequencing error would be stored as 1/1 (present/present) for each tag and would be called a heterozygote). So, we then adopted the TBTByte (.tbt.byte) format which can record read depths per tag per taxon ranging from 0-127 (depths >127 are stored as 127), along with a method for "quantitative" rather than "qualitative" SNP calling (see below). The **-y option** was then added to the TagsToSNPByAlignmentPlugin to indicate that the input TBT (specified by the -i option) is in byte format (.tbt.byte) format, which was preferred over the default of tbt.bin. Later, as explained in the SeqToTBTHDF5Plugin section above, we needed to develop a TBT HDF5 format in order to be able to expand the capacity of our pipeline to allow processing of tens of thousands of samples. As our TBT HDF5 file names end with ‘TBT.h5’, we now use the absence of the -y flag and the presence of the “.h5” extension to determine that the **input TBT (-i option)** is in TBT HDF5 format. So the moral of the story is:

If you are using a:	**-y flag**	**File Name**	**Counts**	**Recommended?**
TBTByte	ON	*.tbt.byte	0-127	Yes
TBT HDF5	OFF	*TBT.h5	0-127	Yes (for very large studies)
TBTBit	OFF	*.tbt.bin	0 or 1	NO!!

If you use a TBT HDF5 as the input to the TagsToSNPByAlignmentPlugin, it needs to be in “tag-optimized” orientation, allowing the fast retrieval of the counts across taxa for a particular tag. The TBT HDF5 created by the SeqToTBTHDF5Plugin (or by using the -i or -c options of the ModifyTBTHDF5Plugin) is in a taxon-optimized orientation (good for adding and merging taxa). A TBT HDF5 in a taxon-optimized orientation can be transposed (pivoted) into to a tag-optimized orientation by using the -p option of the ModifyTBTHDF5Plugin (see above for more details).

This TagsToSNPByAlignmentPlugin performs **quantitative SNP calling** based on an expected **sequencing error rate** specified by the **-errRate option** (default of 0.01). Cutoffs are calculated for the minimum number of reads of the “less tagged allele” needed to call a heterozygote, given the total number of reads across the two most covered alleles in a SNP in an individual taxon. These cutoffs are the minLessTaggedAlleleCounts such that the binomial likelihood ratio `p(Het)/p(Err) > 1`, where `p(Het)` is the binomial probability of the observed counts of the two alleles in an individual, assuming that the individual is in fact a heterozygote and that each allele is equally likely to be sequenced, and `p(Err)` is the binomial probability of the observed counts assuming that the individual is in fact a homozygote and all of the reads of less tagged allele result from sequencing errors, and that sequencing errors to the alternate allele occur at a rate specified by the **-errRate option** (default of 0.01). The cutoffs are calculated at the start, before any SNPs are called, and are written to the console output:

```
Initializing the cutoffs for quantitative SNP calling likelihood ratio
(pHet/pErr) >1

      totalReadsForSNPInIndiv  minLessTaggedAlleleCountForHet
      2                        1
      3                        1
      4                        1
      5                        1
      6                        1
      7                        2
      8                        2
      9                        2
      10                       2
      11                       2
      12                       2
      13                       2
      14                       3
      15                       3
      16                       3
      17                       3
      18                       3
      19                       3
      20                       3
      ETC…
```

From this table (which uses the default -errRate of 0.01), it can be seen that quantitative SNP calling only matters when a SNP in an individual sample has been covered by at least 7 reads in total. At less than 7 reads, it only takes one read of the less tagged allele to call a heterozygote. For a genotype covered by 20 reads in total for the the two most covered nucleotides, in order for a heterozygote to be called, the less tagged allele must be covered by at least 3 reads.

The multiple sequence alignments produced for each TagLocus sometimes contain gaps. In such cases, if the expected tag from the reference genome is not present in the alignment, and if the gaps are caused by small insertions relative to the reference, SNPs downstream of the insertion (toward the common adapter end of the tags) can end up with slightly wrong chromosomal positions. The expected tag from the reference genome may not be present either because the reference genome haplotype is not well-represented among your samples, or because the reference genome has a null allele for that GBS tag. A null allele could occur if the restriction enzyme cut site is absent, or if the restriction fragment is too large for efficient GBS. To remedy this problem, we added the **-ref (reference genome) option**, which ensures that a tag from the corresponding position and strand in the reference genome is always included in the multiple sequence alignment for each TagLocus. This ensures that small insertions relative to the reference do not result in slight errors in the positions of SNPs downstream of those insertions. The argument of the **-ref option** is the relative path and name of the reference genome file in FASTA format, including all chromosomes of interest, and where the header lines preceding the sequence of each chromosome consisting of nothing but “>1”, “>2”, “>3”, etc or “>chr1”, “>chr2”, “>chr3” etc.

We recommend that you DO NOT invoke the **-inclRare** option, so that 3rd and 4th allelic states (*i.e.*, triallelic and quadra-allelic SNPs) are ignored (genotypes set to missing). Any 3rd and 4th allelic states are far more likely to result from sequencing error than biological reality.

Similarly, we recommend that you do not invoke the **-inclGaps** option, so that small indels are not scored. Because of alignment issues for small indels (multiple, equally likely alignments), they can end up being positioned slightly differently in replicate runs of the plugin. Also, because our tags are all 64 bases (or smaller) in length, small indels in the middle of a tag alignment always result in artifactual, compensatory small indels of equal size at or near the end of the tag alignment. However, if you are interested in maximizing marker saturation (for example, for GWAS or for fine-mapping of a QTL), then you might want to invoke inclGaps: there will very likely be numerous sets of tag alignments that contain no SNPs but do contain a small indel. Note that with inclGaps invoked, a three base indel (for example) will be output as three consecutive single base gaps in the HapMap file (plus an additional three artifactual, single base gaps). If the insertion is not present in the reference genome, the three real gaps will all have the same position (the base in the reference genome immediately preceding the insertion). Essentially, they are redundant scorings of the same indel.

If you invoke the **-callBiSNPsWGap (call biallelic SNPs with a gap) option**, then, for SNPs where the major and minor alleles are both nucleotides (A, C, G, or T) but there is an apparent third allele that is a gap (-), the gap alleles will be included in the genotype calls (where “-” in the hmp.txt output file represents homozygous for a gap, and “0” represents a heterozygote for the gap and one of the other nucleotides). Our thinking behind including this option is related to the problem of imputing missing data. If actual homozygotes for a gap were scored as missing (the default when -callBiSNPsWGap is NOT invoked), then we will likely end up mis-imputing a nucleotide where one does not in fact exist. In practice, we have NOT found this option to be very helpful in maize. Furthermore, Tassel3 still has some issues encoding gaps: 0 = +/- which creates a new “+” allele and we no longer know whether the + means A, C, G, or T. Therefore we do not recommend invoking this option.

If you plan to use the Production Pipeline (RawReadsToHapMapPlugin) to perform production SNP calling on raw GBS sequence files (FASTQ files) produced AFTER running the Discovery Pipeline, then you will need to invoke the **-mUpd (update TOPM with variants) option**. If you invoke this option, then, for every SNP that is output to the HapMap (.hmp.txt or .hmp.txt.gz) genotypes file, variants will be added to the corresponding tags in the TOPM that comprise the TagLocus covering that SNP. Up to eight variants (SNPs) are stored per tag, with both the relative position of the variant within the tag (VariantPositionOffset) and the particular allele represented by that tag (VariantDefinition) being recorded for each variant. The same set of variants (VariantPositionOffsets) are stored for each tag within a TagLocus, but the VariantDefinitions will vary among the tags within a TagLocus, depend on the sequence of each tag. The argument to the -mUpd option is a new name for the modified TOPM file -- this will be a copy of the input TOPM (from the -m option), but with the variants now recorded. To avoid overwriting the input TOPM, we recommend that you provide a different name for the -mUpd option than that for the input TOPM (-m option).

The **HapMap genotype files** that we generate save disk space and memory by using single letters to represent phase unknown, diploid genotypes. Heterozygotes are represented by IUPAC nucleotide codes:

```
A = A/A
C = C/C
G = G/G
T = T/T
M = A/C
R = A/G
W = A/T
S = C/G
Y = C/T
K = G/T
N = missing data
```

Genotypes from tags matching the minus strand of the reference genome are complemented, so that they are recorded relative to the plus strand. Hence, all SNPs in the output are relative to the plus strand. For restriction fragment smaller than 128bp, the (plus and minus strand) reads from opposite ends can overlap and assay the same SNPs. Hence, the output of TagsToSNPByAlignmentPlugin will contain some duplicate SNPs, each with different patterns of missing data. These duplicate SNPs can be merged in the next step of the analysis, with the MergeDuplicateSNPsPlugin.

MergeDuplicateSNPsPlugin

Summary:
Finds duplicate SNPs in the input HapMap file, and merges them if they have the same pair of alleles (not necessarily in the same major/minor order) and if their mismatch rate is no greater than the threshold specified by **-maxMisMat**. If **-callHets** is on, then genotypic disagreements will be called heterozygotes; otherwise they will be set to missing (callHets is off by default).

Input:

- HapMap genotype files (.hmp.txt or .hmp.txt.gz). Use a plus sign (+) as a wild card character to specify multiple chromosome numbers (each chromosome in a separate file).

Output:

- HapMap genotype files (.hmp.txt or .hmp.txt.gz) (one per chromosome) in which duplicate SNPs have been merged

Arguments:

MergeDuplicateSNPsPlugin	
-hmp	Input HapMap genotype file(s) (.hmp.txt or .hmp.txt.gz). Use a plus sign (+) as a wildcard character to specify multiple chromosome numbers (each chromosome in a separate file).
-o	Output HapMap genotype file(s) (.hmp.txt or .hmp.txt.gz). Use a plus sign (+) as a wildcard character to specify multiple chromosome numbers (each chromosome in a separate file). If you use a ".gz" suffix at the very end of the filename, the output genotype files will be gzip compressed.

-misMat	Threshold genotypic mismatch rate above which the duplicate SNPs won't be merged. Default: 0.05
-p	Optional pedigree file containing full sample names & expected inbreeding coefficient (F) for each. Only taxa (samples) with expected F >= 0.8 (*i.e.*, S3 or more) will be used to test if two duplicate SNPs agree with each other. See Appendix 2 for an example pedigree file. Default: use ALL taxa to compare duplicate SNPs.
-callHets	When two genotypes at a replicate SNP disagree for a taxon, call it a heterozygote. Defaults to false (=set to missing).
-kpUnmergDups	When a pair of duplicate SNPs are not merged (because they have different alleles, too many mismatches, or the major or minor allele for one of them is a gap), keep them. Defaults to false (=delete them).
-sC	Start chromosome. Must be an integer.
-eC	End chromosome. Must be an integer.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -MergeDuplicateSNPsPlugin
-hmp hapmap/raw/myGBSGenos_chr+.hmp.txt -o
hapmap/mergedSNPs/myGBSGenos_mergedSNPs_chr+.hmp.txt -misMat 0.1 -p
myPedigreeFile.ped –callHets -sC 1 -eC 10 -endPlugin -runfork1
```

Gory Details:

This step is usually run directly after TagsToSNPByAlignmentPlugin, using the HapMap file(s) from that step as input. Duplicate SNPs arise from overlapping, but separate TagLoci that cover the same SNP. These overlapping TagLoci are usually on different strands, starting on either end of a restriction fragment that is less than 128 bp in length.

If the germplasm is not fully inbred, and still contains residual heterozygosity (like the maize NAM or IBM populations do) then **-callHets** should be on and **-maxMisMat** should be set fairly high (0.1 to 0.2, or even higher, depending on the amount of heterozygosity). Because the sequencing coverage is usually less than 1x, most of the time only one allele at a heterozygous SNP will be detected (particularly for *Ape*KI). Hence, duplicate SNPs genotypes from a true heterozygote may disagree simply because different alleles were sampled by the duplicate assays. Hence, these disagreements are not necessarily errors, and should not necessarily be used to prevent duplicate SNPs from being merged (unless your germplasm *is* highly inbred, with very little residual heterozygosity).

Indels (gaps) are ignored by this plugin: it makes no attempt to merge apparent duplicate sites with the same chromosomal position where either the major or minor allele is a gap.

GBSHapMapFiltersPlugin

Summary:

Reads HapMap format genotype files (one per chromosome) and filters out SNPs with low taxon coverage (missing data at most taxa), high heterozygosity, low (and/or high) minor allele frequency, or that are not in LD with at least one neighboring SNP. Taxa with low SNP coverage (missing data at most SNPs) can also be removed. All filters are off by default and all cutoffs are adjustable.

Input:

- HapMap genotype file(s) (.hmp.txt or .hmp.txt.gz)

Output:

- HapMap genotype file(s) (.hmp.txt or .hmp.txt.gz) with some SNPs and/or taxa filtered out

Arguments:

GBSHapMapFiltersPlugin	
-hmp	Input HapMap file(s) (.hmp.txt or .hmp.txt.gz). Use a plus sign (+) as a wild card character to specify multiple chromosome numbers (each chromosome in a separate file).
-o	Output HapMap file(s) (.hmp.txt or .hmp.txt.gz). Use a plus sign (+) as a wildcard character to specify multiple chromosome numbers (each chromosome in a separate file). If you use a ".gz" suffix at very end of the filename, the output genotype files will be gzip compressed.
-mnTCov	Minimum taxon coverage. The minimum SNP call rate for a taxon to be included in the output, where call rate is the proportion of the SNP genotypes for a taxon that are not "N" (where N = missing). Default: no filter.
-mnScov	Minimum site coverage. The minimum taxon call rate for a SNP to be included in the output, where taxon call rate is the proportion of the taxa with genotypes that are not "N" for that SNP (where N = missing). Default: no filter
-mnF	Minimum value of F (inbreeding coefficient). Not tested by default.
-p	Optional pedigree file containing full sample names & expected inbreeding coefficient (F) for each. Only taxa (samples) with expected F >= mnF used to calculate F (= 1-Ho/He) when applying the -mnF filter. See Appendix 2 for an example pedigree file. Default: use ALL taxa to calculate F.
-mnMAF	Minimum minor allele frequency Default: 0.0 (no filtering).
-mxMAF	Maximum minor allele frequency. Default: 1.0 (no filtering).
-hLD	Specifies that SNPs should be filtered for those in statistically significant LD with at least one neighboring SNP. Default: Off.
-mnR2	Minimum R-square value for the LD filter. Default: 0.01
-mnBonP	Minimum Bonferroni-corrected p-value for the LD filter. Default: 0.01
-sC	Start chromosome. Must be an integer.
-eC	End chromosome. Must be an integer.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -GBSHapMapFiltersPlugin
-hmp hapmap/mergedSNPs/myGBSGenos_mergedSNPs_chr+.hmp.txt -o
hapmap/filt/myGBSGenos_mergedSNPsFilt_chr+.hmp.txt -mnTCov 0.01 -mnSCov 0.2
-mnMAF 0.01 -hLD -mnR2 0.2 -mnBonP 0.005 -sC 1 -eC 10 -endPlugin -runfork1
```

Gory Details:

The **-mnTCov** and **-mnSCov options** allow you to filter out taxa and/or SNPs, respectively, with call rates lower than the specified cutoffs. These filters are off by default. If the -mnTCov (taxon filter) is invoked, it is applied first, so that taxa with very low call rates (*i.e.*, blanks and/or failed samples) are removed prior to applying any of the other filters. Taxa with low call rates are identified based only on the starting chromosome (specified by the -sC option), and then this same set of low call rate taxa is filtered from the output of all the chromosome. This is done to avoid the possibility of output genotype files for different chromosomes containing different sets of taxa, which could happen if some taxa hover above or below the -mnTCov cutoff on different chromosomes.

Filtering based on the **-mnF (minimum F) option**, and the optional use of the **-p (pedigree file) option** to specify which samples should be included in the calculation of F, are the same as described above for the TagsToSNPByAlignmentPlugin.

The **-mnMAF and -mxMAF options** allow you to select for those SNPs whose minor allele frequencies fall into an expected range. For example, if you are working in a backcross (or psuedo-testcross) family, with an expected minor allele frequency (MAF) of 0.25, you might set the -mnMAF at 0.15 and the -mxMAF at 0.35.

If your study samples are from a single, biparental cross (or from another type of population in which LD is fairly extensive along a chromosome), then the **-hLD (high LD) filter** (off by default) can be very useful to filter out bad SNPs with high genotyping error or incorrect physical genomic positions. If you invoke the -hLD filter, the cutoff minimum R^2 and Bonferroni-corrected *p*-value can be adjusted using the **-mnR2 and -mnBonP options** (both of these default to 0.01).   To pass through the LD filter, a SNP must be in statistically significant LD (Bonferroni corrected *p*-value less than that specified by the -mnBonP option) with at least one SNP that is a minimum of 128 bp away (*i.e.*, not from the same TagLocus or cut site) but within a window of 50 SNPs on either side. In Tassel3, **the LD filter only works properly for inbred lines** (*e.g.*, RILs). This will be fixed in Tassel4, so that the LD filter can be applied to outbred (highly heterozygous) populations as well.

BiParentalErrorCorrectionPlugin

Summary:
Takes advantage of the presence of biparental families among the germplasm in your discovery build (input HapMap file) as a powerful aid to filter out SNPs that either display high rates of genotyping error or are not in linkage disequilibrium with other nearby SNPs on the same chromosome.

Input:

- A HapMap genotype file (.hmp.txt or .hmp.txt.gz) in which a subset of the samples (taxa) belong to one or more biparental families

Output:

- A HapMap genotype file with SNPs removed (filtered out) that are either (1) error-prone, and/or (2) are not in LD in the biparental families with surrounding SNPs on the same chromosome.

Arguments:

BiParentalErrorCorrectionPlugin	
-hmp	Input HapMap file. Use a plus sign (+) as a wildcard character in place of the chromosome number to allow processing of multiple files from different chromosomes. File(s) can either be compressed (.hmp.txt.gz) or uncompressed (.hmp.txt).
-o	Output HapMap file. Use a plus sign (+) as a wild card character in place of the chromosome number to allow processing of multiple files from different chromosomes. File(s) can either be compressed (.hmp.txt.gz) or uncompressed (.hmp.txt).
-mxE	Maximum error rate (default: 0.05)
-mnD	Minimum segregation distortion factor (default: 2.5)
-mnPLD	Minimum median population LD (R^2) (default: -1.0 = don’t test for LD)
One of -popM, -popF, or -pedF	

-popM	Population mask: a regular expression specifying the biparental family-specific prefixes (or suffixes) in the names of samples that are part of a biparental population.
-popF	Population file: the name of a file containing the biparental family-specific prefixes (or suffixes) in the names of samples that are part of a biparental population. One population prefix per line.
-pedF	Pedigree file: the name of a file that lists the population (family) names, the full samples names, parents, parental contributions and the expected F for each taxon (sample) in the input Hapmap file. Taxa that are not part of a biparental family have "NA" as their family name. See Appendix 2 for more details.
-sC	Start chromosome. Must be an integer. Ignored if -hmp and -o arguments do not contain '+'.
-eC	End chromosome. Must be an integer. Ignored if -hmp and -o arguments do not contain '+'.
-kpUT	Keep SNPs that could not be tested for high error rate (off by default = remove them)

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -BiParentalErrorCorrectionPlugin
-hmp myMaizeGenosIncludingNAM_chr+.hmp.txt.gz -o
myMaizeGenosIncludingNAM_bpec_chr+.hmp.txt.gz -mxE 0.03 -mnD 2.0 -mnPLD 0.2
-popM Z[0-9]{3} -sC 1 -eC 10 -endPlugin -runfork1
```

Gory Details:

The BiParentalErrorCorrectionPlugin takes advantage of the presence of biparental families (populations) among the germplasm in your discovery build (experiment) as a powerful aid to filter out SNPs that either display high rates of genotyping error or are not in linkage disequilibrium with other nearby SNPs on the same chromosome. To use this plugin, not all of the samples in the input hapmap genotype file need to belong to a biparental population, and the presence of multiple biparental populations is accommodated for and taken advantage of. Currently, the expected segregation ratio of these biparental families is hard coded as 1:1 (*e.g.*, F_2 progeny or RILs derived therefrom).

Specifying which samples belong to which biparental families

There are three (mutually exclusive) ways to indicate which samples in the input hapmap genotypes file belong to which biparental families, by using either (1) the -popM, or "population mask" option, (2) the -popF, or "population file" option, or (3) the -pedF, or "pedigree file" option.

If the taxa (samples) that belong to each biparental population all have a unique prefix (or suffix) in their short names that indicates which population they belong to, then you can use the **-popM ("population mask") option** to provide a regular expression that will match these prefixes (or suffixes). For example, our maize discovery builds always contain the 25 biparental families that make up the maize Nested Association Mapping population (5000 RILs in total). The 200 RILs in each family are named Z###E####, where the Z### part indicates the particular biparental family (from Z001 to Z025) and the E#### part indicates the particular RIL ("entry") within that family (from E0001 to E0200). An example sample name for a NAM RIL is Z011E0099, which is the 99th RIL in the 11th NAM family. Samples belong to the NAM population can thus be classified by the BiParentalErrorCorrectionPlugin to their particular NAM family by using the following population mask:

```
    -popM Z[0-9]{3}
```

This regular expression ("`Z[0-9]{3}`") matches the prefixes Z001 through Z025 in the sample names (it looks for a "Z" followed by three numerical digits). The plugin then keeps track of which family each RIL belongs to (*e.g.*, Z011E0099 belongs to family Z011). Regular expressions are quite powerful, so, if you have particular prefixes (or suffixes) in your sample names that indicate which family each sample belongs to, it is likely that you will be able to supply a regular expression to match them (Google "regular expression" for more info).

A second way to indicate which samples belong to which biparental family is by using the **-popF ("population file") option** to provide the name of a text file containing all of the prefixes (or suffixes) for each biparental family, with one prefix (or suffix) per line. Using the above example of the NAM population, the corresponding population file would consist of 25 lines, as follows:

```
Z001
Z002
…
Z025
```

The third alternative, and most flexible, method to specify which samples are part of a biparental family is to use the **-pedF ("pedigree file") option** to indicate the name of a pedigree file in tab-delimited text format. See Appendix 2 for a description and example of the pedigree file.

Filtering of SNPs based on linkage disequilibrium
If your biparental families consist of predominantly homozygous RILs, then you can take advantage of the ability of the BiParentalErrorCorrectionPlugin to filter out SNPs that do not exhibit linkage disequilibrium (LD) in those families with surrounding SNPs on the same chromosome. Families consisting of highly heterozygous progeny should not be used for LD filtering, however, because the manner in which LD is calculated in Tassel3 assumes that the samples are inbred lines (this assumption has been lifted in Tassel4).

If your biparental families do consist of RILs, then you can invoke the LD filtering function of the BiParentalErrorCorrectionPlugin by setting the **-mnPLD (Minimum median population LD (R^2))** parameter to something larger than 0.0 but less than 1.0 (it is -1.0 by default = no LD-based filtering). The higher the value of the -mnPLD parameter, the more stringent is the LD filtering. For our maize discovery builds, which include the maize NAM set of biparental families, we typically set -mnPLD to 0.1. If LD filtering is invoked, then LD (R^2) is calculated for every SNP in every biparental family, provided that there are at least 10 non-missing genotypes for the SNP in that family, and that the minor allele frequency in that family is at least 0.15. If these criteria are met for a SNP in a particular family, then R^2 is calculated in that family for that SNP versus the 5% of the remaining SNPs from the same chromosome that most closely flank the SNP being tested, excluding SNPs that are within 100Kb of the site being tested. (This 100Kb exclusion is designed to prevent SNPs that fall within local mis-assemblies of the genome [or paralagous regions] from internally reinforcing one another.) If from among those flanking SNPs there are at least 5 that can be tested for LD versus the target SNP, then the median (within family) R^2 of those valid tests is recorded. Across multiple families, the median of the median within-family R^2 value is then determined. SNPs for which this median population R^2 (across families) is less than the value of the **-mnPLD parameter** will then be filtered from the output HapMap genotypes file. Note that SNPs that did not have valid within-family median R^2 values for any of the families (*e.g.*, SNPs that did not segregate with a MAF >= 0.15 in any of the families) are considered as "untested for LD" but are not removed from the output HapMap genotypes file.

Unlike the error detection portion of the BiParentalErrorCorrectionPlugin (see below), the LD filtering portion does not require expected segregation ratios of 1:1 in the biparental families, but only requires that there is some level of LD present with each biparental family at the local chromosomal scale, and that the population-specific minor allele frequency is at least 0.15. However, as mentioned above, the LD filtering does require that the progeny are predominantly homozygous RILs.

Detection of Error-Prone SNPs
For detection of error-prone SNPs, these requirements are reversed: the progeny need not be predominantly homozygous RILs, but the expected segregation ratio for each biparental families should be 1:1. Hence, the error detection part of the BiParentalErrorCorrectionPlugin can be used on either F2 families or families consisting of F2-derived RILs.

To detect and filter out error-prone SNPs, each SNP is tested in each biparental family for highly significant deviation (p<0.001) from the expected 1:1 segregation pattern using a binomial test. If you use a pedigree file (-pedF option) to denote the biparental families, only families where the expected parental contributions are 50% will be used for this test. Alternatively, if you use the -popM or -popF option to denote biparental families, this error detection part of the plugin will assume that all the families have expected segregation ratios of 1:1. Each SNP is only tested in a family if the number of non-missing allele calls for that SNP in the family is at least 19 (with homozygotes counted once and heterozygotes counted twice) -- this is to ensure adequate power for detection of significant (p<0.001) deviation from 1:1 segregation. Hence, depending on the amount of missing data, small biparental families (say, with fewer than 40 progeny) may not be useful for this error-detection part of the plugin. If a SNP is found to (1) significantly deviate from 1:1 segregation at p<0.001 in a family, **and** (2) to display a degree of segregation distortion such that the family-specific minor allele frequency is less than `expSegregation/minDistortionRatio` (where `expSegregation` is 0.5 and `minDistortionRatio` is the value set by the **-minD parameter** [default: 2.5]), then the number of allele calls for the family-specific minor allele and the total number of allele calls in that family are included in the calculation of an overall error rate across the families for each SNP. Essentially, if a SNP is polymorphic in a biparental family but deviates very strongly from the expected segregation ratio, then it is very likely that the family-specific minor allele calls result from sequencing errors (or other errors, such as alignment errors). Note that SNPs that do not segregate (are monomorphic) in a family (*i.e.*, no errors detected) are still included in the calculation of the overall error rate across families, as long as the number of non-missing genotype calls for that SNP in the family is at least 19.

Once all SNPs have been tested in each biparental family for highly significant segregation distortion, and overall error rates tallied across all of the families for each SNP, then the SNPs with an overall error rate greater than the `maxErrorRate` set by the **-mxE parameter** (default: 0.05) are filtered from the output HapMap genotype file. In addition, unless the **-kpUT ("keepUnTested for Error") option** is invoked (off by default), all SNPs for which the number of non-missing allele calls (with homozygotes counted once and heterozygotes counted twice) is less than 19 in all of the biparental families will be removed from the output HapMap genotype file (these are SNPs with undefined overall error rates because of very high amounts of missing data).

For SNPs that were not removed from the output, but that displayed high error rates (*i.e.*, severe segregation distortion) in particular biparental families, the apparent polymorphisms in those particular families are corrected, providing that the likelihood ratio `probErr/probSegDist >= 1` for the corresponding SNP in that family, where `probErr` is the binomial likelihood of the observed number of apparently erroneous allele calls in that family, based on the overall error rate, and `probSegDist` is binomial likelihood of the observed degree of segregation distortion, assuming an expected segregation ratio of 1:1. To correct the genotypes in such families, homozygotes for the family-specific minor alleles are set to missing, and heterozygotes are set to homozygous for the family-specific major allele, so that the SNP in question is no longer polymorphic in the family in question. Genotypes in taxa that are not part of a specific biparental family displaying high error rates for a SNP are not affected by this step.

Note that, in contrast to the LD filtering function of the BiParentalErrorCorrectionPlugin, which requires that the progeny of the biparental populations are predominantly homozygous RILs, the error detection part of this plugin should work even if the progeny in the biparental families are highly heterozygous, provided that the expected segregation ratio is 1:1 (*e.g.*, an F2 family). Although multiple biparental families are preferred, the error detection portion should also work even if there is only one biparental family amongst your samples (as should the LD filter).

MergeIdenticalTaxaPlugin

Summary:
Merges the genotypes of samples ("taxa") with identical short names (up to the first colon of their full name).

Input:

- A hapmap format genotype file (.hmp.txt or .hmp.txt.gz) containing replicate taxa

Output:

- A hapmap format genotype file in which the replicate taxa have been merged

Arguments:

<u>MergeIdenticalTaxaPlugin</u>	
-hmp	Input HapMap file. Use a plus sign (+) as a wildcard character in place of the chromosome number to allow processing of multiple files from different chromosomes. File(s) can either be compressed (.hmp.txt.gz) or uncompressed (.hmp.txt).
-o	Output HapMap file. Use a plus sign (+) as a wild card character in place of the chromosome number to allow processing of multiple files from different chromosomes. File(s) can either be compressed (.hmp.txt.gz) or uncompressed (.hmp.txt).
-xHet	Exclude heterozygote calls (default: false = call hets)
-hetFreq	Cutoff frequency between het vs. homozygote calls (default: 0.8)
-sC	Start chromosome. Must be an integer. Ignored if -hmp and -o arguments do not contain '+'.
-eC	End chromosome. Must be an integer. Ignored if -hmp and -o arguments do not contain '+'.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -MergeIdenticalTaxaPlugin
-hmp myGenos_chr+.hmp.txt.gz -o myGenos_taxaMerged_chr+.hmp.txt.gz -hetFreq
0.75 -sC 1 -eC 10 -endPlugin -runfork
```

Gory Details:

The MergeIdenticalTaxaPlugin merges all sets of two or more samples ("taxa") in the input hapmap genotypes file with the same short name (up to the first colon of the full name). For this plugin to work correctly, the remainder of the full sample names (after the first colon) need to be unique (i.e., different flowcell:lane:well) for each replicate sample. For example, three samples in the input hapmap genotype file with the full names "B73:42A87AAXX:2:A01", "B73:42A87AAXX:2:E12", and "B73:58C95AAXX:7:G10" will be merged in the output file, and the merged sample will be named "B73:MERGE".

Invoking the **-xHet** ("Exclude heterozygote calls") **option** sets apparently heterozygous genotypes in merged samples to missing. Invoke this option only if you are highly confident that all of your replicate samples are fully inbred with no heterozygosity whatsoever. Since this is rarely the case, most users will leave the -xHet option off (the default), so that apparent heterozygous sites in a merged sample are called as such. Note that the -xHet option, if invoked, will have no effect on the genotypes of *non-replicate* samples that are not merged with any others (*i.e.*, existing heterozygote calls in unique samples that are not merged with any other samples will not be set to missing even if -xHet is invoked). The -xHet option only affects the genotypes of merged samples.

The **-hetFreq option** is used to control the cutoff allele frequency between heterozygous and homozygous calls with respect to the most frequently observed allele among the replicate individuals to be merged. The value assigned to the -hetFreq parameter is used in the following manner to call heterozygotes in merged samples:

```
if ((nMajGenos+nHetGenos)/(nMajGenos+nMinGenos+2*nHetGenos) > hetFreq)
    geno = homMajor;
else if ((nMinGenos+nHetGenos)/(nMajGenos+nMinGenos+2*nHetGenos) > hetFreq)
```

```
    geno = homMinor;
else if (xHet)
    geno = missing;
else
    geno = heterozygous;
```

Where:
`nMajGenos` = the number of homozygous major allele genotypes observed among the replicate samples to be merged,
`nHetGenos` = the number of heterozygous genotypes, and
`nMinGenos` = the number of homozygous minor allele genotypes.

This algorithm assumes low coverage (~1x or less), so that, if a homozygote was called for a SNP in an individual sample (prior to merging), most of the time it would have been called on the basis of only a single read. In other words, homozygotes are counted only once toward their respective allele frequency, regardless of the actual number of reads behind the SNP call in the individual replicate sample (prior to merging).

Here is an example of the application of this algorithm. If you have 10 replicate samples ("taxa"), 8 of which have genotypes of A/A at a particular SNP, 2 with A/C (="M"), and 0 with C/C, and use the default value of -hetFreq of 0.8, then the genotype of the SNP in the merged sample will be A/A (coded as "A" in the hapmap output), since (8+2)/(8+0+2*2) = 0.833 which is > 0.8.

In contrast, if 7 have a genotype of A/A, 2 have a genotype of A/C ("M") and 1 has a genotype of C/C, then, provided that the -xHet is not invoked, the merged genotype will be called A/C ("M"), since (7+2)/(7+1+2*2) = 0.75 which is <= 0.8. However, if -xHet (exclude heterozygotes) is invoked, then the merged genotype would be called N/N (=N).

RawReadsToHapMapPlugin (the Production SNP Caller)

Summary:
The RawReadsToHapMapPlugin is the "Production Pipeline". You can use this plugin if you have already performed a large-scale "Discovery Build" in your species and have, since then, obtained GBS raw sequence data (FASTQ files) for some new samples that were not included in the Discovery Build. The RawReadsToHapMapPlugin allows you to quickly call genotypes in these new samples without having to redo a whole new Discovery Build, by using information on all the SNPs ascertained in the Discovery Build. This information, regarding which SNP alleles are represented by each tag, needs to have been made available in a "Production-ready" TagsOnPhysicalMap (Production TOPM) by the using the -mUpd option of the TagsToSNPByAlignmentPlugin ("Discovery SNP Caller").

Input:
- FASTQ or QSEQ sequence files
- Barcode key file
- Production TOPM created by the TagsToSNPByAlignmentPlugin ("Discovery SNP Caller") through use of the -mUpd flag

Output:
- HapMap genotype (.hmp.txt) files. One per chromosome per input sequence file.

Arguments:

RawReadsToHapMapPlugin	
-i	Input directory containing FASTQ and/or QSEQ files
-k	Barcode key file (see example in Appendix 1)

-e	Enzyme used to create the GBS library
-m	TagsOnPhysicalMap (.topm) file containing genomic positions of all of the tags of interest as well as which alleles each useful tag represents for the useful SNPs (variants) covered by that tag. This “Production TOPM” should have been populated with variants by using the -mUpd option at the TagsToSNPByAlignment (Discovery SNP calling) step.
-o	Output directory to contain output HapMap genotype (.hmp.txt) files. One output genotype file per chromosome is created per input raw GBS sequence file.

Example command:

```
/programs/tassel/run_pipeline.pl -fork1 -RawReadsToHapMapPlugin -i fastq -k
NewSamples_key.txt -e ApeKI -m topm/myProduction.topm -o hapmap/production
-endPlugin -runfork1
```

Gory Details:

The RawReadsToHapMapPlugin (“Production Pipeline”) allows you to quickly generate genotypes for new samples without having to perform an entirely new “Discovery Build” from scratch. The drawback of this is that any novel tags and associated novel SNPs present in your new samples, but not in the Discovery Build, will not be scored. Hence, it makes the most sense to run the RawReadsToHapMapPlugin (rather than performing a new Discovery Build from scratch that encompasses all samples to date) if the samples in your new raw GBS sequence (FASTQ) files are not expected to contain much novel genetic diversity relative to the samples run in your last Discovery Build, and if your last Discovery Build contained a large number (thousands) of samples. **The majority of users probably do not need to run the Production Pipeline (RawReadsToHapMapPlugin)**. Furthermore, the GBS Production Pipeline is more mature in Tassel4 than in Tassel3.

The list of available **restriction enzymes (-e option)** is the same as that provided in the FastqToTagCountPlugin section, above.

To run the RawReadsToHapMapPlugin, you need to have created a “Production TOPM” out of the TOPM used in your last Discovery Build. This should have been done by invoking the -mUpd option at the TagsToSNPByAlignmentPlugin (Discovery SNP Calling) step. This would have resulted in a copy of the input TOPM being produced in which variants were recorded for each useful SNP in each useful tag. See the above section on the TagsToSNPByAlignmentPlugin for more details. In the RawReadsToHapMapPlugin, the **Production TOPM** is specified by the **-m option**.

One issue that is not resolved in the Tassel3 GBS pipeline is that the Production TOPM produced by the -mUpd option of the TagsToSNPByAlignmentPlugin contains all of the SNPs called at that step. If you subsequently used either the GBSHapMapFiltersPlugin or the BiParentalErrorCorrectionPlugin to filter out “bad” SNPs, the only way to remove them from the Production TOPM is by using the KeepSpecifiedSitesInTOPMPlugin **from Tassel4**. As is the case for any Tassel plugin, if you call the Tassel4 KeepSpecifiedSitesInTOPMPlugin without any options, the options will be provided:

```
The options for the KeepSpecifiedSitesInTOPMPlugin are:
    -input   Input directory containing Site List files
    -orig    Original TOPM
    -result  Output, site-filtered TOPM
```

Here, the “Original TOPM” (-orig option) is the Production TOPM produced by the -mUpd option of the Tassel3 TagsToSNPByAlignmentPlugin, containing all the SNPs output by that step, the “Output, site-filtered TOPM” (-result option) is a Production TOPM with only the sites (SNPs) of interest retained, and the “Input directory containing Site List files” (-input option) specifies a folder that contains one file per chromosome specifying which sites you want to keep in the Production TOPM (*i.e.*, which sites passed through the filters applied by the

GBSHapMapFiltersPlugin and/or BiParentalErrorCorrectionPlugin). These Site List files are tab-delimited text (.txt) files with only two columns: the first specifies the chromosome, and the second the site to be retained. The Site List files can be easily generated from the final .hmp.txt files produced after running the GBSHapMapFiltersPlugin or the BiParentalErrorCorrectionPlugin during the Discovery Build.

Another shortcoming of the RawReadsToHapMapPlugin in Tassel3 is that SNP calling is “qualitative” rather than “quantitative”. This means that the read depth for each allele within each sample (taxon) is not taken into consideration when calling genotypes. Therefore, an individual sample with, for example, 20 reads of one allele and only 1 read of the alternate allele will be called a heterozygote. This is in contrast with the TagsToSNPByAlignmentPlugin (“Discovery SNP Caller”) (see above), where SNP calling is quantitative (as long as a TBTByte or TBT HDF5 is used instead of a TBTBit). This shortcoming is resolved in the Tassel4 GBS pipeline, where the Production SNP Caller (aptly named the ProductionSNPCallerPlugin!) performs quantitative SNP calling.

The output HapMap genotype (.hmp.txt) files are named after each input FASTQ (or QSEQ) file, with the ending “_fastq.gz” part of the name (or acceptable variant thereof) being replaced by “_chr#.hmp.txt”, where # stands for an integer representing the chromosome number. One output genotype file is produced per chromosome per input FASTQ (or QSEQ) file.

Since the RawReadsToHapMapPlugin produces separate HapMap genotype files for each input FASTQ file (and for each chromosome), if your new samples are spread across multiple lanes, you will very likely need to merge the output genotype files. This is another area where Tassel3 is not “up to snuff”. Merging multiple genotype files (called “Alignments” in Tassel jargon) is best performed with Tassel4. To do this with the Tassel4 GUI (Graphical User Interface), first load the genotype files to be merged (with Data|Load), then select them under the Sequence folder, and then use Data|Merge Alignments. To merge genotype files with the Tassel4 command line consult the following document:
http://www.maizegenetics.net/tassel/docs/Tassel4MergeAlignments.pdf

BinaryToTextPlugin

Summary:
Reads a binary GBS file and outputs the equivalent text file.

Input:

- Binary File

Output:

- Text File

Arguments:

BinaryToTextPlugin	
-i <filename>	Input binary file name.
-o <filename>	Output text file name.
-t <type>	Type of input file (TagCounts, TBTBit, TBTByte, TOPM). Does not work with TBT HDF5 files.

Example commands:

```
/programs/tassel/run_pipeline.pl -fork1 -BinaryToTextPlugin
-i tagCounts/rice.cnt -o tagCounts/rice_cnt.txt -t TagCounts
-endPlugin -runfork1
```

```
/programs/tassel/run_pipeline.pl -fork1 -BinaryToTextPlugin
-i tbt/rice.tbt.byte -o tbt/rice_tbt.txt -t TBTByte
-endPlugin -runfork1
```

```
/programs/tassel/run_pipeline.pl -fork1 -BinaryToTextPlugin
-i topm/rice.topm -o topm/rice_topm.txt -t TOPM
-endPlugin -runfork1
```

TextToBinaryPlugin

Summary:
Reads a Text GBS File and outputs the equivalent binary file.

Input:

- Text File

Output:

- Binary File

Arguments:

TextToBinaryPlugin	
-i <filename>	Input text file name.
-o <filename>	Output binary file name.
-t <type>	Type of file (TagCounts, TBTBit, TBTByte, TOPM).

Example commands:

```
/programs/tassel/run_pipeline.pl -fork1 -TextToBinaryPlugin
-i tagCounts/rice_tagCounts.txt -o tagCounts/rice.cnt -t TagCounts
-endPlugin -runfork1
```

```
/programs/tassel/run_pipeline.pl -fork1 -TextToBinaryPlugin
-i tbt/rice.tbt.txt -o tbt/rice_tbt.bin -t TBTBit
-endPlugin -runfork1
```

```
/programs/tassel/run_pipeline.pl -fork1 -TextToBinaryPlugin
-i topm/rice.topm.txt -o topm/rice.topm -t TOPM
-endPlugin -runfork1
```

Appendix 1: Key file example

The barcode key file is formatted as tab-delimited text. You can create it from Excel if you save it as tab-delimited text. In the example key below there are two lanes, each at 96 plex. The barcodes correspond to our original 96-plex *Ape*KI layout (we now work with 384-plex). You can combine lanes from multiple flow cells in a single key file and GBS analysis if you wish (we recommend analyzing all of your samples together for discovery builds). Note that there is a "Blank" in each plate, in different positions (G10 and H03 in the example). This facilitates diagnosis of accidental plate swaps. Since well H12 is overused for this, we recommend putting your blanks in wells other than H12.

Only the first seven columns (A-G) are mandatory. If there is an eighth column present (column H) and it contains integers, these will be interpreted as LibraryPrepID's, which should be unique for every Sample/Barcode/Well combination (where Well = Row+Column). These LibraryPrepID's are used to facilitate merging of the TagsByTaxa counts from replicate runs of the same library preps (on multiple flow cell lanes). For more information on merging of TagsByTaxa counts based upon LibraryPrepIDs, see the ModifyTBTHDF5Plugin (-c option) above.

You can add additional columns to the key file as you see fit -- these will be ignored by the pipeline (but may be useful to you). However, if you do not have LibraryPrepID's in Column H (the eighth column), then make sure that none of the cells in column H contain integers.

The key file must not contain spaces or colons (':'). However, it is OK to include dashes, parentheses, or underscores: - ( ) _

Flowcell	Lane	Barcode	Sample	PlateName	Row	Column	LibraryPrepID
ABC12AAXX	1	CTCC	MySample001	MyPlate1	A	1	1234567
ABC12AAXX	1	TGCA	MySample002	MyPlate1	A	2	1234568
ABC12AAXX	1	ACTA	MySample003	MyPlate1	A	3	1234569
ABC12AAXX	1	CAGA	MySample004	MyPlate1	A	4	1234570
ABC12AAXX	1	AACT	MySample005	MyPlate1	A	5	1234571
ABC12AAXX	1	GCGT	MySample006	MyPlate1	A	6	1234572
ABC12AAXX	1	TGCGA	MySample007	MyPlate1	A	7	1234573
ABC12AAXX	1	CGAT	MySample008	MyPlate1	A	8	1234574
ABC12AAXX	1	CGCTT	MySample009	MyPlate1	A	9	1234575
ABC12AAXX	1	TCACC	MySample010	MyPlate1	A	10	1234576
ABC12AAXX	1	CTAGC	MySample011	MyPlate1	A	11	1234577
ABC12AAXX	1	ACAAA	MySample012	MyPlate1	A	12	1234578
ABC12AAXX	1	TTCTC	MySample013	MyPlate1	B	1	1234579
ABC12AAXX	1	AGCCC	MySample014	MyPlate1	B	2	1234580
ABC12AAXX	1	GTATT	MySample015	MyPlate1	B	3	1234581
ABC12AAXX	1	CTGTA	MySample016	MyPlate1	B	4	1234582
ABC12AAXX	1	ACCGT	MySample017	MyPlate1	B	5	1234583
ABC12AAXX	1	GTAA	MySample018	MyPlate1	B	6	1234584
ABC12AAXX	1	GGTTGT	MySample019	MyPlate1	B	7	1234585
ABC12AAXX	1	CCAGCT	MySample020	MyPlate1	B	8	1234586
ABC12AAXX	1	TTCAGA	MySample021	MyPlate1	B	9	1234587
ABC12AAXX	1	TAGGAA	MySample022	MyPlate1	B	10	1234588
ABC12AAXX	1	GCTCTA	MySample023	MyPlate1	B	11	1234589
ABC12AAXX	1	CCACAA	MySample024	MyPlate1	B	12	1234590
ABC12AAXX	1	GCTTA	MySample025	MyPlate1	C	1	1234591
ABC12AAXX	1	CTTCCA	MySample026	MyPlate1	C	2	1234592
ABC12AAXX	1	GAGATA	MySample027	MyPlate1	C	3	1234593
ABC12AAXX	1	ATGCCT	MySample028	MyPlate1	C	4	1234594

ABC12AAXX	1	TATTTTT	MySample029	MyPlate1	C	5	1234595
ABC12AAXX	1	CTTGCTT	MySample030	MyPlate1	C	6	1234596
ABC12AAXX	1	ATGAAAC	MySample031	MyPlate1	C	7	1234597
ABC12AAXX	1	AAAAGTT	MySample032	MyPlate1	C	8	1234598
ABC12AAXX	1	GAATTCA	MySample033	MyPlate1	C	9	1234599
ABC12AAXX	1	GAACTTC	MySample034	MyPlate1	C	10	1234600
ABC12AAXX	1	GGACCTA	MySample035	MyPlate1	C	11	1234601
ABC12AAXX	1	GTCGATT	MySample036	MyPlate1	C	12	1234602
ABC12AAXX	1	AACGCCT	MySample037	MyPlate1	D	1	1234603
ABC12AAXX	1	AATATGC	MySample038	MyPlate1	D	2	1234604
ABC12AAXX	1	ACGACTAC	MySample039	MyPlate1	D	3	1234605
ABC12AAXX	1	GGTGT	MySample040	MyPlate1	D	4	1234606
ABC12AAXX	1	TAGCATGC	MySample041	MyPlate1	D	5	1234607
ABC12AAXX	1	AGTGGA	MySample042	MyPlate1	D	6	1234608
ABC12AAXX	1	TAGGCCAT	MySample043	MyPlate1	D	7	1234609
ABC12AAXX	1	TGCAAGGA	MySample044	MyPlate1	D	8	1234610
ABC12AAXX	1	TGGTACGT	MySample045	MyPlate1	D	9	1234611
ABC12AAXX	1	TCTCAGTC	MySample046	MyPlate1	D	10	1234612
ABC12AAXX	1	CCGGATAT	MySample047	MyPlate1	D	11	1234613
ABC12AAXX	1	CGCCTTAT	MySample048	MyPlate1	D	12	1234614
ABC12AAXX	1	AGGC	MySample049	MyPlate1	E	1	1234615
ABC12AAXX	1	GATC	MySample050	MyPlate1	E	2	1234616
ABC12AAXX	1	TCAC	MySample051	MyPlate1	E	3	1234617
ABC12AAXX	1	AGGAT	MySample052	MyPlate1	E	4	1234618
ABC12AAXX	1	ATTGA	MySample053	MyPlate1	E	5	1234619
ABC12AAXX	1	CATCT	MySample054	MyPlate1	E	6	1234620
ABC12AAXX	1	CCTAC	MySample055	MyPlate1	E	7	1234621
ABC12AAXX	1	GAGGA	MySample056	MyPlate1	E	8	1234622
ABC12AAXX	1	GGAAC	MySample057	MyPlate1	E	9	1234623
ABC12AAXX	1	GTCAA	MySample058	MyPlate1	E	10	1234624
ABC12AAXX	1	TAATA	MySample059	MyPlate1	E	11	1234625
ABC12AAXX	1	TACAT	MySample060	MyPlate1	E	12	1234626
ABC12AAXX	1	TCGTT	MySample061	MyPlate1	F	1	1234627
ABC12AAXX	1	ACCTAA	MySample062	MyPlate1	F	2	1234628
ABC12AAXX	1	ATATGT	MySample063	MyPlate1	F	3	1234629
ABC12AAXX	1	ATCGTA	MySample064	MyPlate1	F	4	1234630
ABC12AAXX	1	CATCGT	MySample065	MyPlate1	F	5	1234631
ABC12AAXX	1	CGCGGT	MySample066	MyPlate1	F	6	1234632
ABC12AAXX	1	CTATTA	MySample067	MyPlate1	F	7	1234633
ABC12AAXX	1	GCCAGT	MySample068	MyPlate1	F	8	1234634
ABC12AAXX	1	GGAAGA	MySample069	MyPlate1	F	9	1234635
ABC12AAXX	1	GTACTT	MySample070	MyPlate1	F	10	1234636
ABC12AAXX	1	GTTGAA	MySample071	MyPlate1	F	11	1234637
ABC12AAXX	1	TAACGA	MySample072	MyPlate1	F	12	1234638
ABC12AAXX	1	TGGCTA	MySample073	MyPlate1	G	1	1234639
ABC12AAXX	1	ACGTGTT	MySample074	MyPlate1	G	2	1234640
ABC12AAXX	1	ATTAATT	MySample075	MyPlate1	G	3	1234641
ABC12AAXX	1	ATTGGAT	MySample076	MyPlate1	G	4	1234642
ABC12AAXX	1	CATAAGT	MySample077	MyPlate1	G	5	1234643
ABC12AAXX	1	CGCTGAT	MySample078	MyPlate1	G	6	1234644
ABC12AAXX	1	CGGTAGA	MySample079	MyPlate1	G	7	1234645
ABC12AAXX	1	CTACGGA	MySample080	MyPlate1	G	8	1234646
ABC12AAXX	1	GCGGAAT	MySample081	MyPlate1	G	9	1234647
ABC12AAXX	1	TAGCGGA	Blank	MyPlate1	G	10	1234648
ABC12AAXX	1	TCGAAGA	MySample082	MyPlate1	G	11	1234649

ABC12AAXX	1	TCTGTGA	MySample083	MyPlate1	G	12	1234650
ABC12AAXX	1	TGCTGGA	MySample084	MyPlate1	H	1	1234651
ABC12AAXX	1	AACCGAGA	MySample085	MyPlate1	H	2	1234652
ABC12AAXX	1	ACAGGGAA	MySample086	MyPlate1	H	3	1234653
ABC12AAXX	1	ACGTGGTA	MySample087	MyPlate1	H	4	1234654
ABC12AAXX	1	CCATGGGT	MySample088	MyPlate1	H	5	1234655
ABC12AAXX	1	CGCGGAGA	MySample089	MyPlate1	H	6	1234656
ABC12AAXX	1	CGTGTGGT	MySample090	MyPlate1	H	7	1234657
ABC12AAXX	1	GCTGTGGA	MySample091	MyPlate1	H	8	1234658
ABC12AAXX	1	GGATTGGT	MySample092	MyPlate1	H	9	1234659
ABC12AAXX	1	GTGAGGGT	MySample093	MyPlate1	H	10	1234660
ABC12AAXX	1	TATCGGGA	MySample094	MyPlate1	H	11	1234661
ABC12AAXX	1	TTCCTGGA	MySample095	MyPlate1	H	12	1234662
ABC12AAXX	2	CTCC	MySample096	MyPlate2	A	1	1234663
ABC12AAXX	2	TGCA	MySample097	MyPlate2	A	2	1234664
ABC12AAXX	2	ACTA	MySample098	MyPlate2	A	3	1234665
ABC12AAXX	2	CAGA	MySample099	MyPlate2	A	4	1234666
ABC12AAXX	2	AACT	MySample100	MyPlate2	A	5	1234667
ABC12AAXX	2	GCGT	MySample101	MyPlate2	A	6	1234668
ABC12AAXX	2	TGCGA	MySample102	MyPlate2	A	7	1234669
ABC12AAXX	2	CGAT	MySample103	MyPlate2	A	8	1234670
ABC12AAXX	2	CGCTT	MySample104	MyPlate2	A	9	1234671
ABC12AAXX	2	TCACC	MySample105	MyPlate2	A	10	1234672
ABC12AAXX	2	CTAGC	MySample106	MyPlate2	A	11	1234673
ABC12AAXX	2	ACAAA	MySample107	MyPlate2	A	12	1234674
ABC12AAXX	2	TTCTC	MySample108	MyPlate2	B	1	1234675
ABC12AAXX	2	AGCCC	MySample109	MyPlate2	B	2	1234676
ABC12AAXX	2	GTATT	MySample110	MyPlate2	B	3	1234677
ABC12AAXX	2	CTGTA	MySample111	MyPlate2	B	4	1234678
ABC12AAXX	2	ACCGT	MySample112	MyPlate2	B	5	1234679
ABC12AAXX	2	GTAA	MySample113	MyPlate2	B	6	1234680
ABC12AAXX	2	GGTTGT	MySample114	MyPlate2	B	7	1234681
ABC12AAXX	2	CCAGCT	MySample115	MyPlate2	B	8	1234682
ABC12AAXX	2	TTCAGA	MySample116	MyPlate2	B	9	1234683
ABC12AAXX	2	TAGGAA	MySample117	MyPlate2	B	10	1234684
ABC12AAXX	2	GCTCTA	MySample118	MyPlate2	B	11	1234685
ABC12AAXX	2	CCACAA	MySample119	MyPlate2	B	12	1234686
ABC12AAXX	2	GCTTA	MySample120	MyPlate2	C	1	1234687
ABC12AAXX	2	CTTCCA	MySample121	MyPlate2	C	2	1234688
ABC12AAXX	2	GAGATA	MySample122	MyPlate2	C	3	1234689
ABC12AAXX	2	ATGCCT	MySample123	MyPlate2	C	4	1234690
ABC12AAXX	2	TATTTTT	MySample124	MyPlate2	C	5	1234691
ABC12AAXX	2	CTTGCTT	MySample125	MyPlate2	C	6	1234692
ABC12AAXX	2	ATGAAAC	MySample126	MyPlate2	C	7	1234693
ABC12AAXX	2	AAAAGTT	MySample127	MyPlate2	C	8	1234694
ABC12AAXX	2	GAATTCA	MySample128	MyPlate2	C	9	1234695
ABC12AAXX	2	GAACTTC	MySample129	MyPlate2	C	10	1234696
ABC12AAXX	2	GGACCTA	MySample130	MyPlate2	C	11	1234697
ABC12AAXX	2	GTCGATT	MySample131	MyPlate2	C	12	1234698
ABC12AAXX	2	AACGCCT	MySample132	MyPlate2	D	1	1234699
ABC12AAXX	2	AATATGC	MySample133	MyPlate2	D	2	1234700
ABC12AAXX	2	ACGACTAC	MySample134	MyPlate2	D	3	1234701
ABC12AAXX	2	GGTGT	MySample135	MyPlate2	D	4	1234702
ABC12AAXX	2	TAGCATGC	MySample136	MyPlate2	D	5	1234703
ABC12AAXX	2	AGTGGA	MySample137	MyPlate2	D	6	1234704

ABC12AAXX	2	TAGGCCAT	MySample138	MyPlate2	D	7	1234705
ABC12AAXX	2	TGCAAGGA	MySample139	MyPlate2	D	8	1234706
ABC12AAXX	2	TGGTACGT	MySample140	MyPlate2	D	9	1234707
ABC12AAXX	2	TCTCAGTC	MySample141	MyPlate2	D	10	1234708
ABC12AAXX	2	CCGGATAT	MySample142	MyPlate2	D	11	1234709
ABC12AAXX	2	CGCCTTAT	MySample143	MyPlate2	D	12	1234710
ABC12AAXX	2	AGGC	MySample144	MyPlate2	E	1	1234711
ABC12AAXX	2	GATC	MySample145	MyPlate2	E	2	1234712
ABC12AAXX	2	TCAC	MySample146	MyPlate2	E	3	1234713
ABC12AAXX	2	AGGAT	MySample147	MyPlate2	E	4	1234714
ABC12AAXX	2	ATTGA	MySample148	MyPlate2	E	5	1234715
ABC12AAXX	2	CATCT	MySample149	MyPlate2	E	6	1234716
ABC12AAXX	2	CCTAC	MySample150	MyPlate2	E	7	1234717
ABC12AAXX	2	GAGGA	MySample151	MyPlate2	E	8	1234718
ABC12AAXX	2	GGAAC	MySample152	MyPlate2	E	9	1234719
ABC12AAXX	2	GTCAA	MySample153	MyPlate2	E	10	1234720
ABC12AAXX	2	TAATA	MySample154	MyPlate2	E	11	1234721
ABC12AAXX	2	TACAT	MySample155	MyPlate2	E	12	1234722
ABC12AAXX	2	TCGTT	MySample156	MyPlate2	F	1	1234723
ABC12AAXX	2	ACCTAA	MySample157	MyPlate2	F	2	1234724
ABC12AAXX	2	ATATGT	MySample158	MyPlate2	F	3	1234725
ABC12AAXX	2	ATCGTA	MySample159	MyPlate2	F	4	1234726
ABC12AAXX	2	CATCGT	MySample160	MyPlate2	F	5	1234727
ABC12AAXX	2	CGCGGT	MySample161	MyPlate2	F	6	1234728
ABC12AAXX	2	CTATTA	MySample162	MyPlate2	F	7	1234729
ABC12AAXX	2	GCCAGT	MySample163	MyPlate2	F	8	1234730
ABC12AAXX	2	GGAAGA	MySample164	MyPlate2	F	9	1234731
ABC12AAXX	2	GTACTT	MySample165	MyPlate2	F	10	1234732
ABC12AAXX	2	GTTGAA	MySample166	MyPlate2	F	11	1234733
ABC12AAXX	2	TAACGA	MySample167	MyPlate2	F	12	1234734
ABC12AAXX	2	TGGCTA	MySample168	MyPlate2	G	1	1234735
ABC12AAXX	2	ACGTGTT	MySample169	MyPlate2	G	2	1234736
ABC12AAXX	2	ATTAATT	MySample170	MyPlate2	G	3	1234737
ABC12AAXX	2	ATTGGAT	MySample171	MyPlate2	G	4	1234738
ABC12AAXX	2	CATAAGT	MySample172	MyPlate2	G	5	1234739
ABC12AAXX	2	CGCTGAT	MySample173	MyPlate2	G	6	1234740
ABC12AAXX	2	CGGTAGA	MySample174	MyPlate2	G	7	1234741
ABC12AAXX	2	CTACGGA	MySample175	MyPlate2	G	8	1234742
ABC12AAXX	2	GCGGAAT	MySample176	MyPlate2	G	9	1234743
ABC12AAXX	2	TAGCGGA	MySample177	MyPlate2	G	10	1234744
ABC12AAXX	2	TCGAAGA	MySample178	MyPlate2	G	11	1234745
ABC12AAXX	2	TCTGTGA	MySample179	MyPlate2	G	12	1234746
ABC12AAXX	2	TGCTGGA	MySample180	MyPlate2	H	1	1234747
ABC12AAXX	2	AACCGAGA	MySample181	MyPlate2	H	2	1234748
ABC12AAXX	2	ACAGGGAA	Blank	MyPlate2	H	3	1234749
ABC12AAXX	2	ACGTGGTA	MySample182	MyPlate2	H	4	1234750
ABC12AAXX	2	CCATGGGT	MySample183	MyPlate2	H	5	1234751
ABC12AAXX	2	CGCGGAGA	MySample184	MyPlate2	H	6	1234752
ABC12AAXX	2	CGTGTGGT	MySample185	MyPlate2	H	7	1234753
ABC12AAXX	2	GCTGTGGA	MySample186	MyPlate2	H	8	1234754
ABC12AAXX	2	GGATTGGT	MySample187	MyPlate2	H	9	1234755
ABC12AAXX	2	GTGAGGGT	MySample188	MyPlate2	H	10	1234756
ABC12AAXX	2	TATCGGGA	MySample189	MyPlate2	H	11	1234757
ABC12AAXX	2	TTCCTGGA	MySample190	MyPlate2	H	12	1234758

Appendix 2: Pedigree file example

A pedigree file contains the following columns:

Family = name of the family to which the sample belongs (use “NA” if it is not part of a family)
Name = full name of the sample (*e.g.*, “Z002E0001:61VBRAAXX:6:250021377”)
Parent1 = short name of the first parent (*e.g.*, “B73”)
Parent2 = short name of the second parent (*e.g.*, “CML103”)
Contribution1 = the expected genetic contribution of Parent1 (e.g., 0.5 for an F2-derived RIL)
Contribution2 = the expected genetic contribution of Parent2 (e.g., 0.5 for an F2-derived RIL)
f = an estimate of the inbreeding coefficient of the sample (e.g., 0.75 for an S3)
Comments = any comments about that sample for your own purposes (e.g. “sample mix-up?”)

The pedigree file must contain an entry (row) for every sample (taxon) in the input hapmap genotype file. If a particular sample is not part of a biparental family, enter “NA” for Family, Parent1, Parent2, Contribution1, and Contribution2. If you do not know (or trust) the level of inbreeding of one or more of your samples, then set the value for **f** as “NA”. For 1:1 segregation (e.g., F2 progeny or RILs derived therefrom), both Contribution1 and Contribution2 should be set at 0.5 (= expected allele frequency). For a backcross or pseudo-testcross, Contribution1 and Contribution2 should be 0.75 and 0.25 respectively. For a BC$_2$ family (or BC$_2$-derived RILs), Contribution1 and Contribution2 should be 0.875 and 0.125 respectively.

The example pedigree file below contains, for illustrative purposes, the 26 inbred founders of the maize Nested Association Mapping (NAM) population, along with 5 RILs per NAM family. The full NAM population actually consists of 5000 RILs (200 RILs per family).

Family	Name	Parent1	Parent2	Contribution1	Contribution2	f	Comments
NA	B73:MRG:2:250040143	B73	NA	1	0	0.9	inbred
NA	B97:MRG:2:250039795	B97	NA	1	0	0.9	inbred
NA	CML103:MRG:4:250056356	CML103	NA	1	0	0.9	inbred
NA	CML228:MRG:4:250056512	CML228	NA	1	0	0.9	inbred
NA	CML247:MRG:4:250056531	CML247	NA	1	0	0.9	inbred
NA	CML277:MRG:4:250057053	CML277	NA	1	0	0.9	inbred
NA	CML322:MRG:4:250056637	CML322	NA	1	0	0.9	inbred
NA	CML333:MRG:4:250056648	CML333	NA	1	0	0.9	inbred
NA	CML52:MRG:4:250056274	CML52	NA	1	0	0.9	inbred
NA	CML69:MRG:4:250056291	CML69	NA	1	0	0.9	inbred
NA	HP301:MRG:2:250040193	HP301	NA	1	0	0.9	inbred
NA	Il14H:MRG:2:250040130	IL14H	NA	1	0	0.9	inbred
NA	Ki11:MRG:2:250039815	Ki11	NA	1	0	0.9	inbred
NA	Ki3:MRG:2:250040211	Ki3	NA	1	0	0.9	inbred
NA	Ky21:MRG:2:250040003	Ky21	NA	1	0	0.9	inbred
NA	M162W:MRG:2:250040157	M162W	NA	1	0	0.9	inbred
NA	M37W:MRG:2:250040142	M37W	NA	1	0	0.9	inbred
NA	Mo18W:MRG:2:250040011	Mo18W	NA	1	0	0.9	Inbred
NA	MS71:MRG:2:250040020	MS71	NA	1	0	0.9	Inbred
NA	NC350:MRG:2:250040135	NC350	NA	1	0	0.9	inbred
NA	NC358:MRG:2:250040063	NC358	NA	1	0	0.9	inbred
NA	Oh43:MRG:2:250040141	Oh43	NA	1	0	0.9	inbred
NA	OH7B:MRG:2:250040208	OH7B	NA	1	0	0.9	inbred
NA	P39:MRG:2:250040161	PI587133	NA	1	0	0.9	inbred
NA	Tx303:MRG:2:250040016	Tx303	NA	1	0	0.9	inbred
NA	Tzi8:MRG:2:250040137	Tzi8	NA	1	0	0.9	inbred
NAM_B97	Z001E0001:628NHAAXX:1:250021125	B73	B97	0.5	0.5	0.9	RIL
NAM_B97	Z001E0002:628NHAAXX:1:250021137	B73	B97	0.5	0.5	0.9	RIL

NAM_B97	Z001E0003:628NHAAXX:1:250021149	B73	B97	0.5	0.5	0.9	RIL
NAM_B97	Z001E0004:628NHAAXX:2:250021176	B73	B97	0.5	0.5	0.9	RIL
NAM_B97	Z001E0005:628NHAAXX:2:250021188	B73	B97	0.5	0.5	0.9	RIL
NAM_CML103	Z002E0001:61VBRAAXX:6:250021377	B73	CML103	0.5	0.5	0.9	RIL
NAM_CML103	Z002E0002:61VBRAAXX:6:250021389	B73	CML103	0.5	0.5	0.9	RIL
NAM_CML103	Z002E0003:61VBRAAXX:6:250021401	B73	CML103	0.5	0.5	0.9	RIL
NAM_CML103	Z002E0004:61VBRAAXX:7:250021428	B73	CML103	0.5	0.5	0.9	RIL
NAM_CML103	Z002E0005:61VBRAAXX:7:250021440	B73	CML103	0.5	0.5	0.9	RIL
NAM_CML228	Z003E0001:705VVAAXX:1:250021629	B73	CML228	0.5	0.5	0.9	RIL
NAM_CML228	Z003E0002:705VVAAXX:1:250021641	B73	CML228	0.5	0.5	0.9	RIL
NAM_CML228	Z003E0003:705VVAAXX:1:250021653	B73	CML228	0.5	0.5	0.9	RIL
NAM_CML228	Z003E0004:705VVAAXX:2:250021680	B73	CML228	0.5	0.5	0.9	RIL
NAM_CML228	Z003E0005:705VVAAXX:2:250021692	B73	CML228	0.5	0.5	0.9	RIL
NAM_CML247	Z004E0001:705VVAAXX:5:250021881	B73	CML247	0.5	0.5	0.9	RIL
NAM_CML247	Z004E0002:705VVAAXX:5:250021893	B73	CML247	0.5	0.5	0.9	RIL
NAM_CML247	Z004E0003:705VVAAXX:5:250021905	B73	CML247	0.5	0.5	0.9	RIL
NAM_CML247	Z004E0004:705VVAAXX:6:250021932	B73	CML247	0.5	0.5	0.9	RIL
NAM_CML247	Z004E0005:705VVAAXX:6:250021944	B73	CML247	0.5	0.5	0.9	RIL
NAM_CML277	Z005E0001:61VBPAAXX:1:250022133	B73	CML277	0.5	0.5	0.9	RIL
NAM_CML277	Z005E0003:61VBPAAXX:1:250022145	B73	CML277	0.5	0.5	0.9	RIL
NAM_CML277	Z005E0004:61VBPAAXX:1:250022157	B73	CML277	0.5	0.5	0.9	RIL
NAM_CML277	Z005E0005:61VBPAAXX:2:250022184	B73	CML277	0.5	0.5	0.9	RIL
NAM_CML322	Z006E0001:61VE7AAXX:1:250022385	B73	CML322	0.5	0.5	0.9	RIL
NAM_CML322	Z006E0002:61VE7AAXX:1:250022397	B73	CML322	0.5	0.5	0.9	RIL
NAM_CML322	Z006E0003:61VE7AAXX:1:250022409	B73	CML322	0.5	0.5	0.9	RIL
NAM_CML322	Z006E0005:61VE7AAXX:2:250022436	B73	CML322	0.5	0.5	0.9	RIL
NAM_CML333	Z007E0001:61VE7AAXX:5:250022637	B73	CML333	0.5	0.5	0.9	RIL
NAM_CML333	Z007E0002:62P7LAAXX:6:250028327	B73	CML333	0.5	0.5	0.9	RIL
NAM_CML333	Z007E0003:61VE7AAXX:5:250022661	B73	CML333	0.5	0.5	0.9	RIL
NAM_CML333	Z007E0004:61VE7AAXX:6:250022688	B73	CML333	0.5	0.5	0.9	RIL
NAM_CML333	Z007E0005:62P7LAAXX:6:250028339	B73	CML333	0.5	0.5	0.9	RIL
NAM_CML52	Z008E0001:61VBPAAXX:5:250022889	B73	CML52	0.5	0.5	0.9	RIL
NAM_CML52	Z008E0002:61VBPAAXX:5:250022901	B73	CML52	0.5	0.5	0.9	RIL
NAM_CML52	Z008E0003:61VBPAAXX:5:250022913	B73	CML52	0.5	0.5	0.9	RIL
NAM_CML52	Z008E0004:61VBPAAXX:6:250022940	B73	CML52	0.5	0.5	0.9	RIL
NAM_CML52	Z008E0005:61VBPAAXX:6:250022952	B73	CML52	0.5	0.5	0.9	RIL
NAM_CML69	Z009E0001:61VE9AAXX:1:250023141	B73	CML69	0.5	0.5	0.9	RIL
NAM_CML69	Z009E0002:61VE9AAXX:1:250023153	B73	CML69	0.5	0.5	0.9	RIL
NAM_CML69	Z009E0003:61VE9AAXX:1:250023165	B73	CML69	0.5	0.5	0.9	RIL
NAM_CML69	Z009E0004:61VE9AAXX:2:250023192	B73	CML69	0.5	0.5	0.9	RIL
NAM_CML69	Z009E0005:61VE9AAXX:2:250023204	B73	CML69	0.5	0.5	0.9	RIL
NAM_Hp301	Z010E0001:627C3AAXX:3:250028523	B73	Hp301	0.5	0.5	0.9	RIL
NAM_Hp301	Z010E0002:628NVAAXX:6:250023405	B73	Hp301	0.5	0.5	0.9	RIL
NAM_Hp301	Z010E0003:628NVAAXX:6:250023417	B73	Hp301	0.5	0.5	0.9	RIL
NAM_Hp301	Z010E0004:628NVAAXX:7:250023444	B73	Hp301	0.5	0.5	0.9	RIL
NAM_Il14H	Z011E0001:61VE9AAXX:3:250028900	B73	Il14H	0.5	0.5	0.9	RIL
NAM_Il14H	Z011E0002:61VE9AAXX:3:250028912	B73	Il14H	0.5	0.5	0.9	RIL
NAM_Il14H	Z011E0003:61VE9AAXX:3:250028924	B73	Il14H	0.5	0.5	0.9	RIL
NAM_Il14H	Z011E0004:61VE9AAXX:4:250024075	B73	Il14H	0.5	0.5	0.9	RIL
NAM_Il14H	Z011E0005:61VE9AAXX:4:250024087	B73	Il14H	0.5	0.5	0.9	RIL
NAM_Ki11	Z012E0001:628DJAAXX:3:250024276	B73	Ki11	0.5	0.5	0.9	RIL
NAM_Ki11	Z012E0002:628DJAAXX:3:250024288	B73	Ki11	0.5	0.5	0.9	RIL
NAM_Ki11	Z012E0003:628DJAAXX:3:250024300	B73	Ki11	0.5	0.5	0.9	RIL
NAM_Ki11	Z012E0004:627C3AAXX:4:250028711	B73	Ki11	0.5	0.5	0.9	RIL
NAM_Ki11	Z012E0005:628DJAAXX:4:250024339	B73	Ki11	0.5	0.5	0.9	RIL

NAM_Ki3	Z013E0001:628DJAAXX:7:250024528	B73	Ki3	0.5	0.5	0.9	RIL
NAM_Ki3	Z013E0002:628DJAAXX:7:250024540	B73	Ki3	0.5	0.5	0.9	RIL
NAM_Ki3	Z013E0003:628NJAAXX:4:250024659	B73	Ki3	0.5	0.5	0.9	RIL
NAM_Ki3	Z013E0004:628NJAAXX:3:250024579	B73	Ki3	0.5	0.5	0.9	RIL
NAM_Ki3	Z013E0005:628NJAAXX:3:250024591	B73	Ki3	0.5	0.5	0.9	RIL
NAM_Ky21	Z014E0001:628NJAAXX:6:250024780	B73	Ky21	0.5	0.5	0.9	RIL
NAM_Ky21	Z014E0002:628NJAAXX:4:250024676	B73	Ky21	0.5	0.5	0.9	RIL
NAM_Ky21	Z014E0003:628NJAAXX:6:250024804	B73	Ky21	0.5	0.5	0.9	RIL
NAM_Ky21	Z014E0004:628NJAAXX:7:250024894	B73	Ky21	0.5	0.5	0.9	RIL
NAM_Ky21	Z014E0005:628NJAAXX:7:250024906	B73	Ky21	0.5	0.5	0.9	RIL
NAM_M162W	Z015E0001:62P7LAAXX:6:250028289	B73	M162W	0.5	0.5	0.9	RIL
NAM_M162W	Z015E0002:62P7LAAXX:6:250028311	B73	M162W	0.5	0.5	0.9	RIL
NAM_M162W	Z015E0003:62P7LAAXX:6:250028323	B73	M162W	0.5	0.5	0.9	RIL
NAM_M162W	Z015E0004:62P7LAAXX:6:250028335	B73	M162W	0.5	0.5	0.9	RIL
NAM_M162W	Z015E0005:62P7LAAXX:6:250028347	B73	M162W	0.5	0.5	0.9	RIL
NAM_M37W	Z016E0001:61VE9AAXX:5:250025347	B73	M37W	0.5	0.5	0.9	RIL
NAM_M37W	Z016E0002:61VE9AAXX:5:250025359	B73	M37W	0.5	0.5	0.9	RIL
NAM_M37W	Z016E0003:61VE9AAXX:5:250025371	B73	M37W	0.5	0.5	0.9	RIL
NAM_M37W	Z016E0004:61VE9AAXX:6:250025398	B73	M37W	0.5	0.5	0.9	RIL
NAM_M37W	Z016E0005:61VE9AAXX:6:250025410	B73	M37W	0.5	0.5	0.9	RIL
NAM_Mo18W	Z018E0001:628AGAAXX:2:250025721	B73	Mo18W	0.5	0.5	0.9	RIL
NAM_Mo18W	Z018E0002:628AGAAXX:2:250025758	B73	Mo18W	0.5	0.5	0.9	RIL
NAM_Mo18W	Z018E0003:628AGAAXX:2:250025741	B73	Mo18W	0.5	0.5	0.9	RIL
NAM_Mo18W	Z018E0004:628AGAAXX:2:250025761	B73	Mo18W	0.5	0.5	0.9	RIL
NAM_Mo18W	Z018E0005:628AGAAXX:2:250025773	B73	Mo18W	0.5	0.5	0.9	RIL
NAM_MS71	Z019E0002:709G4AAXX:2:250025848	B73	MS71	0.5	0.5	0.9	RIL
NAM_MS71	Z019E0003:709G4AAXX:2:250025885	B73	MS71	0.5	0.5	0.9	RIL
NAM_MS71	Z019E0004:709G4AAXX:2:250025868	B73	MS71	0.5	0.5	0.9	RIL
NAM_MS71	Z019E0005:709G4AAXX:2:250025888	B73	MS71	0.5	0.5	0.9	RIL
NAM_NC350	Z020E0001:628NJAAXX:2:250026102	B73	NC350	0.5	0.5	0.9	RIL
NAM_NC350	Z020E0002:628NJAAXX:2:250026139	B73	NC350	0.5	0.5	0.9	RIL
NAM_NC350	Z020E0003:628NJAAXX:2:250026122	B73	NC350	0.5	0.5	0.9	RIL
NAM_NC350	Z020E0004:628NJAAXX:2:250026142	B73	NC350	0.5	0.5	0.9	RIL
NAM_NC350	Z020E0005:627C3AAXX:2:250028422	B73	NC350	0.5	0.5	0.9	RIL
NAM_NC358	Z021E0001:709G4AAXX:3:250026356	B73	NC358	0.5	0.5	0.9	RIL
NAM_NC358	Z021E0002:709G4AAXX:3:250026393	B73	NC358	0.5	0.5	0.9	RIL
NAM_NC358	Z021E0003:709G4AAXX:3:250026376	B73	NC358	0.5	0.5	0.9	RIL
NAM_NC358	Z021E0004:709G4AAXX:3:250026396	B73	NC358	0.5	0.5	0.9	RIL
NAM_NC358	Z021E0005:709G4AAXX:3:250026408	B73	NC358	0.5	0.5	0.9	RIL
NAM_Oh43	Z022E0001:709G4AAXX:5:250026610	B73	Oh43	0.5	0.5	0.9	RIL
NAM_Oh43	Z022E0002:709G4AAXX:5:250026647	B73	Oh43	0.5	0.5	0.9	RIL
NAM_Oh43	Z022E0003:709G4AAXX:5:250026630	B73	Oh43	0.5	0.5	0.9	RIL
NAM_Oh43	Z022E0004:709G4AAXX:5:250026650	B73	Oh43	0.5	0.5	0.9	RIL
NAM_Oh43	Z022E0005:709G4AAXX:5:250026662	B73	Oh43	0.5	0.5	0.9	RIL
NAM_Oh7B	Z023E0001:709G4AAXX:7:250026864	B73	Oh7B	0.5	0.5	0.9	RIL
NAM_Oh7B	Z023E0003:709G4AAXX:7:250026901	B73	Oh7B	0.5	0.5	0.9	RIL
NAM_Oh7B	Z023E0004:709G4AAXX:7:250026884	B73	Oh7B	0.5	0.5	0.9	RIL
NAM_P39	Z024E0001:MRG:2:250027118	B73	P39	0.5	0.5	0.9	RIL
NAM_P39	Z024E0002:627C3AAXX:4:250028702	B73	P39	0.5	0.5	0.9	RIL
NAM_P39	Z024E0004:MRG:2:250027138	B73	P39	0.5	0.5	0.9	RIL
NAM_P39	Z024E0005:MRG:2:250027158	B73	P39	0.5	0.5	0.9	RIL
NAM_Tx303	Z025E0001:70980AAXX:4:250028959	B73	Tx303	0.5	0.5	0.9	RIL
NAM_Tx303	Z025E0002:70980AAXX:4:250028996	B73	Tx303	0.5	0.5	0.9	RIL
NAM_Tx303	Z025E0003:70980AAXX:4:250028979	B73	Tx303	0.5	0.5	0.9	RIL
NAM_Tx303	Z025E0004:70980AAXX:4:250028999	B73	Tx303	0.5	0.5	0.9	RIL

NAM_Tx303	Z025E0005:70980AAXX:4:250029011	B73	Tx303	0.5	0.5	0.9	RIL
NAM_Tzi8	Z026E0001:MRG:2:250027626	B73	Tzi8	0.5	0.5	0.9	RIL
NAM_Tzi8	Z026E0002:MRG:2:250027663	B73	Tzi8	0.5	0.5	0.9	RIL
NAM_Tzi8	Z026E0003:MRG:2:250027646	B73	Tzi8	0.5	0.5	0.9	RIL
NAM_Tzi8	Z026E0004:MRG:2:250027666	B73	Tzi8	0.5	0.5	0.9	RIL
NAM_Tzi8	Z026E0005:MRG:2:250027678	B73	Tzi8	0.5	0.5	0.9	RIL

Appendix 3: Contents of a TagsOnPhysicalMap (TOPM) file

Although a TagsOnPhysicalMap (.topm or .topm.bin) file is binary, if you want to have a look "under the hood" it is possible to convert it to a human-readable, tab-delimited text format by using the BinaryToTextPlugin. If the resulting .topm.txt file is too large to open in Excel (or a text editor), you can look at part of it by using the "`head`" command in Linux (or a combination of `head` and `tail` -- see the Introduction). This Appendix is provided to answer the question that comes next: "What am I looking at?"

The first line of a .topm.txt file consists of three numbers:

1. **tagNum**: the total number of tags contained in the TOPM
2. **tagLengthInLong**: how long is each tag in multiples of 32 bases. Typically 2 (= 64 bases)
3. **maxVariants**: the maximum number of variants (SNPs) that can be stored for each tag. These are populated by the -mUpd option of the TagsToSNPByAlignmentPlugin.

Each subsequent line (row) of a .topm.txt file consists of the record for each tag, with the lines (rows) sorted by the sequence of each tag. Because they are sorted by tag sequence rather than by chromosome, start position, and strand, it is possible that tags that align to the same start position and strand (*i.e.*, that together comprise a TagLocus) will not be adjacent in the .topm.txt file. Each tag row contains the following information:

1. **tagLength**: the actual length of the tag (before padding with polyA). This is always less than or equal to the *tagLengthInLong* × 32 bases.
2. **multimaps**: the number of equally good, optimal chromosomal positions reported by the sequence aligner (BWA or bowtie2). If you used bowtie2 to align your tags to the reference genome, then tags with two or more equally good chromosomal positions will have "99" for the value of *multimaps* (because bowtie2 does report the total number). A "*" character indicates that no good alignments were found.
3. **chromosome**: If *multimaps* = 1, the chromosome number of the unique best alignment for the tag. Otherwise, a "*" = undefined (in that case, all remaining fields are undefined too).
4. **strand**: the strand to which the tag aligns ("1" = plus strand, "-1" = minus strand, "*" = undefined).
5. **startPosition**: the chromosomal position of the first base of the tag (barcoded end). "*" = undefined.
6. **endPostion**: the chromosomal position of the last base of the tag. "*" = undefined.
7. **divergence**: the edit distance to the reference genome of the unique best alignment of that tag. "*" = undefined.
8. For each variant up to *maxVariants*, a pair of integers consisting of the following (these are all "*" if the tag does not have a unique best alignment or if there are no variants recorded for the tag):
    a. **variantPosOff:** the position (offset) of the variant (SNP) relative to the *startPosition* of the tag.
    b. **variantDef:** the allele represented by the tag at that position, in decimal ASCII code, where 65 = "A", 67 = "C", 71 = "G", 84 = "T", 45 = "-" (gap), and 78 = "N".
9. **dcoP**: placeholder for a *p*-value from a binomial test of the genetic mapping support for the chromosome and startPosition. Genetic testing of physical positions (obtained by sequence alignment) is not part of the standard pipeline (it requires custom code), so this is usually undefined (*).
10. **mapP**: placeholder for a *p*-value from a linkage disequilibrium test of the genetic mapping support for the chromosome and startPosition. Genetic testing of physical positions (obtained by sequence alignment) is not part of the standard pipeline (it requires custom code), so this is usually undefined (*).