

# MacVector 17

for Mac OS X

## **Tutorial: Next Generation Reference Alignments Using Bowtie**

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## Introduction

Generating sequencing data is cheaper than it has ever been. However, with this increase in data has come challenges with analyzing this data using a desktop computer.

To help make this achievable, *MacVector with Assembler* can create reference assemblies from next generation sequencing data with just a few mouse clicks. Instead of sending millions of reads away to be assembled or delving into complicated software tools, you can align millions of NGS reads to multi megabase reference sequences in just a few minutes.

Assembler uses the popular *Bowtie2* algorithm to create reference assemblies. Bowtie is a sequence aligner capable of extremely fast alignments of short and longer reads against a much larger reference sequence. Bowtie is extremely fast. You can assemble your data even on a low powered laptop computer with only two or three GB of RAM.

Assembler allows easy point and click assembly of reads against a reference using Bowtie. It will generate reports of SNPs and other variants. It supports the Variant Calling Format (VCF) and BAM file formats. Reads can be assembled against multiple references in a single analysis. Consensus and contig sequences can be exported in Fasta and Fastq formats for further analysis.

This tutorial will show how you can align a set of reads against a single reference sequence and how to analyze the results.

If you are interested in analyzing RNASeq data, you should also read the companion “RNASeq Expression Analysis Tutorial”.

## Sample Files

MacVector comes with a small set of tutorial sequences and reads. It's a "contrived" sample designed to assemble very quickly and small enough to download as part of the installer. The reference sequence is a single contig that is part of the *L. paracasei* genome and the reads are a small subset of the sequencing project that was used to sequence that genome using a 454 sequencer (from a single *de novo* assembled contig). They come from the SRR015575 set (the full set contains 6 such files). Note that although the reference sequence is the full contig sequence the reads will align only against the region from 1 to 427,800. Also be aware that the reads have been manipulated with two mutations.

The original reference sequence is available from the NCBI:

<http://www.ncbi.nlm.nih.gov/nuccore/DS990486>

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You can also download the original full set of reads direct from the NCBI's Short Read Archive (SRA):

<http://www.ncbi.nlm.nih.gov/sra/?term=SRR015575>

These sample files we use for this tutorial are kept in the following folder after MacVector has been installed;

/Applications/MacVector/Tutorial Files/Contig Assembly/NextGen files/

## Overview/Quickstart

To create a reference assembly, you need to have a reference sequence in any common format (MacVector, GenBank, EMBL, FastA etc) and Read files in either FastA or (preferably) FastQ format.

To quickly map reads against a reference, follow these steps;

1. Start MacVector with Assembler and choose **File | New | Assembly Project**
2. Click on the **Add Ref** button – in the resulting dialog, locate your reference sequence and click **OK**
3. Click on the **Add Seqs** button – in the resulting dialog, find the Fasta or Fastq file(s) containing your reads and click **OK**.
4. Select the reference and the read file in the **Project** window (hold down <shift> or use <command>-click to toggle selections). Click on the **Bowtie** button on the toolbar.

Note: that if no sequences are selected, Bowtie will be run on ALL of the files in the project. However, if any sequences are selected then the reference sequence and at least one read file must be selected.

5. Accept the default Bowtie options and click **OK**. Bowtie analyses can take some time depending on the reference and the number of Reads in your Fasta/q file(s). For example, assembly of 5 million paired end reads against a 5 Mb *E. coli* genome takes about 30 minutes on a Core 2 Duo laptop with 4 GB RAM.
6. You can close the progress window while the job completes.
7. When complete, a new named job object will appear in your project window. Inside this object, you will find a **Reference Contig** object.

8. Double-click on the <yoursequencename> Contig 1 item. This opens up the **Reference Contig Editor** – it shows the original reference sequence across the top and the aligned reads and consensus sequence below.
9. The **Summary** tab displays the summary statistics of the alignment. The **SNP** tab lists the differences between the reference and the consensus, along with any codon and amino acid changes if the SNP lies in a CDS feature. The **VCF** tab displays the raw VCF text data.
10. The **Coverage** tab shows the depth of reads across every gene and CDS feature annotated to the reference. This is useful for RNASeq and any analysis that considers expression levels.
11. Back in the **Project** window, click on the disclosure triangle next to the <yoursequencename> Contig 1 item. This reveals all of the individual contigs within the reference assembly. You can double-click on any of these to open the corresponding **Contig Editor** – this shows just the consensus sequence and the aligned reads.

## Tutorial

### Creating and Populating a New Assembly Project

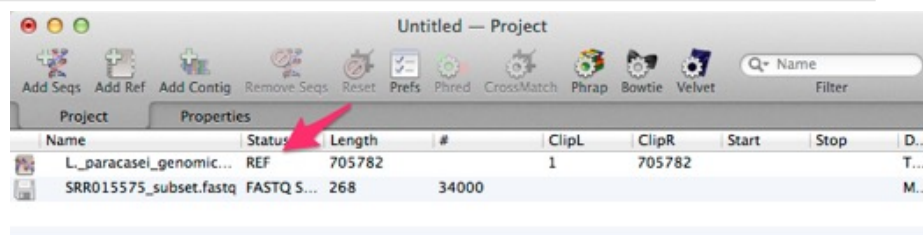
Start MacVector with Assembler and choose **File | New | Assembly Project**.

Click on the **Add Ref** button, select `L.paracasei_genomic_scaffold.nucl` and click **Open**.

Click on the **Add Seqs** button, select the `SRR015575_subset.fastq` and click **Open**.

You can also drag and drop Fasta/q reads files into the assembly project window. However, you must always use the **Add Ref** button to add reference sequences. Note that the **Add Contig** button is for adding existing BAM/SAM or .ace alignment files to a project.

Ensure that the `l.paracasei_genomic_scaffold.nucl` file has REF in the *Status* column (see screenshot below). This indicates it is a reference sequence.



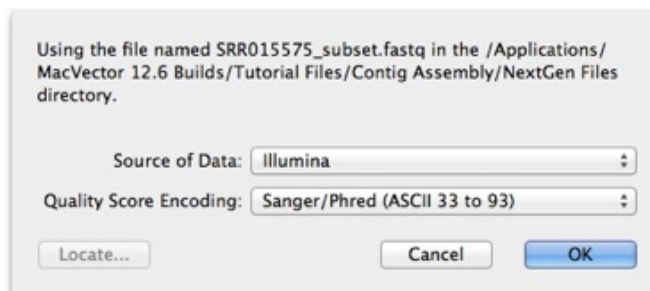
Now is a good opportunity to save the project

Choose **File | Save As...** Select a suitable location and call the file Tutorial Assembly

MacVector uses a *File Package* to store the individual files of the assembly Project. The actual project data is saved as a BSMML file, an XML-based format. This file will also contain trace files. Also note that the individual BAM files and BAM file indexes are also stored within the *File Package*. You can right-click and choose **Show Contents** to view the individual files.

Fasta/q files are added as a reference or “link” to the disk-based file rather than copying the entire file into the project. If you double click on the file it will show the location of the original file. If the file has since been moved you need to use the **Locate** button to specify the location of the moved file.

Double click on the SRR015575\_subset.fastq file in the project window.



Click on **Cancel** to dismiss the dialog.

## Running Bowtie

Select the reference and the read file in the *Project* window (hold down <shift> or use <command>-click to toggle selections). Click on the **Bowtie** icon on the toolbar.

You will see the Bowtie preference dialog. We will mostly use the defaults for this example.

If your Mac has a CPU with more than one core, you can change the *Number of Threads* to a higher value (e.g. **8**). Make sure you have the *Generate Child Contigs* checkbox selected. Click **OK**.

**Bowtie Options**

Preset:

Type of Alignment:

No gaps within first bases of read:

Number of Threads:

Use paired-end alignments

Minimum insert size:

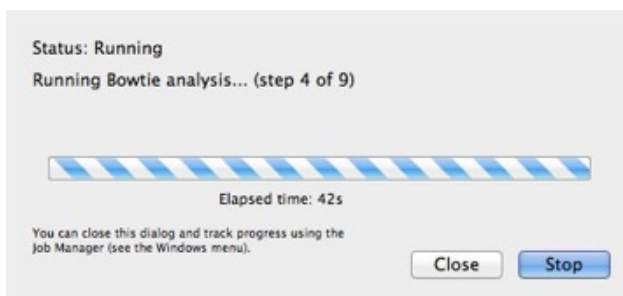
Maximum insert size:

Orientation:

**Generate child contigs**

Check this box if you are using the Reference as a scaffold to assemble related reads, or if you want to 'drill down' into individual alignments. For other tasks (e.g. SNP analysis or RNA-Seq expression analysis) leave this unchecked.

The Bowtie reference alignment job goes through a number of steps. The first few steps will be performed very quickly but the assembly step and the generation of the INDELS and the VCF report (SNPs and other variants) may take a considerably longer time. The status of the alignment will be displayed in the status dialog or in the *Job Manager* if the status dialog is closed.



There are 11 steps to a Bowtie alignment;

1. "Determining the read file encoding (step 1 of 11)"
2. "Creating reference FASTA file (step 2 of 11)"
3. "Creating read FASTQ files (step 3 of 11)"
4. "Running Bowtie indexing and analysis... (step 4 of 11)"
5. "Extracting the consensus sequence and contigs (step 5 of 11)"

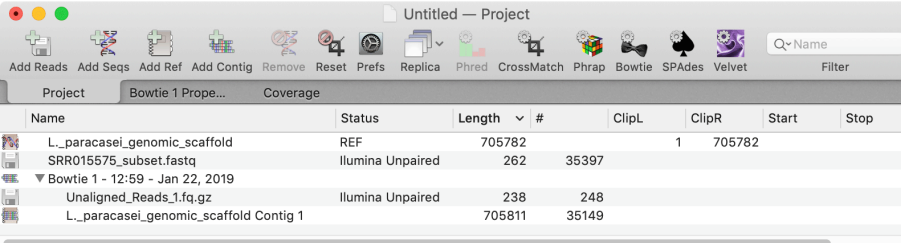


6. "Generating coverage data for (step 6 of 11)"
7. "Generating contig for " (step 7 of 11)"
8. "Generating INDELs for " (step 8 of 11)"
9. "Generating child contigs for " (step 9 of 11)"
10. "Generating SNP report for "(step 10 of 11)"
11. "Gathering unassembled reads (step 11 of 11)"

When complete click view in the **Job Manager** or **Status Dialog** and a new named Bowtie job object will appear in your project window. The original reference and read files are unchanged, so you can re-run the job with different parameters if you wish.

Inside the Bowtie Job Object are two items – (a) a link to a file containing all of the reads the DID NOT align to the reference sequence and (b) a Contig representing the alignment.

*Note that this in the screenshot below the **Name** field has been widened to accommodate the entire Reference Contig name:*



Name	Status	Length	#	ClipL	ClipR	Start	Stop
L_paracasei_genomic_scaffold	REF	705782		1	705782		
SRR015575_subset.fastq	Illumina Unpaired	262	35397				
▼ Bowtie 1 - 12:59 - Jan 22, 2019							
Unaligned_Reads_1.fa.gz	Illumina Unpaired	238	248				
L_paracasei_genomic_scaffold Contig 1		705811	35149				

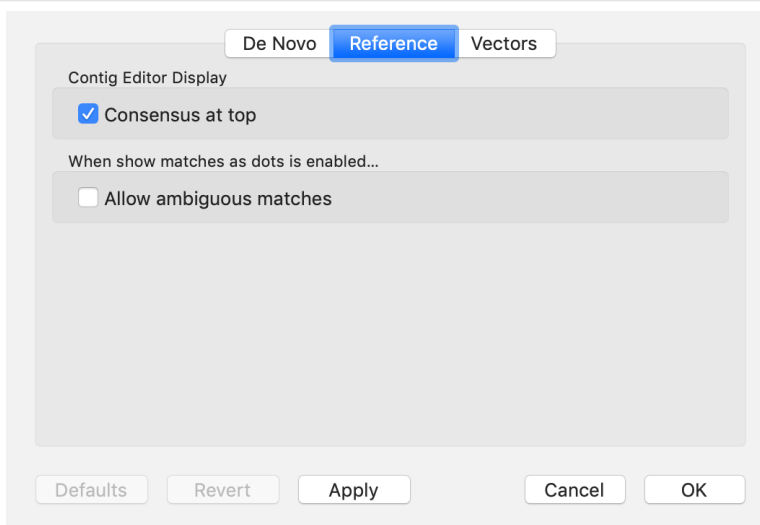
With the assembly project window active, choose **File | Save**

Now we will open the **Reference Contig** to see the results

Double-click on **L\_paracasei\_genomic\_scaffold Contig 1**.

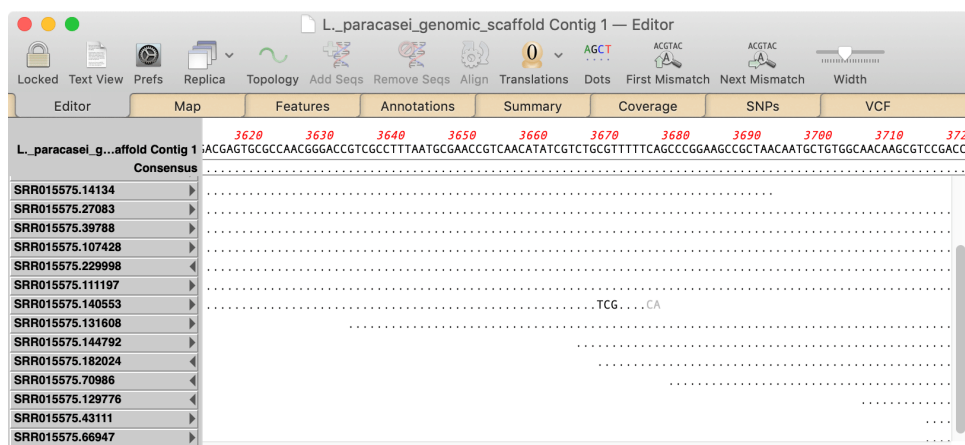
This opens up the **Reference Contig Editor** – it shows the original reference sequence across the top and the aligned reads and consensus sequence below.

Click on the **Prefs** toolbar button, select the **Reference** tab, ensure the **Consensus at top** box is checked and click **OK** (or click **Cancel** if it is already checked).



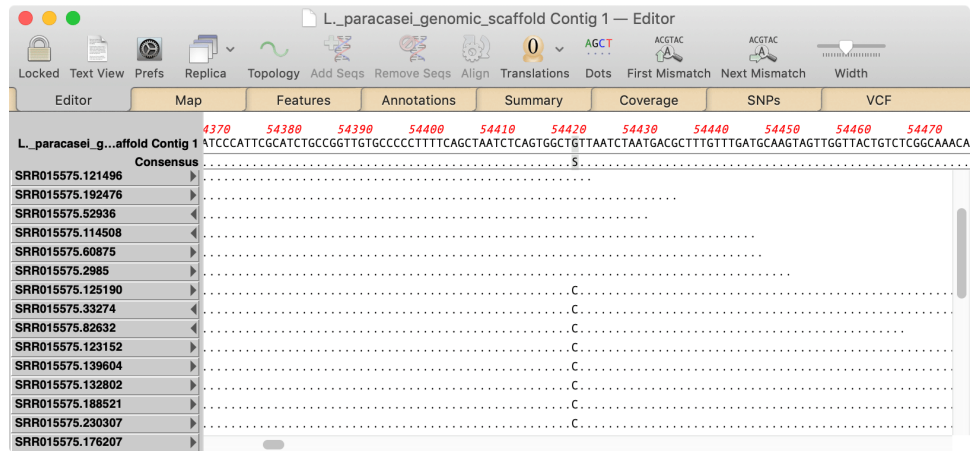
Click the **Dots** button in the toolbar

Now only residues that do not match the reference sequence will be shown as characters;



Now click **First Mismatch**

The cursor will move to and display the first location where the consensus does not agree with the reference sequence. In our example this is near 54,420. Note that only a percentage of the reads are different at this point but sufficient to show a different base in the consensus.



Now click **Next Mismatch** twice

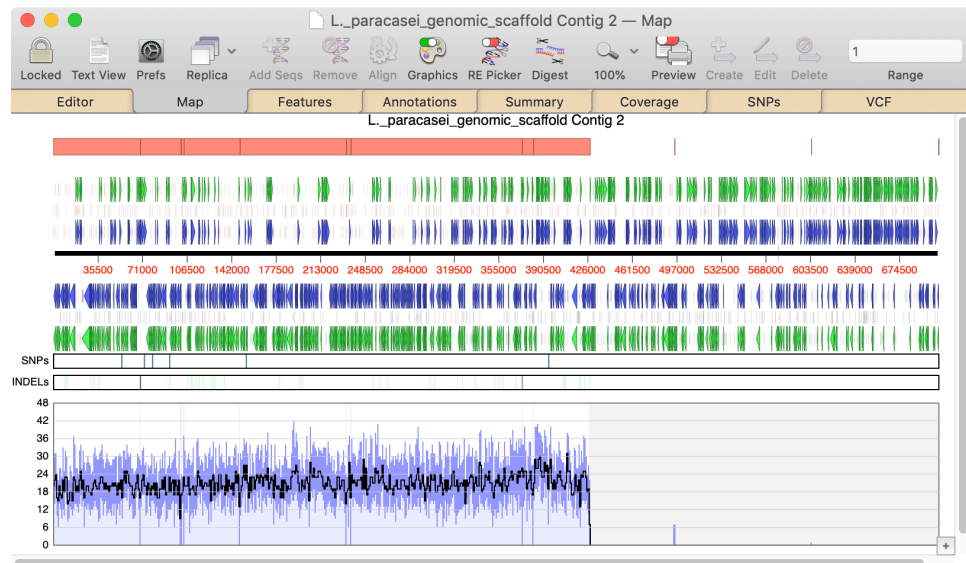
The cursor will move and display the next two mismatches. The second is at 78,933. Here all reads contain the different base.



## Coverage Depth

Click on the **Map** tab. If you see red text warning you that features have been hidden, right-click (or <ctrl>-click) and choose **Show All Features** in the context-sensitive menu.

This shows a graphical representation of the reference contig.  
 NOTE: for performance reasons individual reads are NOT shown in the **Map**.



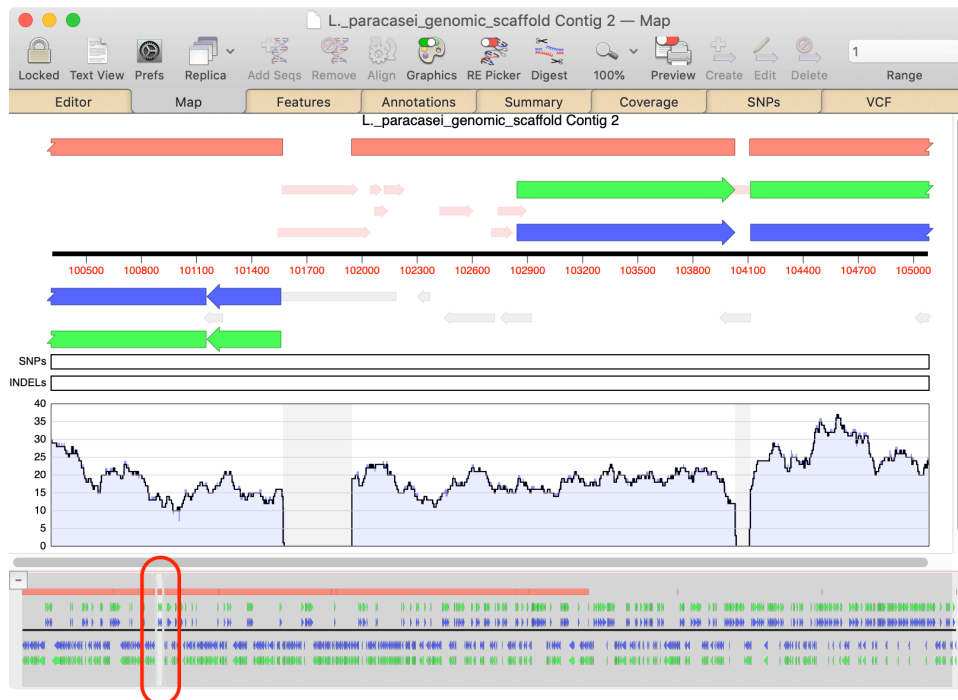
Note the following features of the **Map** view from top to bottom:

- Child contigs are annotated as `misc_feature` features in the reference contig. These will be saved if you export the reference contig as a single sequence MacVector file.
- The original annotation/features are shown above and below the sequence ruler line.
- Any SNPs found during the alignment are represented by a vertical line.
- Any INDELs (insertions or deletions) found during the alignment are represented by a vertical line.
- The *Coverage Depth* plot shows the number of reads aligned against the template at each point (read depth). A single plot line (default color is black) shows a running average of the number of reads at that point, calculated using a moving window of dynamic length depending on the zoom level. The highest value in that window (default color is dark blue) and the lowest value (default color is light blue) are also shown.
- The *Overview* (click on the “+” button in the lower right corner if it is hidden as shown in the image above) shows the full length of the contig with features and child contig features shown. If the main sequence window is zoomed into a region this region will be marked on the overview.

Lets look more closely at the region around 100,000. There are two regions of zero coverage (thin grey vertical bars) you can just make out. Although these regions may be as short as a single nucleotide in length, even at this zoomed out level they are still visible.

Zoom into 100,000 to 104,000 by dragging the cursor along the map. Do this by positioning the cursor along the sequence line somewhere just 100,000. Hold down the mouse button, drag the cursor to just after the 104,000 marker then release the mouse button. You can do this multiple times until you have zoomed into the correct region. To reset the zoom level, simply double click on the white space background. You can also use the right and left cursor keys for fine adjustment of the viewed area.

The **Map** will now look similar to the following screenshot:



Note the following:

- The *Overview* now indicates the location of the region shown in the main window (See the light grey section highlighted by the red oval).
- The three plots in the coverage map have become very close as the window they are calculated from becomes shorter. Compare this with the initial coverage map showing the entire mapped region.
- There are two areas of zero coverage in the coverage map (shown with a grey background).
- The end of one child contigs, a full one and the start of a third are annotated (in red) on the reference contig. Note that these start and stop with the areas of zero coverage (a child contig is defined as a region of the reference sequence

bounded by either end or a region of two or more bases with zero reads aligned.).

- Hovering the mouse over a child contig feature will show the start, stop and name of that child contig.
- The green and blue features are genes and CDS features that existed in the original reference sequence.
- If you hover the mouse over the coverage map it will give the exact number of reads at that position (for example X reads over base XX), along with the range of reads and the number of bases over which the average has been calculated.

Hover the mouse cursor over the larger area of zero coverage.



Note the tooltip showing zero values along with the window size.

Now hover the mouse over the highest peak



Note that the tooltip now shows the height of that peak along with the same window size.

## Mapping Statistics

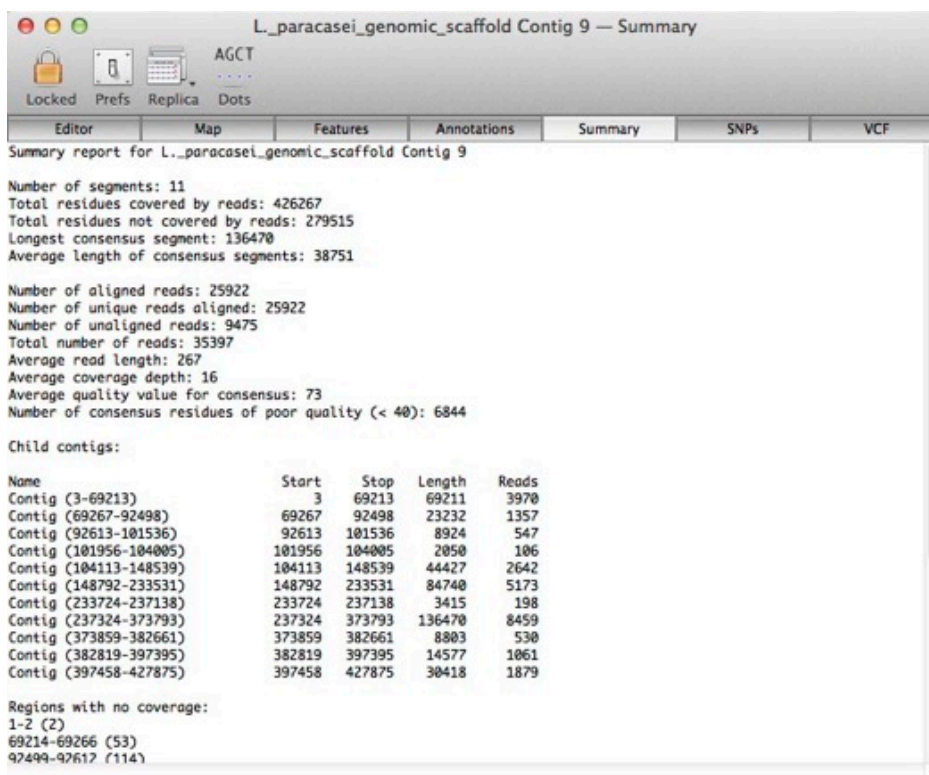
Click on the **Summary** tab.

The **Summary** tab displays a summary of the alignment.

Note the following:

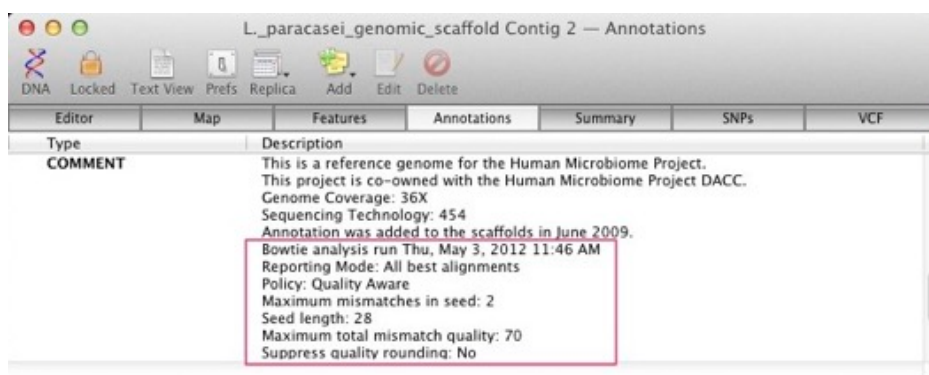
- All the child contigs are listed with details.
- Regions with no coverage are again listed.
- There is also a base composition table of the consensus.

This report can be saved using File | Export As..



Click on the **Annotations** tab, scroll down and view the **COMMENT** annotation

All the settings used to run Bowtie are added, along with the date and time of the run, to the Comment field of the **Annotations** tab.



## Looking for SNPs and other variations

Assembler produces two reports on sequence variations found in the alignment.

Click on the **SNPs** tab

Editor	Map	Features	Annotations	Summary	SNPs	VCF
SNP report for L._paracasei_genomic_scaffold Contig 16						
Differences between the Consensus and Reference Sequences:						
54419	G -> S					
78933	T -> A	lies in CDS	/codon_start=1			
/db_xref="GI:239526721"						
/locus_tag="LBPG_01171"						
/product="malate dehydrogenase"						
/protein_id="EEQ65722.1"						
/transl_table="11"						
/translation="MARTIGIIGIHGVVTTAFNLVSKGVADKLVLDIKKAELAEGESFDLKDALGGLPTYTDIVVNDYDALKDADVVISAVGNIGAISNGRIGETKTSKVAL"						

The SNP tab lists the differences between the Reference and the Consensus, along with any codon and amino acid changes if the SNP lies in a CDS feature.

Note that the two variations that we have already seen above are listed:

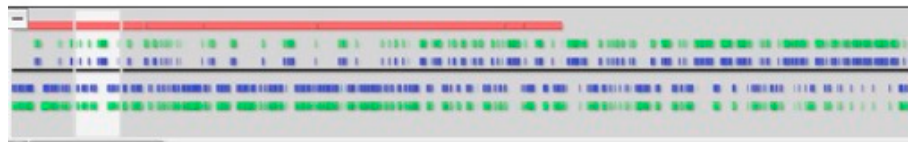
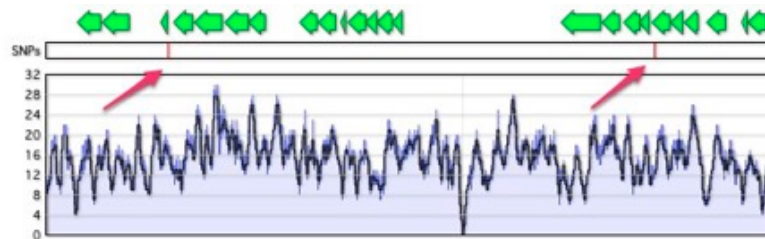
Click on the **VCF** tab

VCF or Variant Calling Format is a popular file format used to store and report variations found in a reference assembly. The **VCF** tab displays the raw VCF data that many other programs can use to evaluate SNPs in the data. Again our two SNPs are reported here.

You can export the VCF file using **File | Export As...** from this tab.

Switch back to the **Map** tab. Drag select to zoom into the section around the first vertical red line in the SNP line

Any reported SNPs are shown in the Map view.



## Child Contigs

Back in the *Assembly Project* window, click on the disclosure triangle next to the *L.\_paracasei\_genomic\_scaffold Contig 1* item.

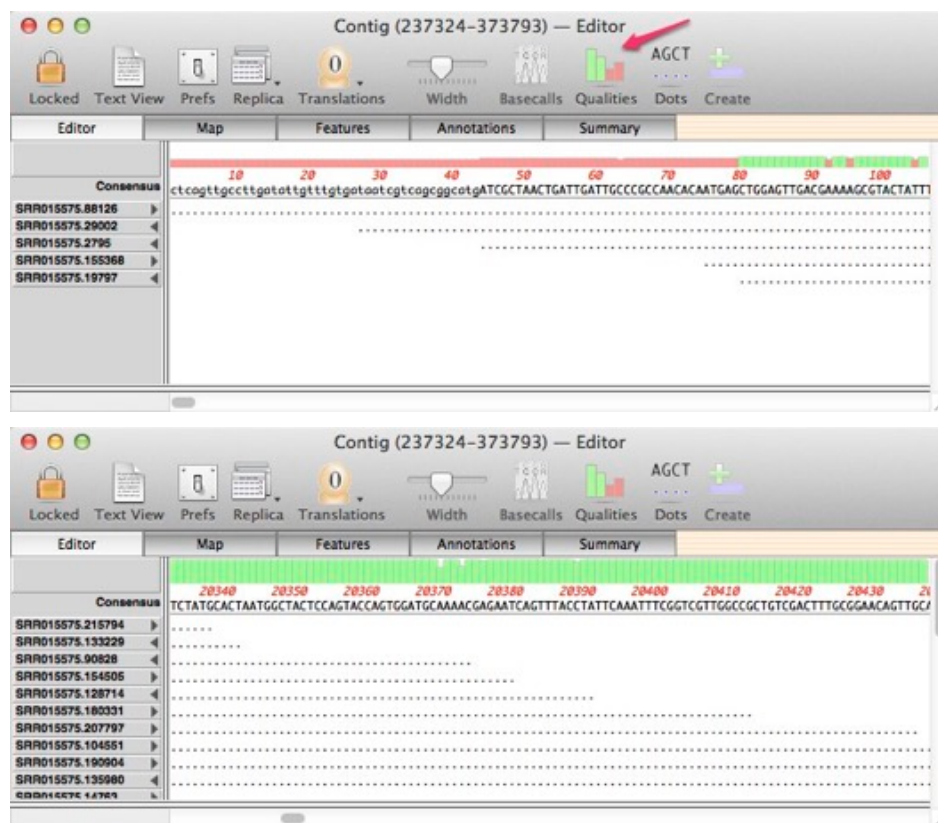
This reveals all of the individual child contigs within the reference assembly. You can double-click on any of these to open the corresponding *Contig Editor* – this shows just the consensus sequence and the aligned reads.



Double click on the longest contig (237,324 to 373,793).

Once open click on the **Qualities** button in the toolbar

Note that the beginning of the contig shows poor quality due to the low number of reads and their poor quality scores. However, as you scroll along the contig in this example the consensus will be shown to have a higher quality score due to the number of high quality reads that the consensus has been calculated from.



## Further Analysis

### Exporting Contigs

The **File | Export As..** menu option allows contigs and consensus sequences to be exported in Fasta or Fastq formats.

- From the Project window, if the reference contig is selected it will save a Fasta or Fastq file containing all child contigs and no reference contig sequence or reference contig consensus. Selection of child contigs is ignored.
- From the Project window with only child contigs selected, it will save a Fasta or Fastq file containing all selected child contigs and no reference contig sequence or reference contig consensus.

- From an open Reference Contig it will save a multiple sequence Fasta or Fastq file containing the reference sequence and the consensus.
- From an open Child Contig it will export a single sequence Fasta or Fastq file with the consensus sequence.
- From the Project window with the unassembled reads file selected it will save a Fasta or Fastq file containing all unaligned reads and no other sequences.

### Working with sequences back in MacVector

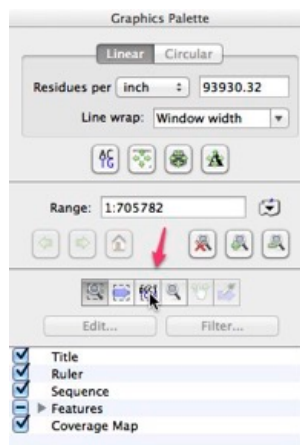
The coverage map makes it very easy to design primers for further sequence, for example, Sanger sequencing for hybrid assembly. Remember that you can run any MacVector analysis function directly on a contig and it will act as if you are running that analysis on a single sequence.

Here's how easy it is to design primers:

Zoom into an area of low coverage using the cursor in the reference contig.

First look for an area of low, or zero, coverage. Remember that areas of 2 or more bases with zero aligned reads are highlighted in grey and will be visible at all levels.

Click the **Select Sequence** button in the *Graphics Palette*



Now back in the **Map** tab of the *Reference Contig* drag the cursor over the sequence spanning the low coverage region to select it.

Now select **Analyze | Primers | Design Primers (Primer3)....**

Check the popup menu in the dialog is set to *Amplify Feature/Region*. This will now take a 200bp region either side of your selected region and design primers to amplify this region.

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Now you can amplify this sequence from your original sample, or instead design some sequencing primers and sequence it directly. For more information and practical examples of designing PCR and sequencing primers with MacVector, read the [Primer Design Tutorial.pdf](#) document in the `/MacVector 12.6/Documentation/` folder.

## Important Concepts

Bowtie2 uses less memory and is generally faster and more sensitive than Bowtie1. There is some advantage to using bowtie1 for reads less than 50bp. But nowadays the reads from all current sequencing technologies are much longer than this.

### Preset

Bowtie2 comes with eight sets of preset parameters. With speed versus accuracy. Note that the presets do change whether you are doing a local or global alignment. Again Assembler deals with this for you and just presents you with four presets:

*very fast*

*fast*

*sensitive (default)*

*very sensitive*

### Type of Alignment (-local, default end to end).

Bowtie2 has local alignment as well as global alignments (end to end). In local alignment mode it will “trim” bases from either end of a read if a better alignment is the result. Global (end to end) alignments will map the entire read against the reference, although do remember that gaps may be inserted (see below).

### No gaps within first bases of read

Bowtie2 will now insert gaps in the reference and reads where doing so will increase the alignment score. Such INDELS are reported in the VCF tab and are also graphically represented in the MAP tab.

By default gaps will not be introduced into the first four bases of a read. This may be changed with the following parameter.

## Threads

The `-p` parameter defines how many threads are used. MacVector defaults to one less thread than the number of CPU cores on your machine.

This does increase speed, but obviously also memory requirement. Note in testing with 8 threads and an *E. coli* reference with 2.5 million Solexa paired reads total memory usage typically stays below 0.5 GB.

## Paired End Alignments

There are two new concepts with regards to pairs.

Bowtie2 now has a “mixed” mode. If it cannot find a paired match, within the given parameters, for a supposedly paired read it will treat each pair mate as single reads.

There is a new concept of concordant and discordant aligned pairs. Concordant means it matches all the given parameters. Discordant means that they still align but with much relaxed limits. This is distinct from the mixed mode stated above. Discordant matches is the default setting and is important to find structural variants. e.g. you are aligning your reads against a reference that has large insertions or deletions. Any pairs that span those insertions or deletions will not match the specific insert limits you have defined. That is each of the pair will be closer or further apart than you would have been expecting, but will still be valid matches. This is turned on.

**USE PAIRED-END ALIGNMENTS:** Default is ON for projects containing two reads files and OFF for all others

**MINIMUM INSERT SIZE** (`-i`, Default: 0). The minimum insert size for valid paired-end alignments. For example if 60 is specified and a paired-end alignment consists of two 20-bp alignments in the appropriate orientation with a 20-bp gap between them, that alignment is considered valid. A 19-bp gap would not be valid in that case.

**MAXIMUM INSERT SIZE** (`-X`, Default: 250): The maximum insert size for valid paired-end alignments. For example if 100 is specified and a paired-end alignment consists of two 20-bp alignments in the proper orientation with a 60-bp gap between them, that alignment is considered valid. A 61-bp gap would not be valid in that case.

**ORIENTATION:**

Forward Reverse (fr): the paired reads are 5' to 3' at one end with 3' to 5' at the other end. i.e. the pair are on opposite strands and pointing to each other.

Forward Forward (ff): Both reads are 5' to 3' and on the same strand

Reverse Forward (rf): the paired reads are 3' to 5' at one end with 5' to 3' at the other end. i.e. the pair are on opposite strands and pointing away from each other.

Note: - most, but not all, Illumina paired end reads are -fr. The Mate Pair protocol for Illumina produces -rf and Solid reads are always -ff.

## Coverage Map

When you generate a reference contig with Bowtie, the **Map** tab of a reference or child contig will show a plot of the depth of reads along the entire reference. This coverage map shows four statistics. A single plot line (default color is black) shows a running average of the number of reads at that point, calculated using a moving window of varying length depending on the zoom level. Such a plot is not sensitive when the window shows a large region of sequence at a high level, for example when viewing megabases of sequence). So, two shaded areas indicate the highest value (default color is dark blue) and the lowest value (default color is light blue) of the reads averaged for that window. When the coverage map is viewed at higher magnifications, the window from which the running average is calculated becomes shorter and so these three values will become closer. Eventually, when viewed at, or close to, residue level, these three plots will become identical.

### Regions of zero coverage

Areas of zero coverage are shown in light grey. These areas are always displayed even when they are disproportionate to the level of magnification to ensure that you can spot even a single residue of no coverage in a 20 Mb contig.

### Regions with low coverage

There are many reasons why regions will have lower than average coverage. These generally are caused by the base composition over that region. For example regulatory elements in a sequence, where proteins such as transcription factors bind, often display abnormally low coverage.

**Regions with high coverage**

Short regions with excessively high coverage are typically indicative of a repeated region, particularly if All Alignments is selected as the search mode. Reads will be piled up on one of the repeated sections rather than being spread out over each repeated region. Paired end reads can go some way to help detect these and allow correct alignment of reads.