CpGviewer *User Guide*

Overview

This programme is designed to automate the process of reading and aligning the DNA sequences of cloned PCR products derived from bisulphite-treated mammalian DNA. It is able to analyse files from the MegaBace and ABI series of sequencers, as well as standard chromatogram format (*.scf) and plain text files. There is no preset minimum hardware specification, but the computer must run the Microsoft .NET Framework 2.0. The length of time needed to analyse each data set will depend on the number of files and the size of the CpG island.

1 Getting Started

Figure 1 shows the main menu used to create the alignments and alter the alignment options.

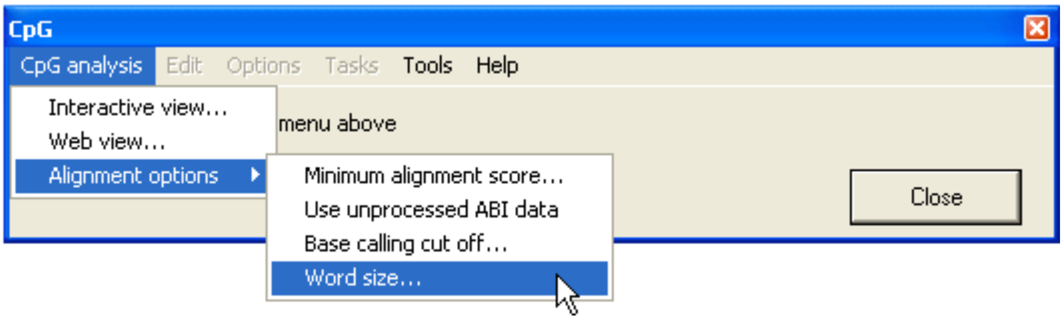


Figure 1

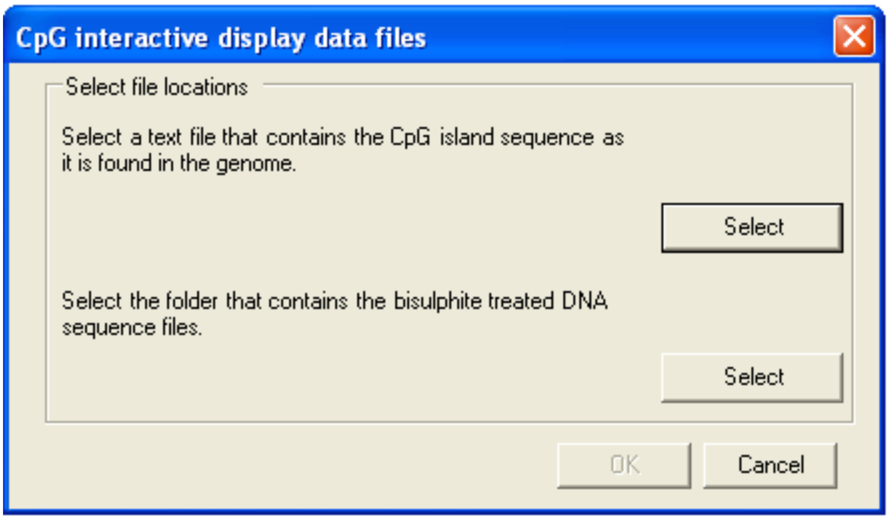
Alignment options

- **Minimum alignment score**...: This sets the cut-off score at which a sequence alignment is accepted. The values range from 20 to 50, with a score of 20 roughly equating to 20% of the aligned sequence segments closely matching the reference sequence.

- **Use unprocessed ABI data**: This option instructs the programme to analyse the peak heights using the raw data in .AB1 files. (If the files have not been previously analysed, the programme will automatically read and process the raw data.) When using this option, the programme will run more slowly, since it has to search the files first for the processed data and then the raw data.
- **Base calling cut off**… : This sets the minimum peak height at which the programme will call a nucleotide. This value is shown as a red horizontal line across all electropherogram images. (See **Figure 7**). This line should ordinarily be close to the bases of the trace peaks unless the sequence is very faint.
- **Word size**… : This sets the initial size of a local alignment from which the global alignment is created. The valid range is between 6 and 15. The optimum value depends on the sequence of the specific CpG island under study. Increasing the value reduces the overall alignment score, but may reduce the insertion of aberrant gaps (**Figure 6**).

Creating an alignment

Alignments can be displayed in two formats, the '**Interactive view**…' and the '**Web view**…' . Each format displays an alignment of the reference and query sequences and identifies the position and status of each CpG dinucleotide in the query sequences. To create an alignment, a reference sequence (plain text) and a folder containing the data files must first be selected; this is done by clicking the appropriate menu and selecting the files via the form (**Figure 2**).



CpG interactive display data files

Select file locations

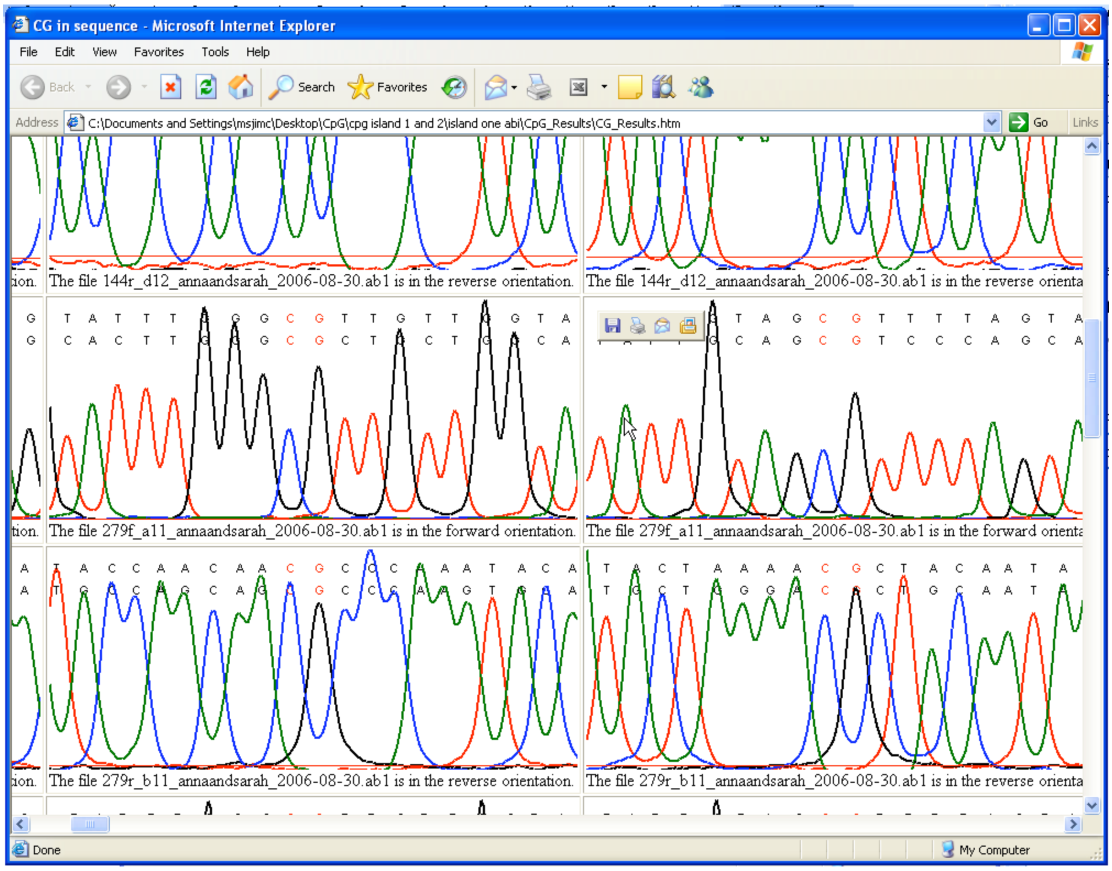
Select a text file that contains the CpG island sequence as it is found in the genome.

Select the folder that contains the bisulphite treated DNA sequence files.

Figure 2

Web view

In the **Web view**, the sequence information is used to create a table of images, each image showing a CpG dinucleotide within the alignment and a local segment of the underlying electropherogram data (**Figure 3**). (Since these images are derived from scan data, this option cannot be used with plain text input files.)



C:\Documents and Settings\msjimc\Desktop\CpG\cpg island 1 and 2\island one abi\CpG_Results\CG_Results.htm

ion. The file 144r_d12_annaandsarah_2006-08-30.ab1 is in the reverse orientation. The file 144r_d12_annaandsarah_2006-08-30.ab1 is in the reverse orienta

tion. The file 279f_a11_annaandsarah_2006-08-30.ab1 is in the forward orientation. The file 279f_a11_annaandsarah_2006-08-30.ab1 is in the forward orienta

ion. The file 279r_b11_annaandsarah_2006-08-30.ab1 is in the reverse orientation. The file 279r_b11_annaandsarah_2006-08-30.ab1 is in the reverse orienta

Figure 3

Within the table, the CpG dinucleotides are ordered by column and the sequence files by row. When creating a **Web view**, it must be remembered that each image is approximately 12 kb in size, so that for large CpG island aligned to many trace files, a very large web page may be created, which will be slow to load. Also, since each image is saved to disk, the alignment is relatively slow to complete.

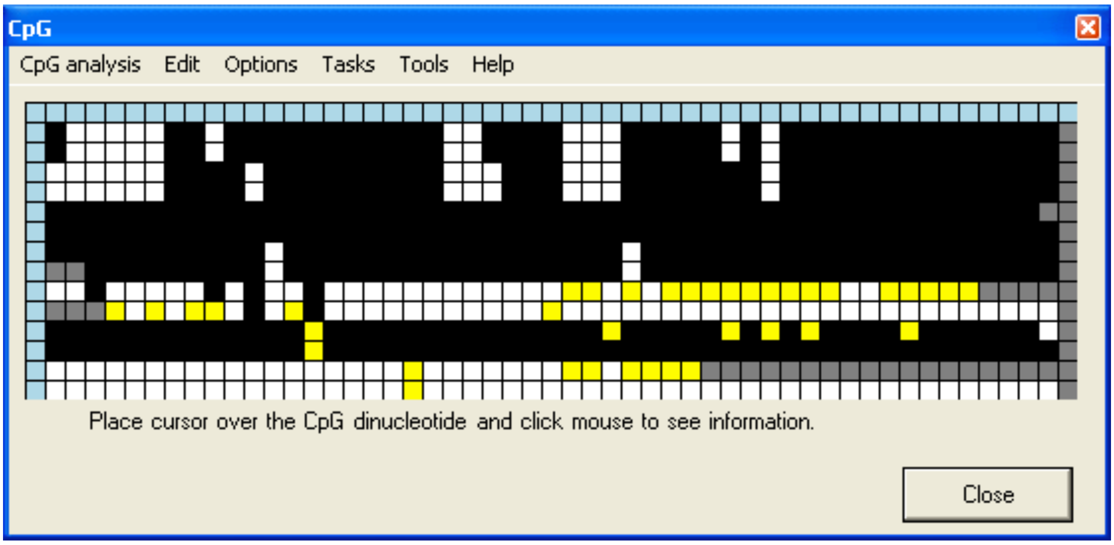
Interactive view

In the **Interactive view**, the alignment data are used to create a data grid, similar to the **Web view** in that the dinucleotides are arranged by column and the files by row (**Figure 4**). However, rather than a local sequence image, each CpG is represented by a cell, which is colour-coded according to the sequence of that dinucleotide in the query sequence (**Table 1**). When an alignment is created, the programme window resizes to fit the newly-generated grid.

Colour	**Sequence**	**Methylation status**
Black	CpG	Methylated
White	TpG or CpA (single colour mode)	Unmethylated
Pale green	TpG (two-colour mode)	Unmethylated
Pink	CpA (two-colour mode)	Unmethylated
Yellow	Not CpG, TpG or CpA	Unknown
Grey	Not aligned	Unknown

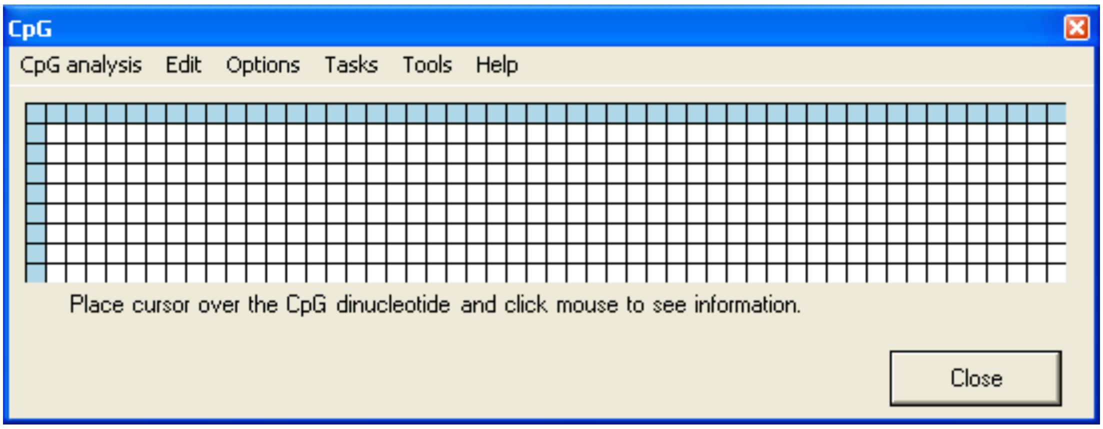
Table 1

The grid display enables the methylation status of each CpG to be identified and checked. Because bisulphite-PCR amplification is specific for one strand of the original template DNA, any unmethylated CpG in a sequence should be converted to the same dinucleotide variant. Therefore, if an individual CpG dinucleotide is found to be represented by TpG within a sequence containing multiple CpG to CpA conversions, an error of some kind is present that requires attention. This kind of error will not be noticed if TpG and CpA dinucleotides are both displayed as white squares (**Figure 4B**). In contrast, if the two-colour option is selected (**Figure 4C**) the unmethylated dinucleotides CpA and TpG are shown in different colours, so that any erroneously called dinucleotides (row 6, column 16 and row 8, column 31) can clearly be seen.



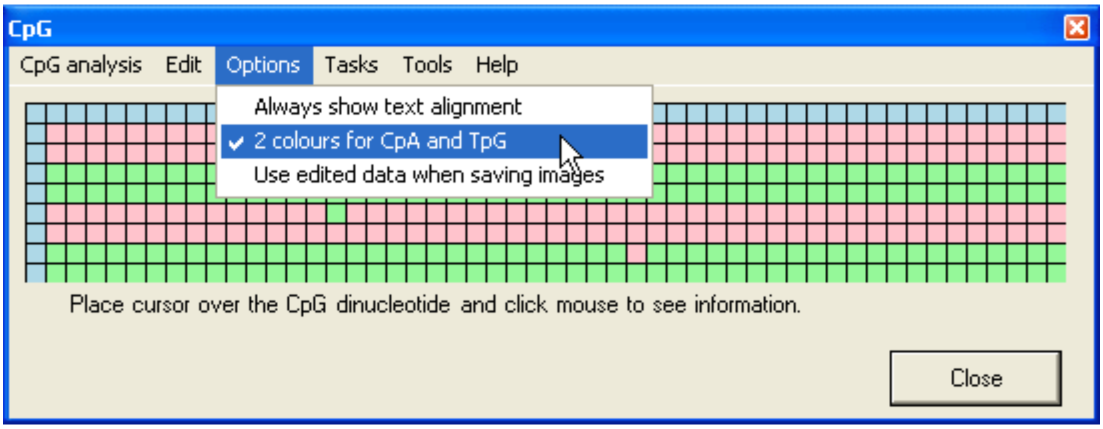
Place cursor over the CpG dinucleotide and click mouse to see information.

A



Place cursor over the CpG dinucleotide and click mouse to see information.

B


CpG
CpG analysis
Edit
Options
Tasks
Tools
Help
Always show text alignment
✓ 2 colours for CpA and TpG
Use edited data when saving images
Close

Place cursor over the CpG dinucleotide and click mouse to see information.

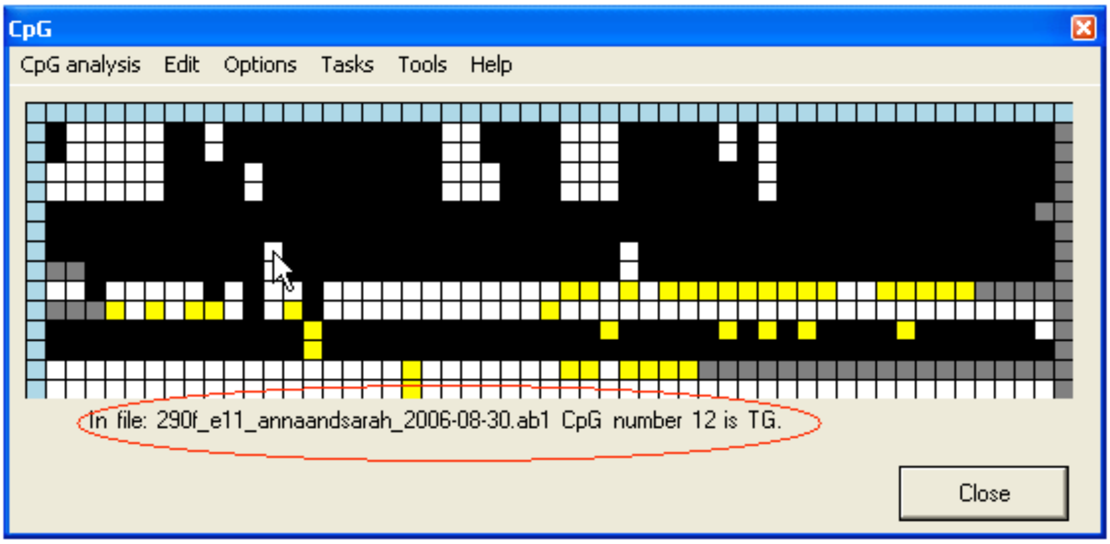
C

Figure 4

The programme will identify all the CpG dinucleotides in the reference sequence; however it will only score those dinucleotides that lie more than 10 bp from the ends of the reference sequence. Any dinucleotide within 10 bp of the end will be unaligned, and shown as a grey square (see the last column in Figure 4a).

File and dinucleotide identification

Left-clicking a grid square causes that dinucleotide’s position in the reference sequence, its status within the query sequence and the query sequence’s filename to appear below the grid (**Figure 5**). The blue squares at the top of each column and start of each row contain more detailed information on the dinucleotide (column header) and the query sequence file (row leader); again, this information can be accessed by left-clicking the square. Alternatively, right-clicking the blue square at the start of each row will open a window displaying the electropherogram trace image. The top left corner blue square contains statistics on the dinucleotide status of the grid as a whole.

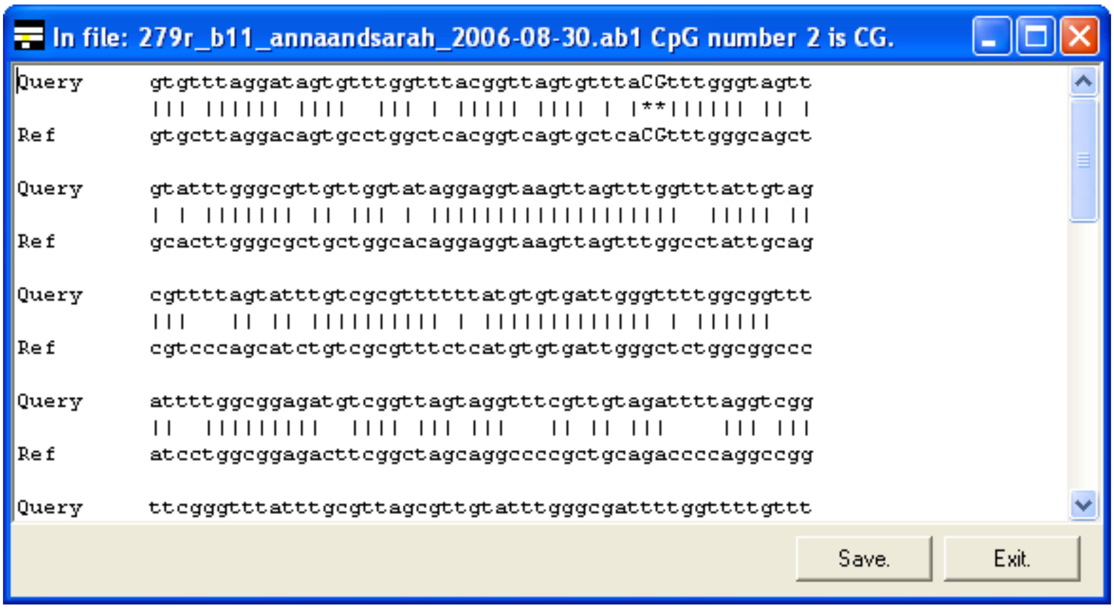


In file: 290f_e11_annaandsarah_2006-08-30.ab1 CpG number 12 is TG.

Figure 5

Viewing the Alignment

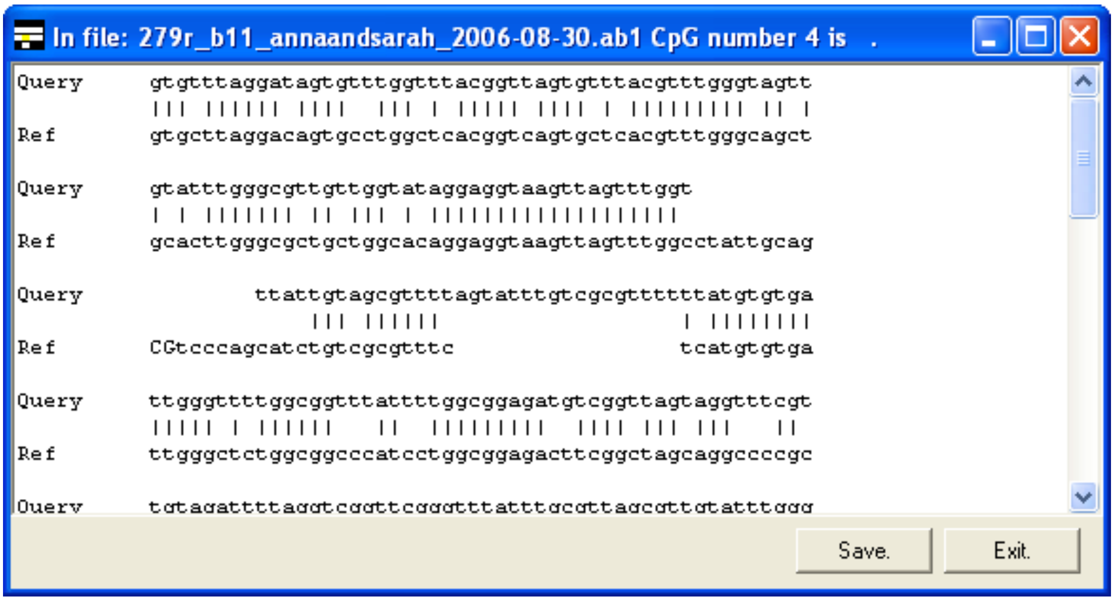
By right-clicking a square (other than blue or grey squares), the underlying sequence alignment can be inspected. This can be either a global text alignment (plain text sequence files) (Figure 6A) or a local section of this alignment along with the corresponding part of the electropherogram trace (Figure 7). In the latter case, the trace image will sometimes represent the reverse complement of the reference sequence (Compare the reverse sequence in Figure 7A to the forward sequence in Figure 7B). By default, the trace image is displayed, but by setting the **Always show text alignment** option (Figure 8) the global (text) alignment will be displayed in preference. If it appears that inappropriate gaps have been inserted in the global alignment, increasing the **Word size** (via the **alignment option**) may eliminate them (*e.g.* Figure 6A uses a word size of 10 compared to a word size of 6 in 6B).



In file: 279r_b11_annaandsarah_2006-08-30.ab1 CpG number 2 is CG.

```
Query       gtgtttaggatagtgtttggtttacggttagtgtttaCGtttgggtagtt
            ||| |||||| ||||  ||| | ||||| |||| | |**|||||| || |
Ref         gtgcttaggacagtgcctggctcacggtcagtgctcaCGtttgggcagct

Query       gtatttgggcgttgttggtataggaggtaagttagtttggtttattgtag
            | | ||||||| || ||| | |||||||||||||||||||  ||||| ||
Ref         gcacttgggcgctgctggcacaggaggtaagttagtttggcctattgcag

Query       cgttttagtatttgtcgcgttttttatgtgtgattgggttttggcggttt
            |||   || || |||||||||| | ||||||||||||| | ||||||
Ref         cgtcccagcatctgtcgcgtttctcatgtgtgattgggctctggcggccc

Query       attttggcggagatgtcggttagtaggtttcgttgtagattttaggtcgg
            ||  |||||||||  |||| ||| |||   || || |||    ||| |||
Ref         atcctggcggagacttcggctagcaggccccgctgcagaccccaggccgg

Query       ttcgggtttatttgcgttagcgttgtatttgggcgattttggttttgttt
```

A

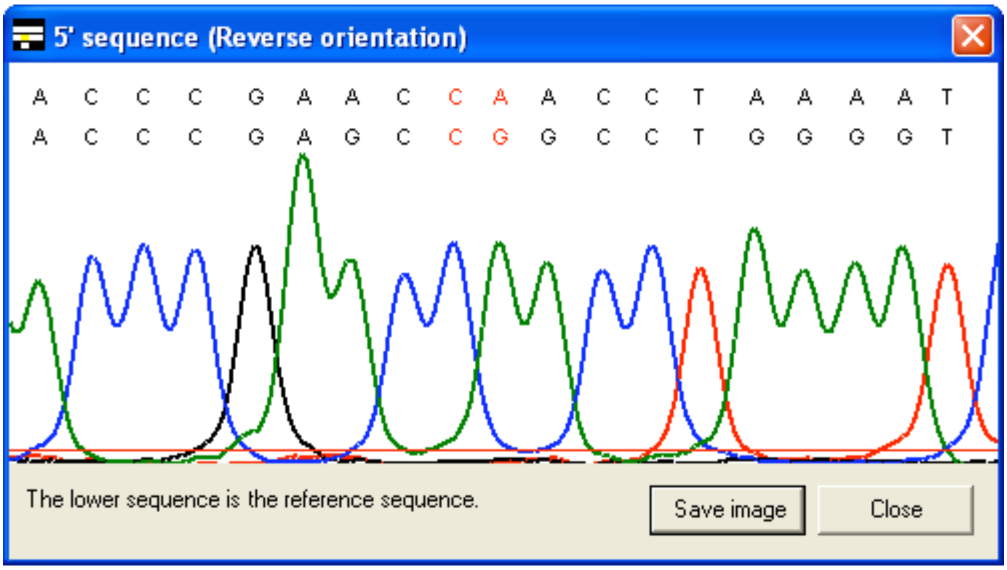


In file: 279r_b11_annaandsarah_2006-08-30.ab1 CpG number 4 is .

```
Query       gtgtttaggatagtgtttggtttacggttagtgtttacgtttgggtagtt
            ||| |||||| ||||  ||| | ||||| |||| | ||||||||| || |
Ref         gtgcttaggacagtgcctggctcacggtcagtgctcacgtttgggcagct

Query       gtatttgggcgttgttggtataggaggtaagttagtttggt
            | | ||||||| || ||| | |||||||||||||||||||
Ref         gcacttgggcgctgctggcacaggaggtaagttagtttggcctattgcag

Query               ttattgtagcgttttagtatttgtcgcgttttttatgtgtga
                        ||| ||||||                  | ||||||||
Ref         CGtcccagcatctgtcgcgtttc                 tcatgtgtga

Query       ttgggttttggcggtttattttggcggagatgtcggttagtaggtttcgt
            ||||| | ||||||   ||  ||||||||| |||| ||| |||   ||
Ref         ttgggctctggcggcccatcctggcggagacttcggctagcaggccccgc

Query       tgtagattttaggtcggttcgggtttatttgcgttagcgttgtatttggg
```

B

Figure 6



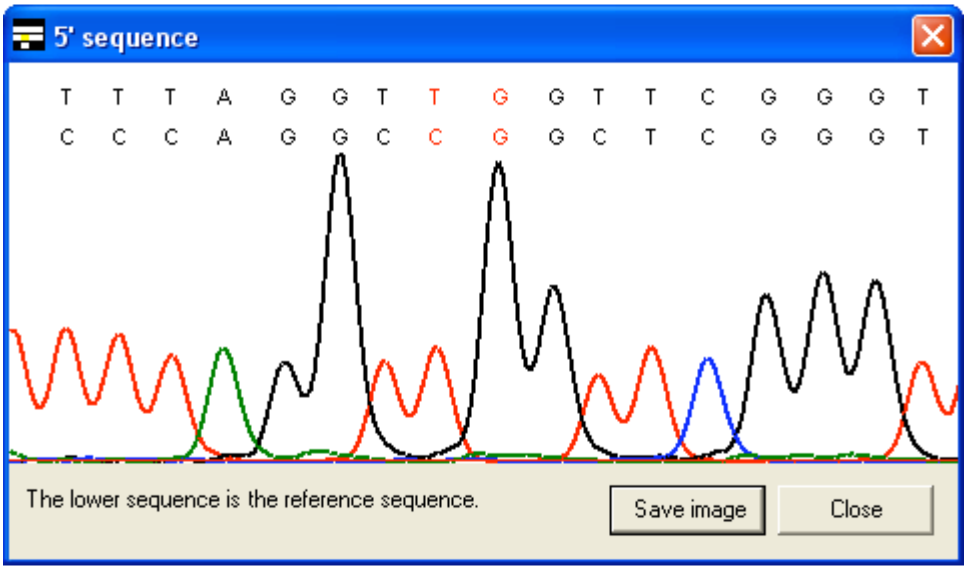
5' sequence (Reverse orientation)

A C C C G A A C C A A C C T A A A A T

A C C C G A G C C G G C C T G G G G T

The lower sequence is the reference sequence.

A



5' sequence

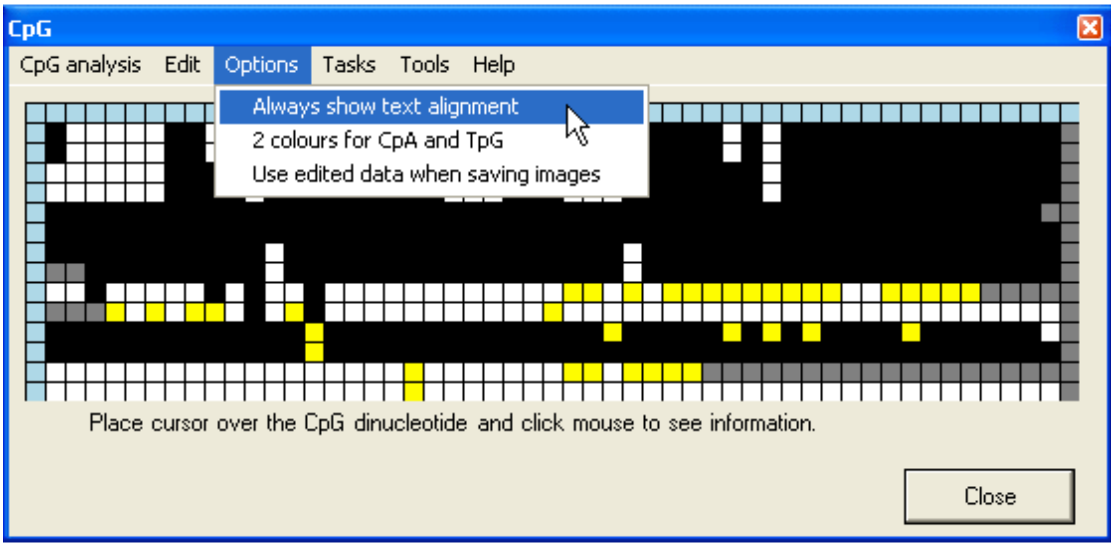
T T T A G G T T G G T T C G G G T

C C C A G G C C G G C T C G G G T

The lower sequence is the reference sequence.

B

Figure 7

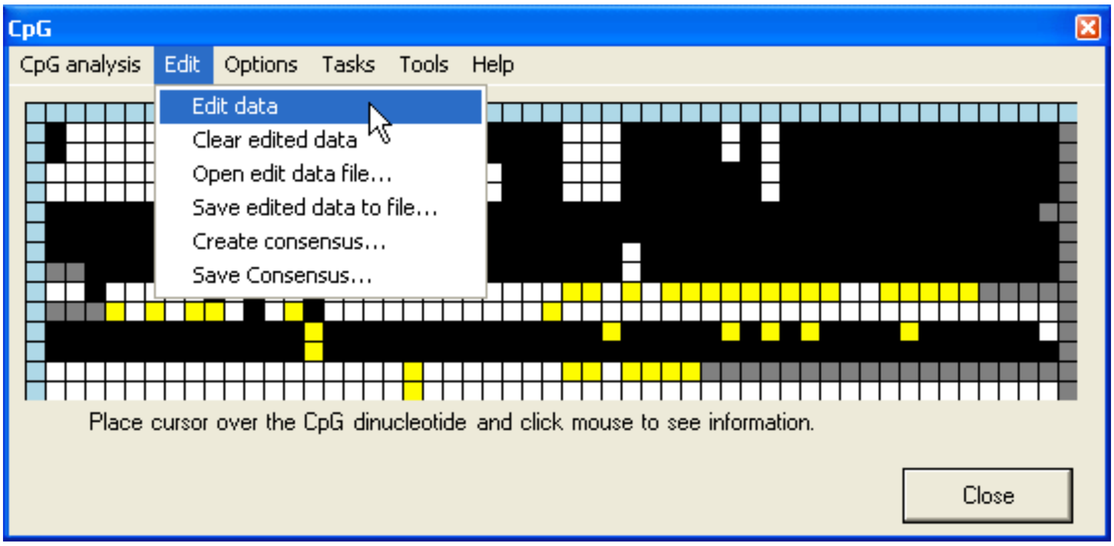

CpG
CpG analysis
Edit
Options
Tasks
Tools
Help
Always show text alignment
2 colours for CpA and TpG
Use edited data when saving images
Close

Place cursor over the CpG dinucleotide and click mouse to see information.

Figure 8

Manual editing

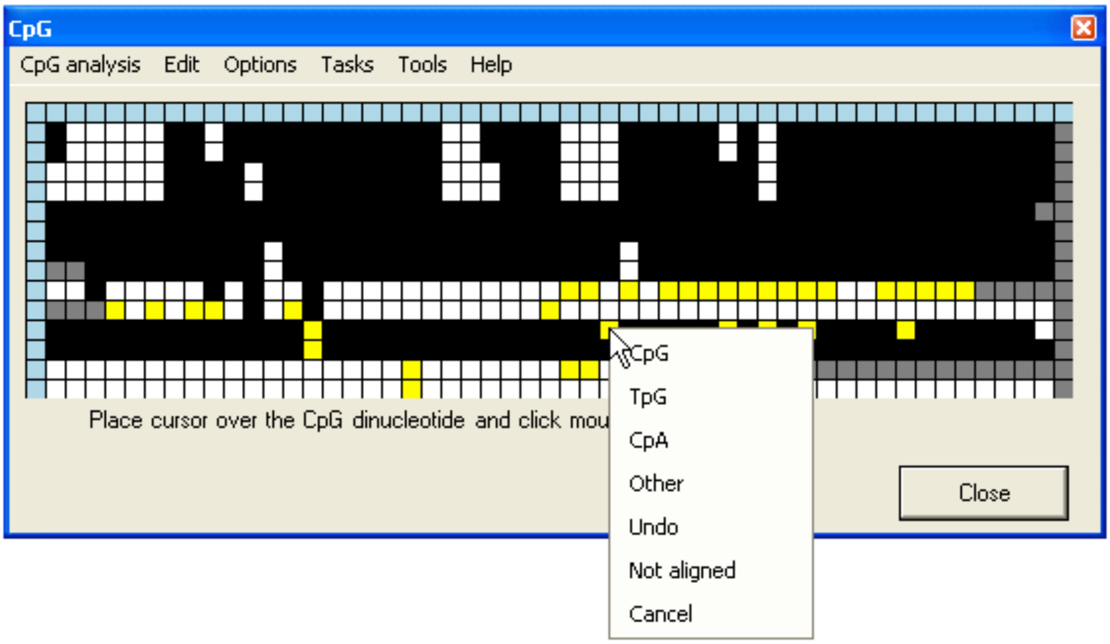
Since bisulphite treated DNA often contains long runs of low complexity sequences, the desired or “correct” alignment between modified sequence and reference sequence may not be the mathematically optimum one. In cases where the programme miscalls a CpG dinucleotide, it is possible to edit the methylation status of a square. To edit the grid select the **Edit data** option (Figure 9) and left click the square to edit.



Place cursor over the CpG dinucleotide and click mouse to see information.

Figure 9

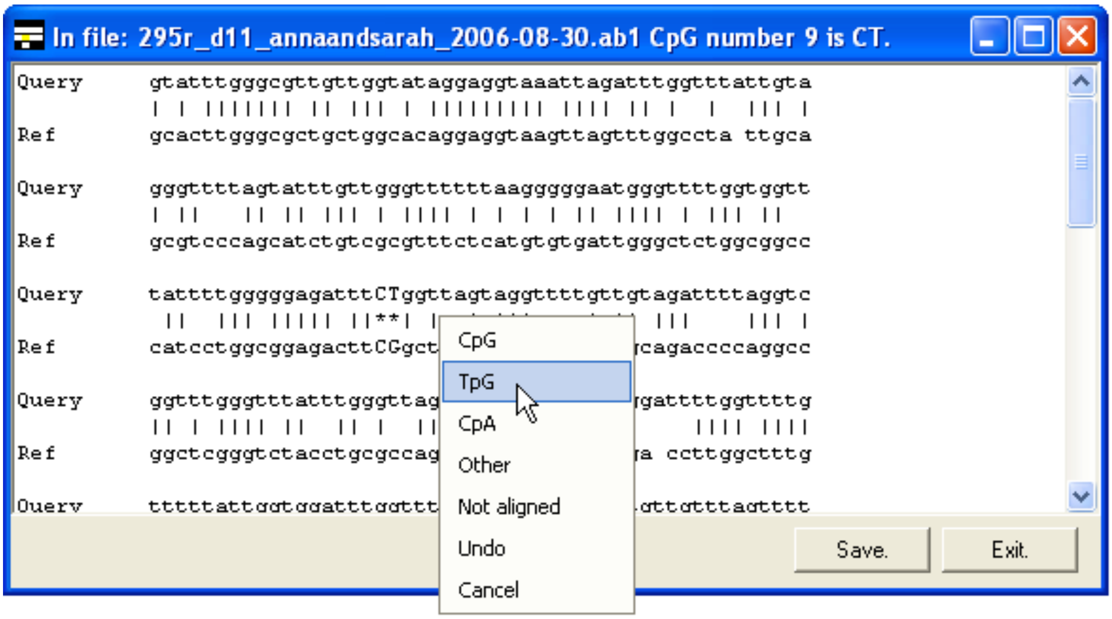
This generates a floating menu that allows you to select the dinucleotide’s assigned status within the bisulphite treated sequence (Figure 10).



Place cursor over the CpG dinucleotide and click mou

Figure 10

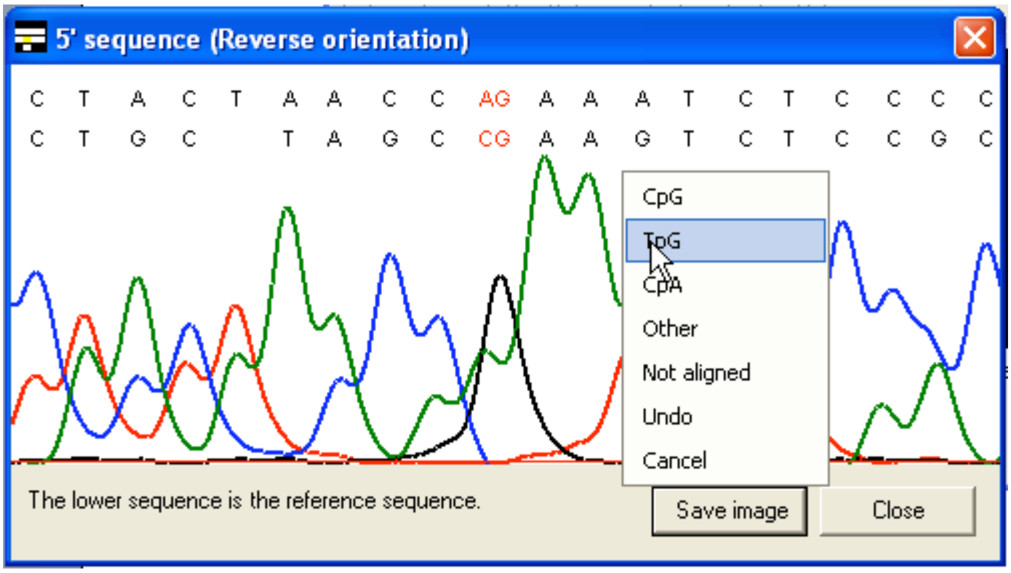
The same menu can also be accessed by clicking the trace image or text area of the global alignment forms (Figure 11 A and B), without the need first to select the **Edit data** option.



In file: 295r_d11_annaandsarah_2006-08-30.ab1 CpG number 9 is CT.

```
Query      gtatttgggcgttgttggtataggaggtaaattagatttggtttattgta
           | | ||||||| || ||| | ||||||||| |||| || |  |  ||| |
Ref        gcacttgggcgctgctggcacaggaggtaagttagtttggccta ttgca

Query      gggttttagtatttgttgggtttttaaggggaatgggttttggtggtt
           | ||   || || ||| | |||| | | | | || |||| | ||| ||
Ref        gcgtcccagcatctgtcgcgtttctcatgtgtgattgggctctggcggcc

Query      tattttggggagatttCTggttagtaggttttgttgtagattttaggtc
            ||  ||| ||||| ||**| |                |||    ||| |
Ref        catcctggcggagacttCGgct                cagaccccaggcc

Query      ggtttgggtttatttgggttag                 gatttggtttg
           || | |||| ||  || |  ||                   |||| ||||
Ref        ggctcgggtctacctgcgccag                a ccttggctttg

Query      tttttattggtggatttggttt                gttgtttagtttt
```

A

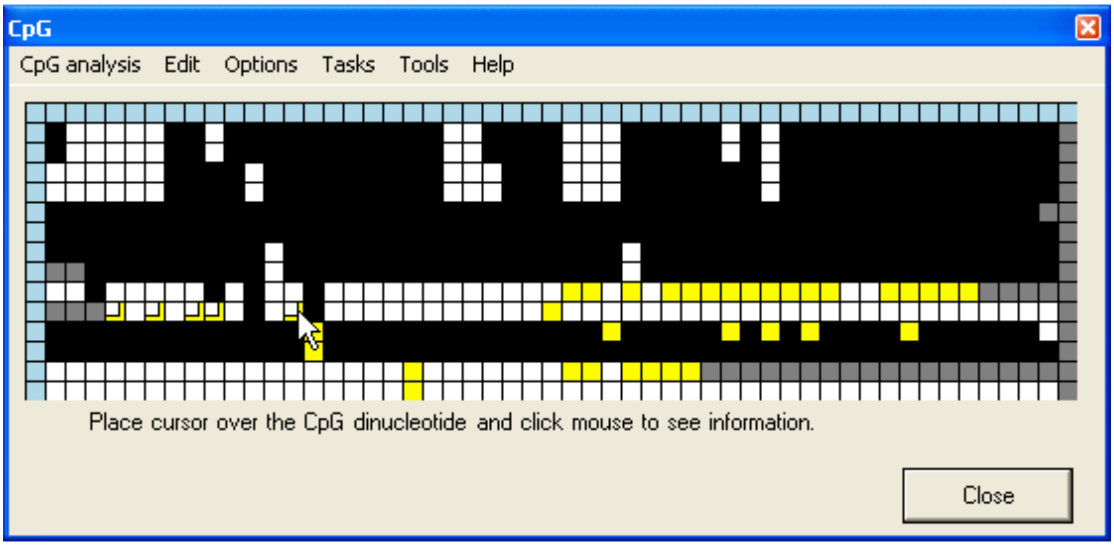

5' sequence (Reverse orientation)
C T A C T A A C C AG A A A T C T C C C C
C T G C T A G C CG A A G T C T C C G C
CpG
TpG
CpA
Other
Not aligned
Undo
Cancel
Save image
Close

The lower sequence is the reference sequence.

B

Figure 11

Squares that have been manually edited are identifiable, as their new edited values are shown as smaller squares overlying the original image (Figure 12). With some limitations, the edited data can be saved to file or recovered from file via the **Edit** menu; however, this file contains only the editing information, and can only be used in conjunction with the correct reference sequence; individual sequence files may, however, be added or removed from the alignment.

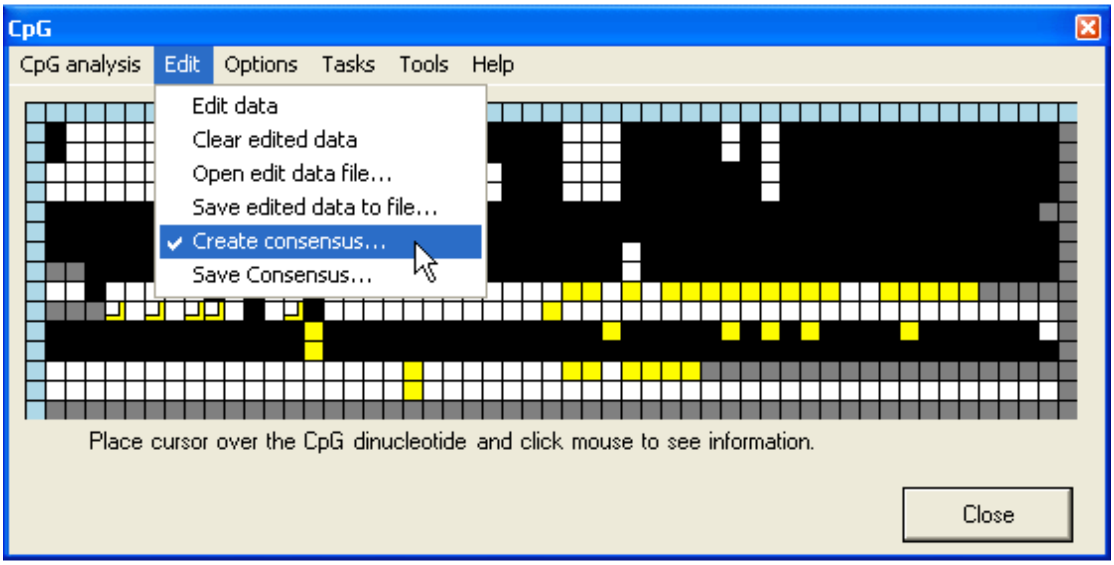


Place cursor over the CpG dinucleotide and click mouse to see information.

Figure 12

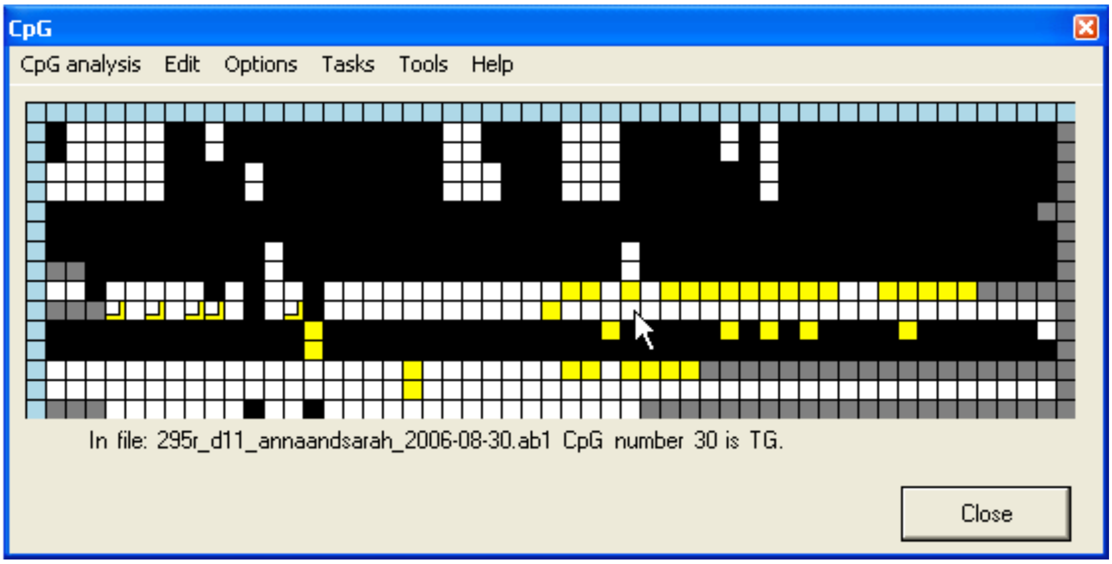
Creating a consensus sequence

If several sequences are derived from a common source (*e.g.* multiple sequences from the same clone, or multiple clones from the same tissue sample) it may be desired to form a consensus sequence from them. Such a consensus sequence can also then be re-loaded into the programme along with other consensus sequences, to form a grid that displays methylation status from multiple sequence files in an abridged manner. The **Create consensus…** option (Figure 13A) adds a new row to the bottom of the grid. The cells in this row are initially grey, but change to match whichever colour is chosen by clicking on any cell in the same column (Figure 13B). Once the consensus row is completed, the underlying sequence can be saved via **Save consensus…** (Figure 13A). If a square has been edited, the consensus sequence takes the colour of the edited value. Once a consensus has been saved, left-clicking the blue square at the start of the row clears the sequence. This allows multiple consensus sequences to be generated from a single alignment.



Place cursor over the CpG dinucleotide and click mouse to see information.

A



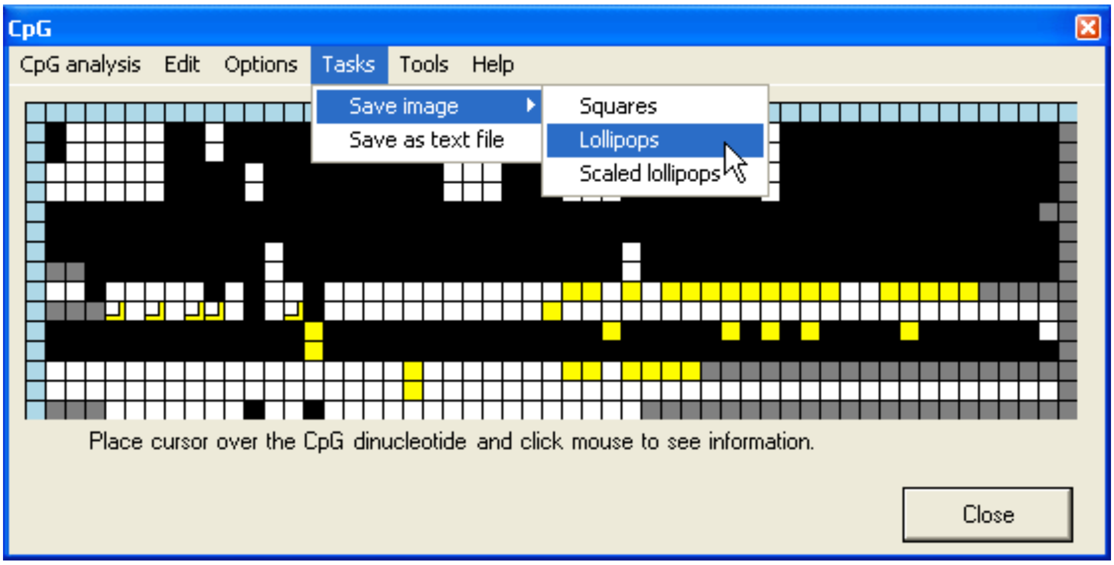
In file: 295r_d11_annaandsarah_2006-08-30.ab1 CpG number 30 is TG.

B

Figure 13

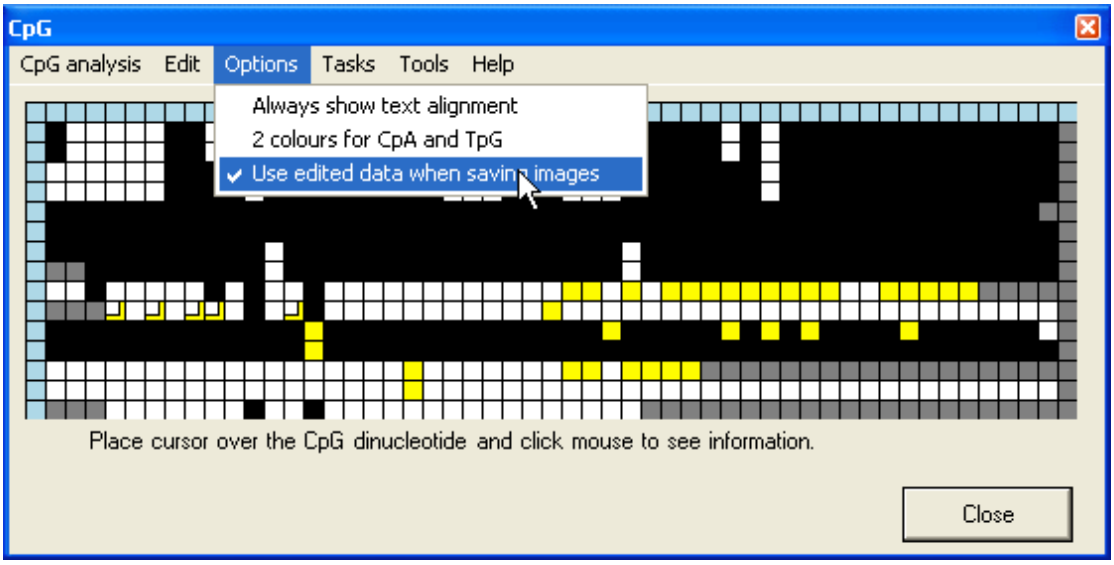
Saving Grid data

Once an alignment has been created and edited, the grid may be saved, either as a text file or an image file, via the **Tasks** menu (Figure 14). If the **Use edited data when saving images** option is selected (Figure 15), the image will be generated from the edited grid, otherwise from the original CpG dinucleotide scores.



Place cursor over the CpG dinucleotide and click mouse to see information.

Figure 14



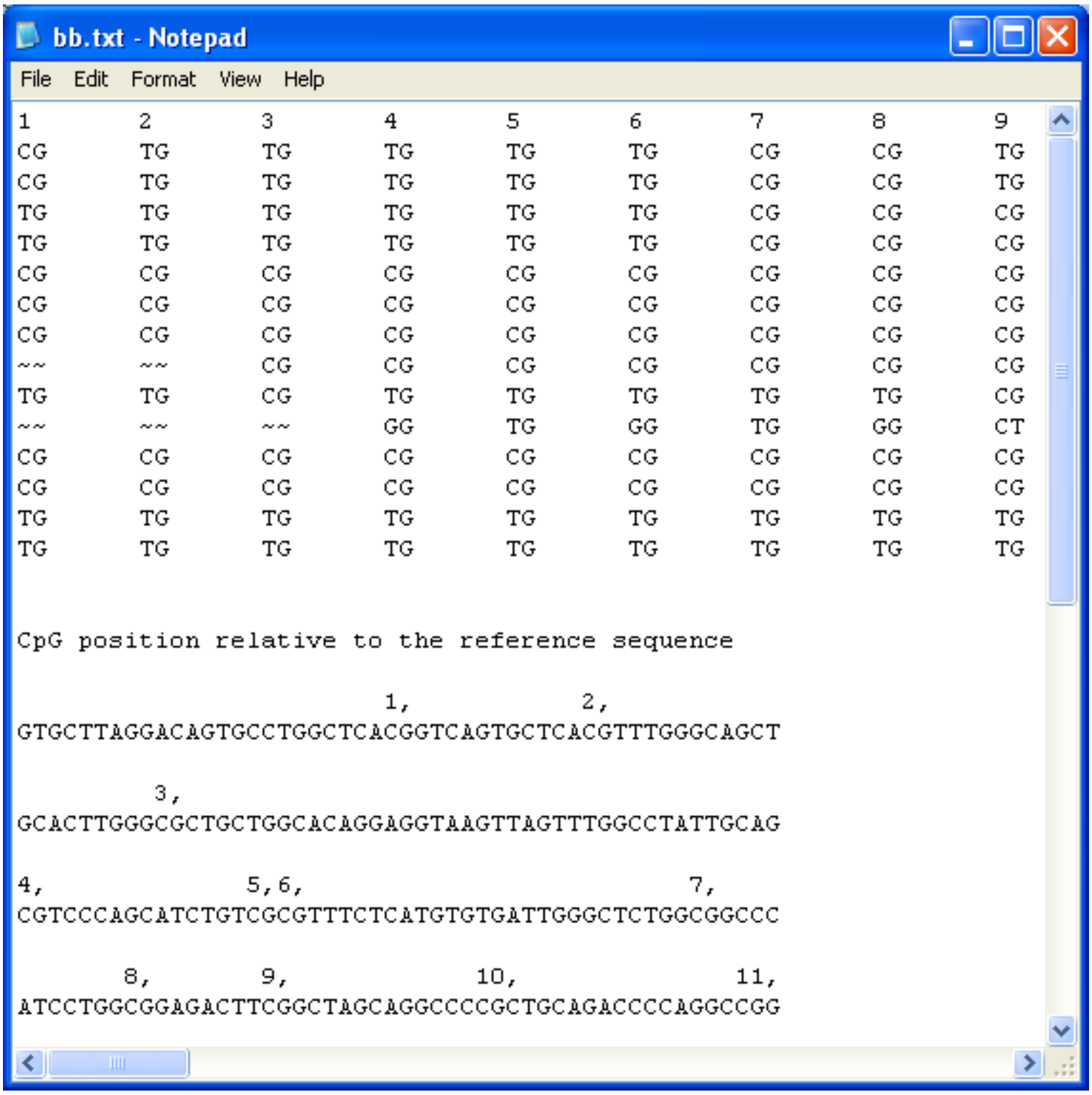
CpG
CpG analysis
Edit
Options
Tasks
Tools
Help
Always show text alignment
2 colours for CpA and TpG
✓ Use edited data when saving images
Close

Place cursor over the CpG dinucleotide and click mouse to see information.

Figure 15

Text format

The text file recreates the grid as a plain text “tab-delimited” table arranged in the same order as the grid, with each dinucleotide sequence replacing its colour-coded square. The ‘~~’ symbol represents unaligned dinucleotides. The file also contains the reference sequence with the CpG dinucleotides numbered in the order that they appear in the table (Figure 16). Each row is identified by the originating sequence file’s name at the end of each row. Since the table is “tab-delimited” it can be opened using a spreadsheet programme, with each individual dinucleotide score placed in a cell.



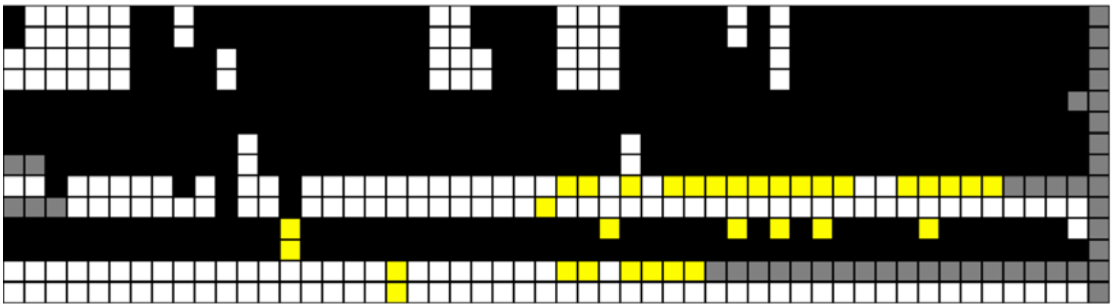
```
1       2       3       4       5       6       7       8       9
CG      TG      TG      TG      TG      TG      CG      CG      TG
CG      TG      TG      TG      TG      TG      CG      CG      TG
TG      TG      TG      TG      TG      TG      CG      CG      CG
TG      TG      TG      TG      TG      TG      CG      CG      CG
CG      CG      CG      CG      CG      CG      CG      CG      CG
CG      CG      CG      CG      CG      CG      CG      CG      CG
CG      CG      CG      CG      CG      CG      CG      CG      CG
~~      ~~      CG      CG      CG      CG      CG      CG      CG
TG      TG      CG      TG      TG      TG      TG      TG      CG
~~      ~~      ~~      GG      TG      GG      TG      GG      CT
CG      CG      CG      CG      CG      CG      CG      CG      CG
CG      CG      CG      CG      CG      CG      CG      CG      CG
TG      TG      TG      TG      TG      TG      TG      TG      TG
TG      TG      TG      TG      TG      TG      TG      TG      TG


CpG position relative to the reference sequence

                        1,            2,
GTGCTTAGGACAGTGCCTGGCTCACGGTCAGTGCTCACGTTTGGGCAGCT

         3,
GCACTTGGGCGCTGCTGGCACAGGAGGTAAGTTAGTTTGGCCTATTGCAG

4,             5,6,                         7,
CGTCCCAGCATCTGTCGCGTTTCTCATGTGTGATTGGGCTCTGGCGGCCC

       8,       9,           10,            11,
ATCCTGGCGGAGACTTCGGCTAGCAGGCCCCGCTGCAGACCCCAGGCCGG
```

Figure 16

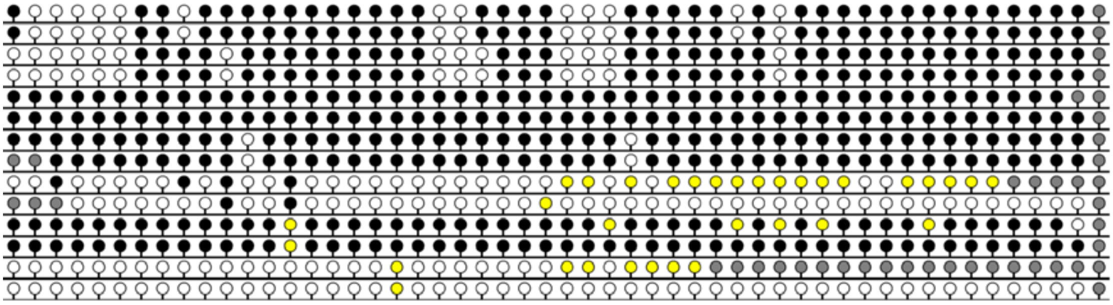
Image Format

The grid image can be saved as a bitmap (*.bmp), portable network graphics (*.png), scalable vector graphics (*.svg) or a Powerpoint presentation (*.ppt) image file. The file format is chosen when entering the image filename. To save as a powerpoint presentation, Microsoft Powerpoint must be installed and the helper file “Interop.PowerPoint.dll” must be located in the same folder as the “CpGViewer.exe” programme. Also, since the two programmes must communicate with each other to create a powerpoint presentation, this option may be slow for large grids (*e.g.* 50 CpG dinucleotides in each of 50 different files).

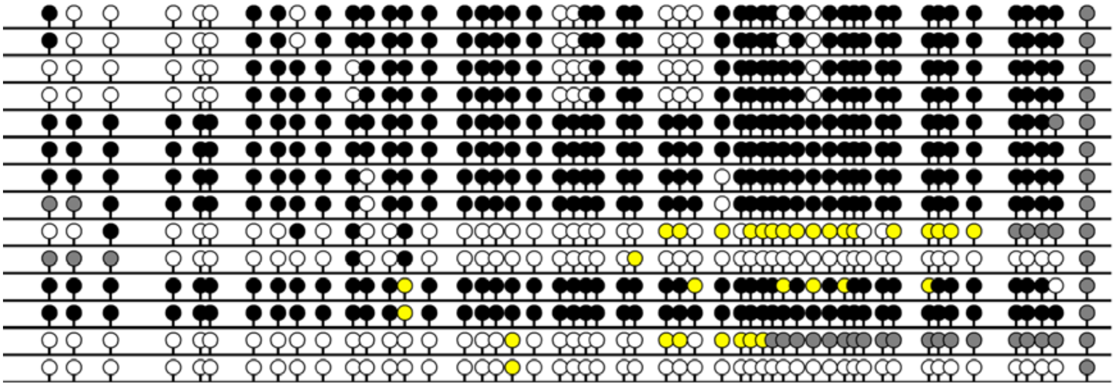
In addition to the square pattern used for screen display of the interactive grid, two “lollipop” styles, as commonly used for publication, are available. These may have either a fixed horizontal spacing or be scaled to show the approximate actual position of each CpG within the sequence (Figure 17A, B and C). (in the scaled view adjacent lollipops are also shifted by a small fixed distance, to ensure that in the final image no two lollipops ever completely overlie one another). In Figure 18 the blue lines shows the minimum width each lollipop will occupy and the red lines represent the spacing if drawn strictly to scale. If the **2 colours for CpA and TpG** option is selected (Figure 4) the exported images inherit the same 2 colour scheme.



A



B



C

Figure 17

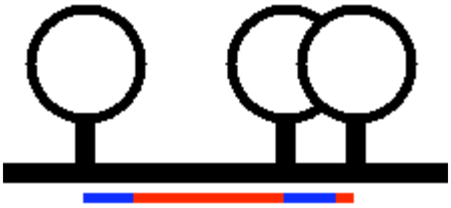
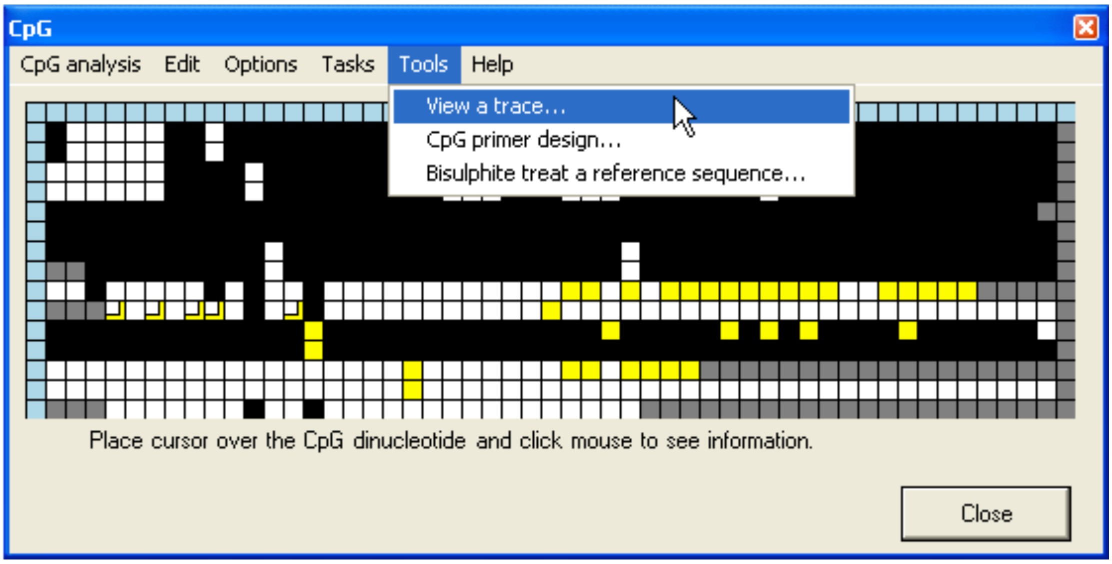


Figure 18

Tools

The programme also contains three other tools which may be useful to anyone engaged in bisulphite genomic sequencing projects (Figure 19). These tools aid primer design, viewing of electropherogram data and creating theoretically bisulphite treated sequences.

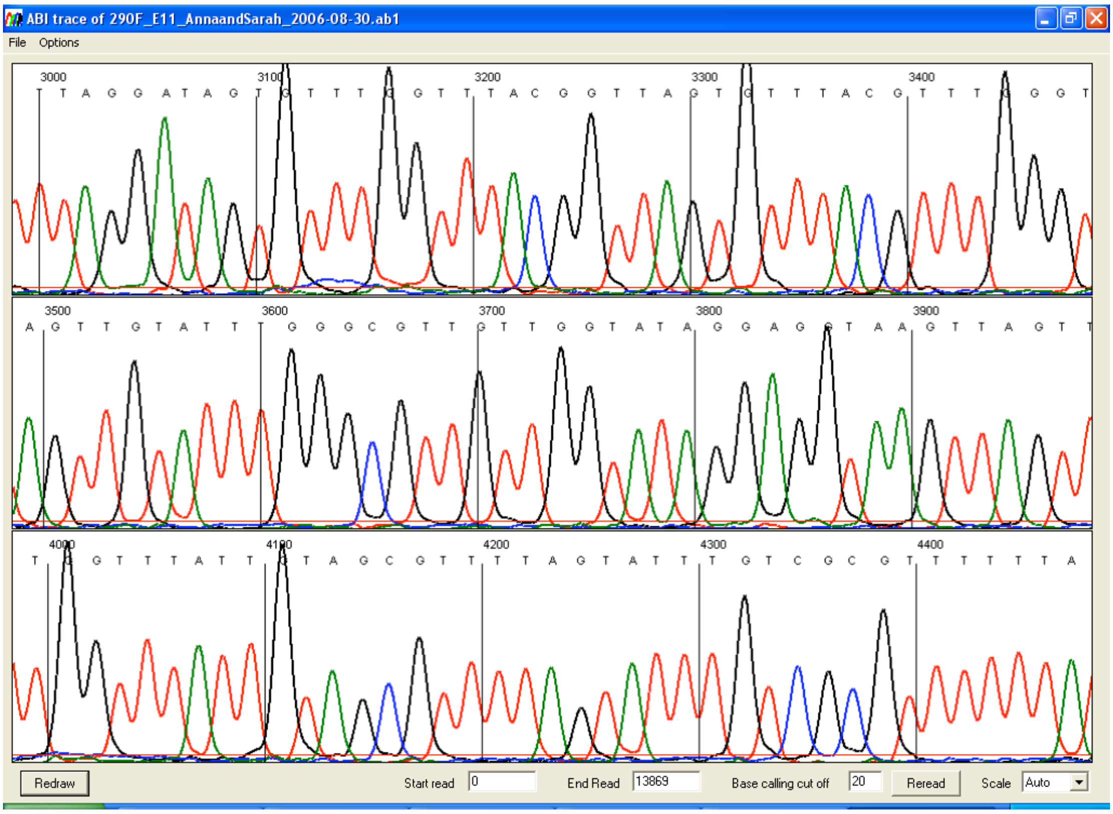


CpG
CpG analysis
Edit
Options
Tasks
Tools
Help
View a trace...
CpG primer design...
Bisulphite treat a reference sequence...
Place cursor over the CpG dinucleotide and click mouse to see information.
Close

Figure 19

Viewing an electropherogram

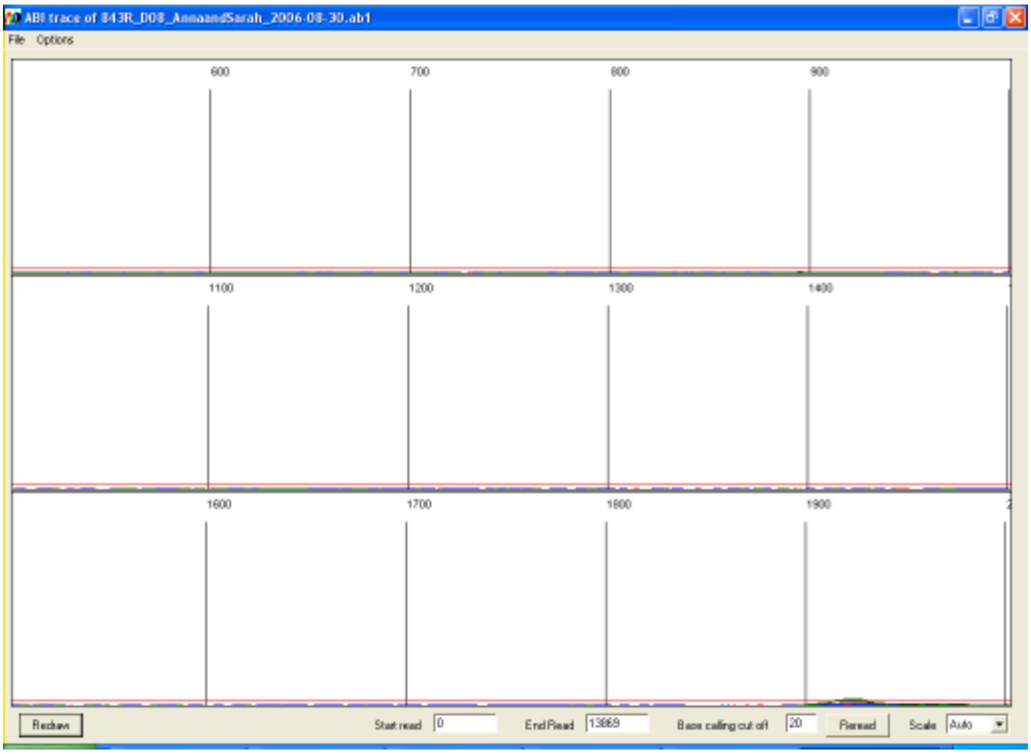
Electropherogram traces can be viewed either by right-clicking a blue square at the start of a row or by selecting **View a trace…** from the **Tools** menu and selecting a trace file. The trace will be displayed in a new window (Figure 20). If the selected file is an unprocessed ABI file (*.ab1), a message will be displayed stating that the file contains no processed data and offering the choice either to view another file or allow the programme to analyse the data. Note that when unprocessed data are viewed (either from *.ab1 or *.rsd file) the display window will initially show the earliest scans from the run, which do not contain sequence data (Figure 21). The trace view can be altered or scrolled using the menu or the controls at the bottom of the window (Figure 22).


ABI trace of 290F_E11_AnnaandSarah_2006-08-30.ab1

Start read 0 End Read 13869 Base calling cut off 20 Scale Auto

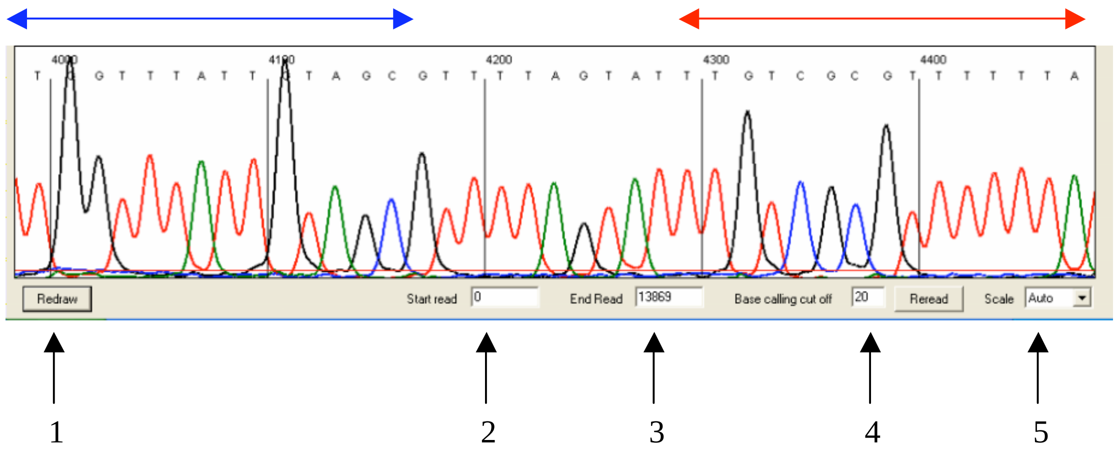
File
Options
3000
3100
3200
3300
3400
T T A G G A T A G T G T T T G G T T T A C G G T T A G T G T T T A C G T T T G G G T
3500
3600
3700
3800
3900
A G T T G T A T T T G G G C G T T G T T G G T A T A G G A G G T A A G T T A G T T
4000
4100
4200
4300
4400
T G G T T T A T T G T A G C G T T T T A G T A T T T G T C G C G T T T T T T A
Redraw
Reread

Figure 20


ABI trace of 843R_D08_AnnaandSarah_2006-08-30.ab1

Start read 0 End Read 13869 Base calling cut off 20 Scale Auto

Figure 21


4000
4100
4200
4300
4400
T G G T T T A T T T G T A G C G T T T T A G T A T T T G T C G C G T T T T T T A
Redraw
Reread
1
2
3
4
5

Start read 0

End Read 13869

Base calling cut off 20

Scale Auto

Figure 22

Navigation

The trace is displayed as a series of panels of which three are shown in the window. To view panels that contain data 3´ of the current view, left-click a panel in the region delimited by the red arrow (Figure 22); similarly, to move 5´ from the current sequence region, left-click the images in the region indicated by the blue arrow.

Saving the Image

Each panel can be saved individually, as a bitmap, by right-clicking the image and choosing the file name and location. If the sequence of interest lies across two panels, adjust the start point of the images as described below in *Changing the start and end points of the images*.

Changing the trace appearance

Applying changes

Since each change requires the images to be redrawn, to save time multiple changes can be made (as described below) and then applied using the **Redraw** or **Reread** buttons. These buttons have similar functions but, whereas the **Redraw** button creates the images by reanalysing the original data, the **Reread** button uses partially analysed data. If the basecalling cut off is changed by a large amount, then it is preferable to use the **Redraw** button.

Changing the start and end points of the images

By default, all of a run's scan lines are analysed by the programme. To limit the range of scan lines used to create the images, enter the start and end points into the text boxes at the bottom of the screen (labelled 2 and 3 respectively in Figure 22) and press **Redraw**.

Adjusting the base calling cut off

The base calling cut off (labelled 4 in Figure 22) adjusts the intensity at which peaks are identified as either true base calls or background noise. When sequences are of good quality, this value is relatively unimportant. However, adjustments may be useful in the analysis of traces that have low amplitude and/or high background. If this value is changed, the new value is stored by the programme and applied to all subsequent base calling when showing other traces or creating a CpG alignment. This value can also be adjusted via the **CpG analysis** > **Alignment options** menu of the main programme window (Figure 1).

Changing the image scale

The vertical scale can be adjusted via the list box (labelled 5 in Figure 22) and applied by pressing **Redraw**. If this is set to “Auto” the programme uses a value calculated from the peak heights across the entire trace.

File menu functions

Figure 23 shows the **File** menu, using which it is possible to open a new trace file, save the sequence as a plain text file, or save the trace as a standard chromatogram format file (*.scf). The SCF file only contains scan data between the start and end values used to create the image (labelled 2 and 3 respectively in Figure 22). This menu also allows the user to print the trace.

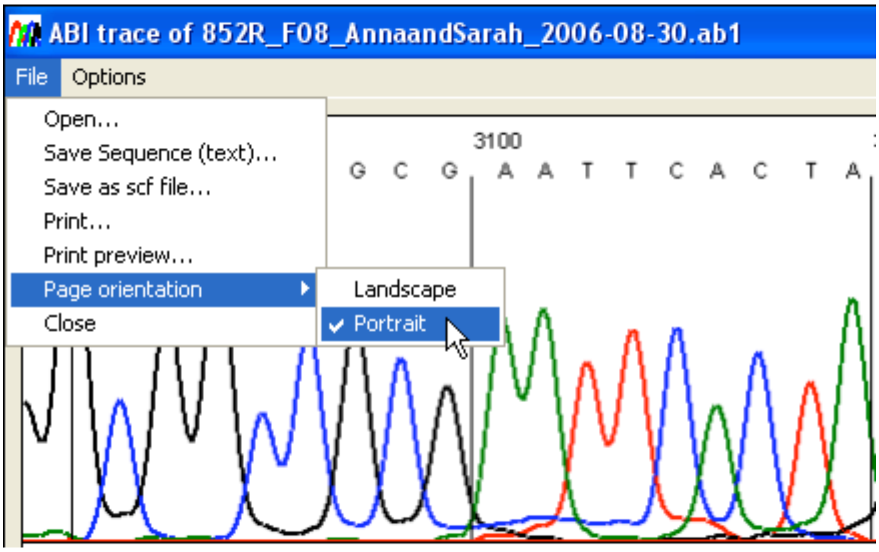


Figure 23

Options menu functions

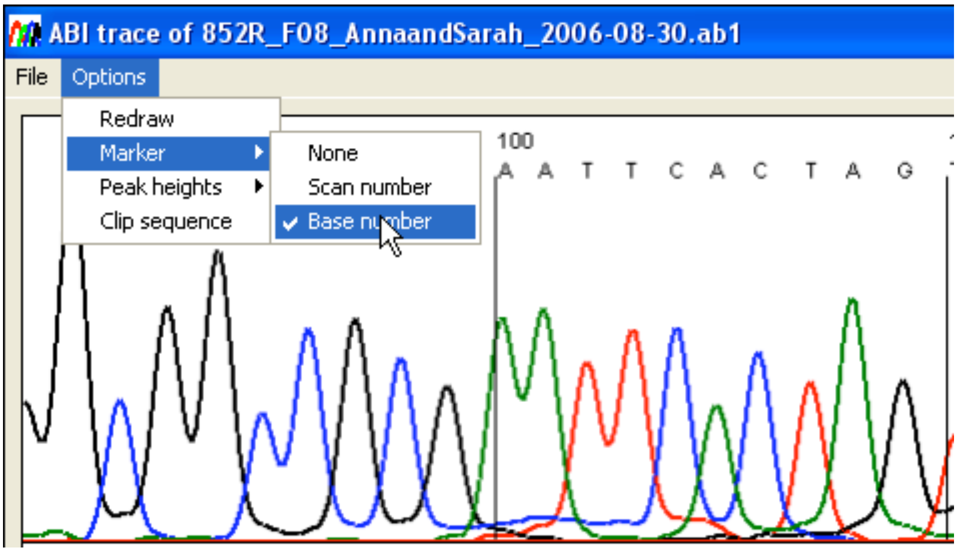


Figure 24

The **Options** menu (Figure 24) enables the user to change the format of the panels as well as to access peak height data.

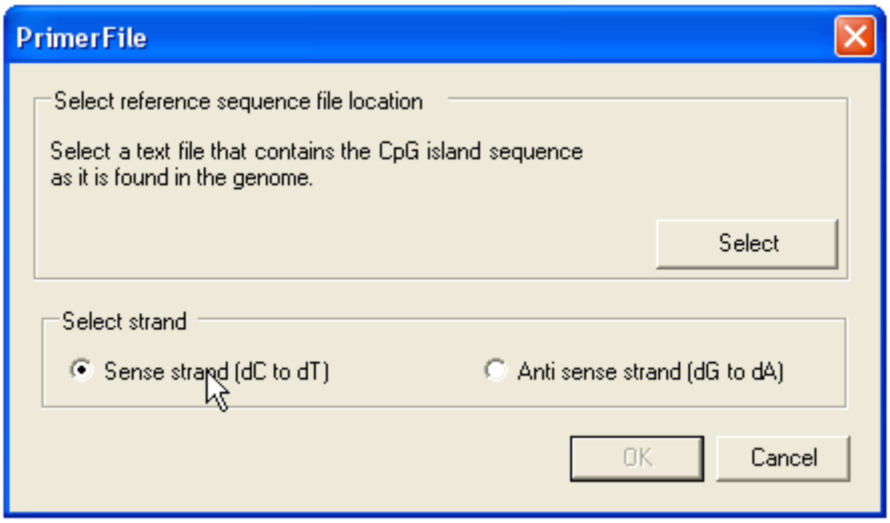
Options

- **Redraw**: this performs the same function as the **redraw** button at the bottom of the form.

- **Marker**: This changes the marker intervals to appear either every 10 nucleotides or every hundred scan lines. It also allows the markers to be hidden.
- **Peak heights**: the average peak heights are calculated as the programme basecalls the trace file. This information can be shown either visually or as text. When the visual option is selected, horizontal lines are drawn to show the average peak height for each nucleotide. The text option displays the average, standard deviation and count for each nucleotide.
- **Clip sequence**: This function removes the 3´ and 5´ end low quality sequence when it is saved to a plain text file.

CpG primer design

Since the sister strands of bisulphite-treated DNA are no longer complementary, before designing primers it is necessary to select the CpG island sequence (plain text file) and which strand the user wishes to work with (Figure 25).


PrimerFile

Select reference sequence file location

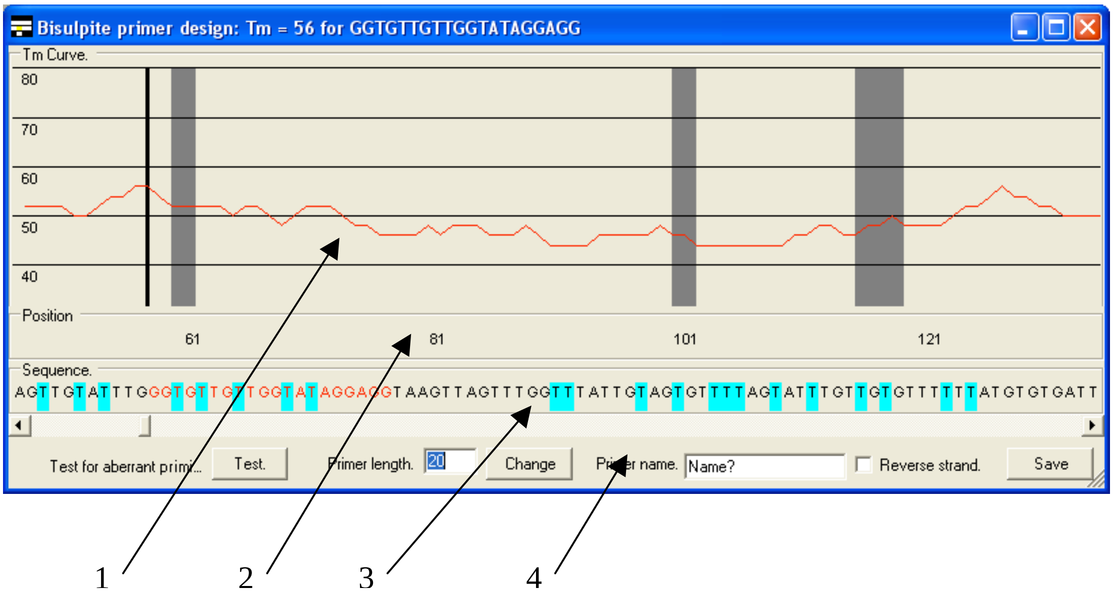
Select a text file that contains the CpG island sequence
as it is found in the genome.

Select strand

● Sense strand (dC to dT)   ○ Anti sense strand (dG to dA)

Figure 25

The primers are then designed using the primer design form (Figure 26) which is composed of four parts as described below.



Bisulpite primer design: Tm = 56 for GGTGTTGTTGGTATAGGAGG

AGTTGTATTTGGGTGTTGTTGGTATAGGAGGTAAGTTAGTTTGGTTTATTGTAGTGTTTTAGTATTTGTTGTGTTTTTTATGTGTGATT

Test for aberrant primi...

Primer length. 20

Primer name. Name?

☐ Reverse strand.

Figure 26

1. **The T_m graph**. This shows the T_m of a primer starting at that position and extending to the right by the length entered in the **Primer length** box. The solid black vertical line shows the start point of the current primer. The grey boxes show the positions of CpG dinucleotides in the reference sequence.
2. **The position panel**. This panel shows the base pair position of the sequence in the T_m graph and sequence panels.
3. **The sequence panel**. The predicted sequence of the bisulphite-treated CpG island sequence if unmethylated. Pale blue boxes indicate where a C residue has been converted to a T and the red text represents the current primer sequence.

4. **The function panel**. This region contains the controls used to create the primers.

How to design a primer

1. To select a primer, first enter the primer length in the **Primer length** number box and press **Change**. This redraws the T_m graph shown in the main panel. (The primer length can be changed at any point during the primer design process).
2. Navigate to the desired region in the CpG island sequence using the scroll bar across the top of the function region and choose a suitable point in the sequence.
3. If the primer is the reverse primer tick the **Reverse strand** box.
4. Click the point you wish the primer to start from (forward primer) or end at (reverse primer) on the T_m graph panel, the position panel or the sequence panel. The primer sequence will be highlighted in red text in the sequence panel and in the form's title bar, along with its calculated T_m. If the **Reverse strand** box is checked, the complementary sequence appears in the title bar, but not in the sequence panel.
5. Pressing the **Test** button (bottom left of the function panel) displays a text field that shows the possible problems that may arise with the chosen primer, from primer self-annealling and positions of (ungapped) homology along the CpG island sequence.
6. Enter the primer name in the relevant text box and press **Save**. When prompted for a filename, if a new filename is entered, a plain text file is

created, while if an existing file is chosen the primer is appended to the end of that file. The primer is saved as tab-delimited plain text as shown in Table 2.

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
CpG1R	Starting at: 367	Primer length: 20	Tm: 46	Reverse direction	CCACAAAAAA AACACTAAAA
CpG1F	Starting at: 71	Primer length: 20	Tm: 52	Forward direction	AGGAGGTAAG TTAGTTTGGT

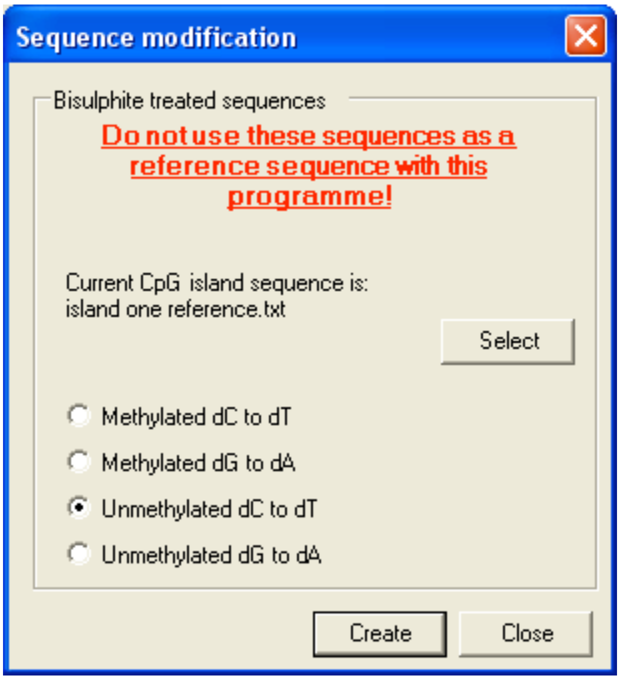
Table 2

Hypothetical bisulphite-treated sequences

The programme also allows the user to create the predicted sequences of bisulphite-treated DNA molecules.

- **It must be noted that these sequences must not be used as reference sequences for this programme**.

To create a bisulphite-modified sequence select the **Bisulphite treat a reference sequence** option (Figure 19). This causes the form in Figure 27 to be displayed. The user must then load the CpG island sequence (as a plain text file) and then choose the methylation status of the DNA (methylated or unmethylated at CpG residues; other C residues always assumed to be unmethylated) and whether the forward (dC to dT) or reverse (dG to dA) strand is to be created. Pressing **Create** prompts the user for a file name and then saves the modified sequence to the file.



Sequence modification

Bisulphite treated sequences

Do not use these sequences as a reference sequence with this programme!

Current CpG island sequence is:
island one reference.txt

○ Methylated dC to dT

○ Methylated dG to dA

● Unmethylated dC to dT

○ Unmethylated dG to dA

Figure 27