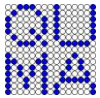

QUantification tool for Methylation Analysis

http://quma.cdb.riken.jp/

QUMA User's manual

April 30, 2008

Version 1.02

Contact information

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Contents

1. About QUMA

Bisulfite sequencing, a standard method for DNA methylation profile analysis, is widely used in basic and clinical studies. This method is limited, however, by the time-consuming data analysis processes required to obtain accurate DNA methylation profiles from the raw sequence output of the DNA sequencer, and by the fact that quality checking of the results can be influenced by a researcher's bias.

We have developed an interactive and easy-to-use web-based tool, QUMA (QUantification tool for Methylation Analysis), for the bisulfite sequencing analysis of CpG methylation. QUMA includes most of the data-processing functions necessary for the analysis of bisulfite sequences. It also provides a platform for consistent quality control of the analysis. QUMA has four major features. First, it is easy-to-use and needs only two types of input: a PCR target genomic sequence and raw bisulfite sequences. With its user-friendly interface, only a few clicks are needed to quickly align, visualize, and quantify the bisulfite sequence data in a comprehensive manner. Almost all the displayed data are downloadable. Second, QUMA is an all-in-one tool that includes most of the data-processing functions necessary for the analysis of bisulfite sequences. In addition, many optional parameters are available to change the output style according to the user's preferences. Third, QUMA provides a helpful feature that allows the user to control the quality of aligned sequences easily, by changing the cutoff parameters; if the input data and cutoff parameters are indicated, anyone can reproduce the analysis, by using the QUMA web server. Fourth, QUMA server can be launch locally, on a personal computer connected to a local network, by using a bootable CD. This feature is especially helpful to the researcher who must analyze sensitive data. The QUMA web server is available at http://quma.cdb.riken.jp/

Overall, we feel confident that QUMA will prove to be of value to the biological community.

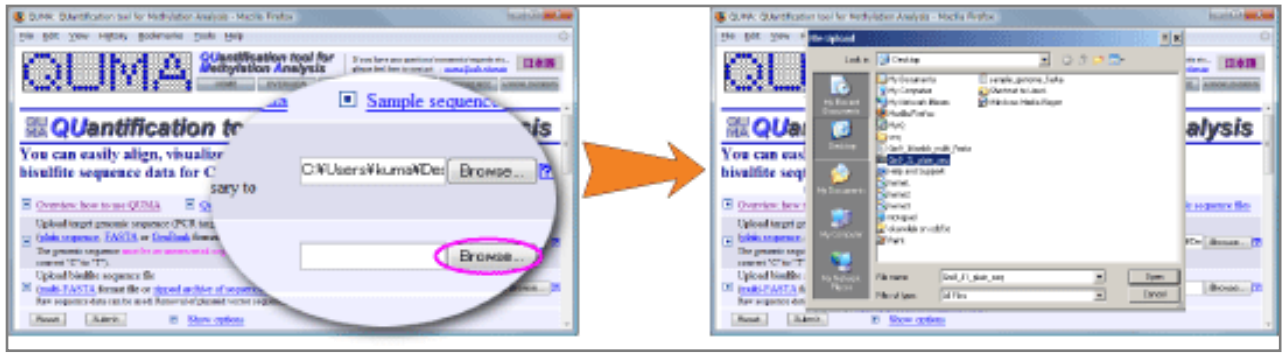
2. Quick start

2.1. Select a genomic sequence file

The genomic sequence must be an unconverted sequence between PCR primer pair (not necessary to convert "C" to "T"). (See "7.1. Genomic sequence" for more details.)

2.2. Select a bisulfite sequence file

Raw sequence data can be used. Removal of plasmid vector sequence is not necessary. Use 8.4. Multi-FASTA file or 8.5. Zipped archive of sequence files. (See "7.2. Bisulfite sequences" for more details.)



2.3. Submit

Typically, only a few seconds are necessary to process sequence data.

3. Supported browsers

We supported the following web browsers.

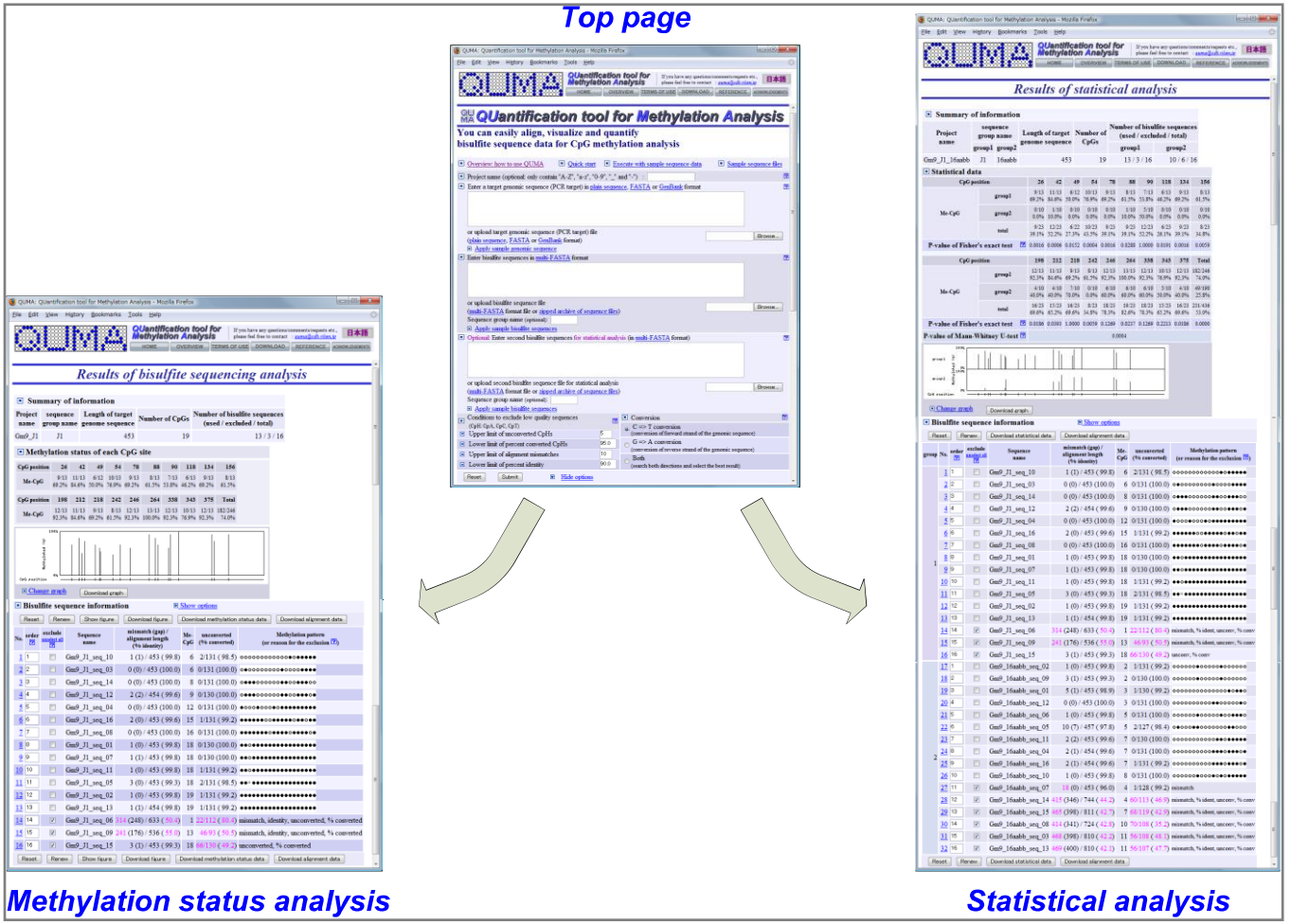
- **Firefox (Mac/Win)**
- **Safari (Mac)**
- **Opera (Mac/Win)**
- **Internet Explorer(IE) 6.0 and higher (Win) (IE 7 is not recommended because it has many bugs)**

Many browsers such as IE 5.0 for Windows, Mozilla, and Netscape 6 and higher may work as well. Some older browsers such as IE for Mac or Netscape 4 will not work.

4. Overview

QUMA is a web-based tool for CpG methylation analysis. You can easily align, visualize and quantify bisulfite sequence data!

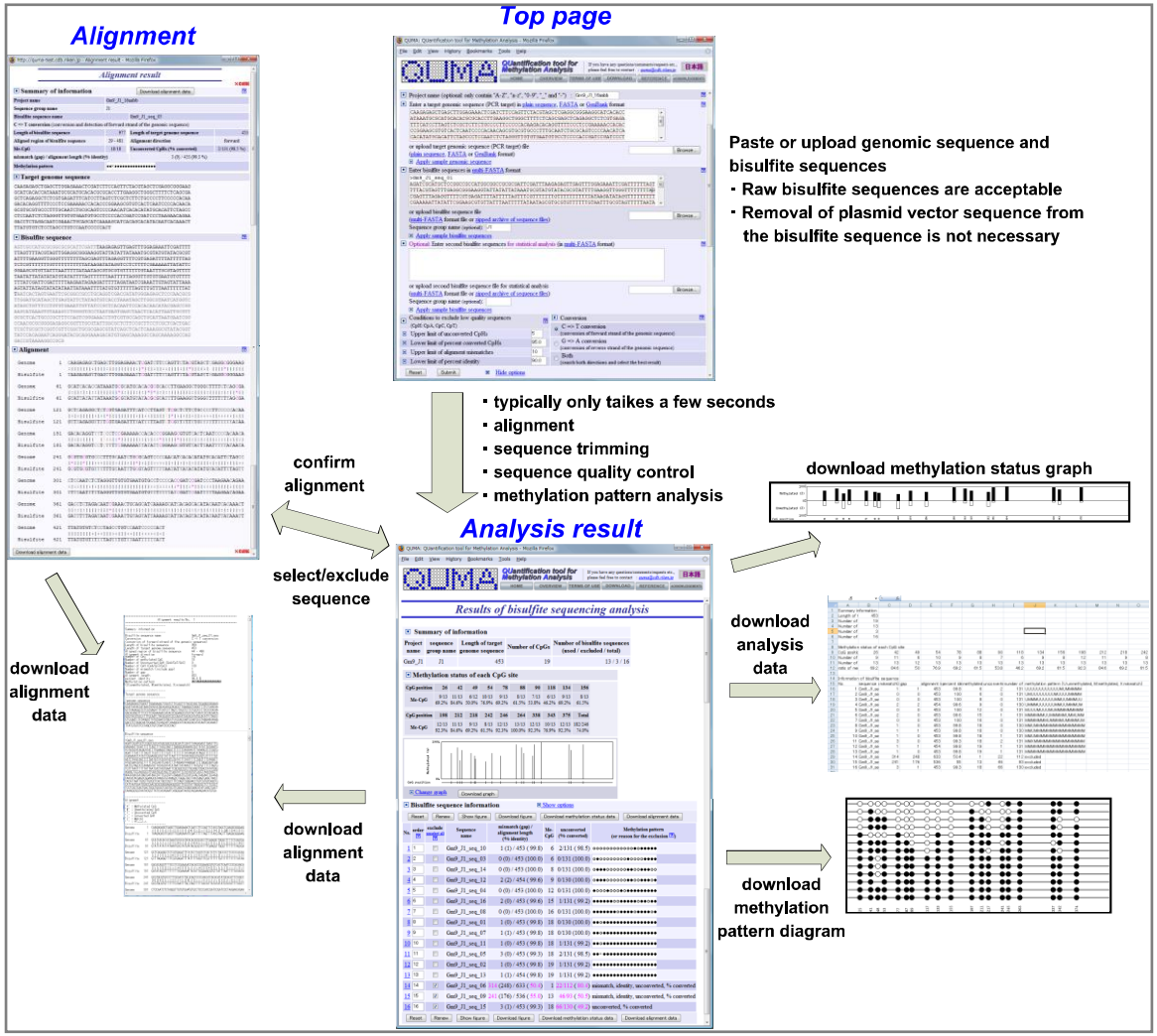
QUMA consists of two separate analyses; a "Methylation status analysis mode" using one group of bisulfite sequences and a "Statistical analysis mode" mode using two groups of bisulfite sequences.



5. Methylation status analysis mode

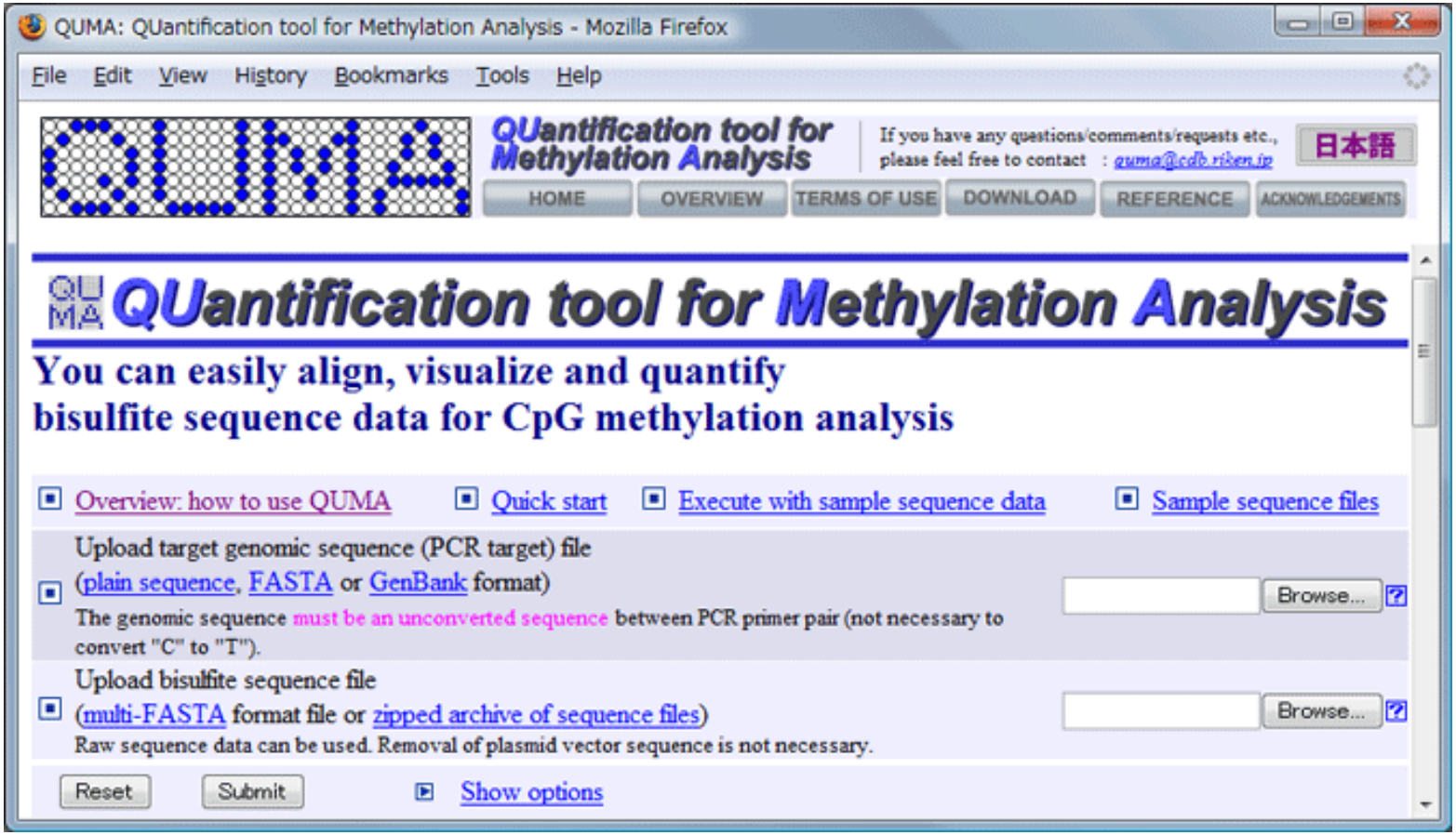
5.1. Main features

- Raw bisulfite sequences are acceptable. No need to exclude plasmid vector sequence
- Typically only a few seconds are necessary for
  - ✓ Bisulfite alignment
  - ✓ Sequence trimming
  - ✓ Sequence quality check
  - ✓ Methylation pattern analysis
  - ✓ Making of figures
- Easy to iterate many alignments with different parameters without difficulties.
- Many optional parameters are available to change the output style to the user's preference.



5.2. Top page

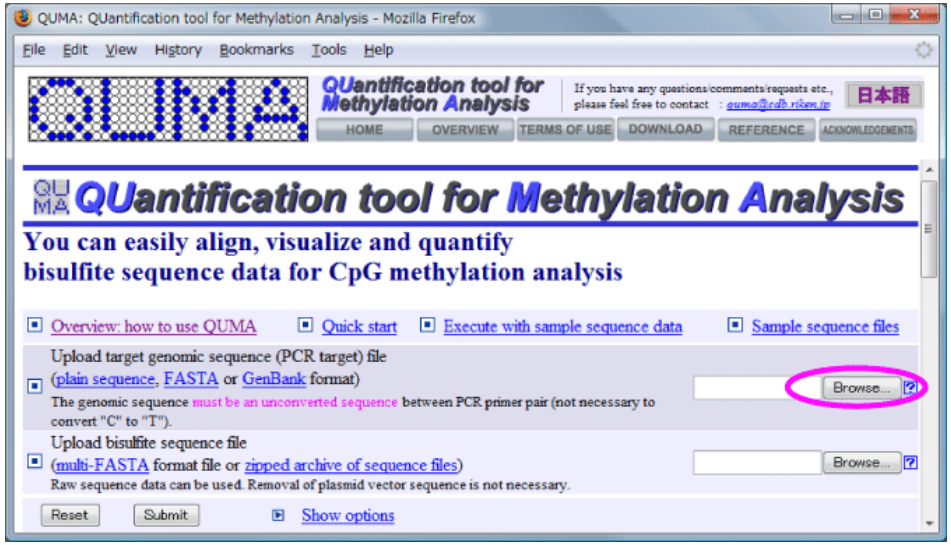
Top page can be switched between two modes, that is, 5.3. Top page simple and 5.4. Top page option.



5.3. Top page simple

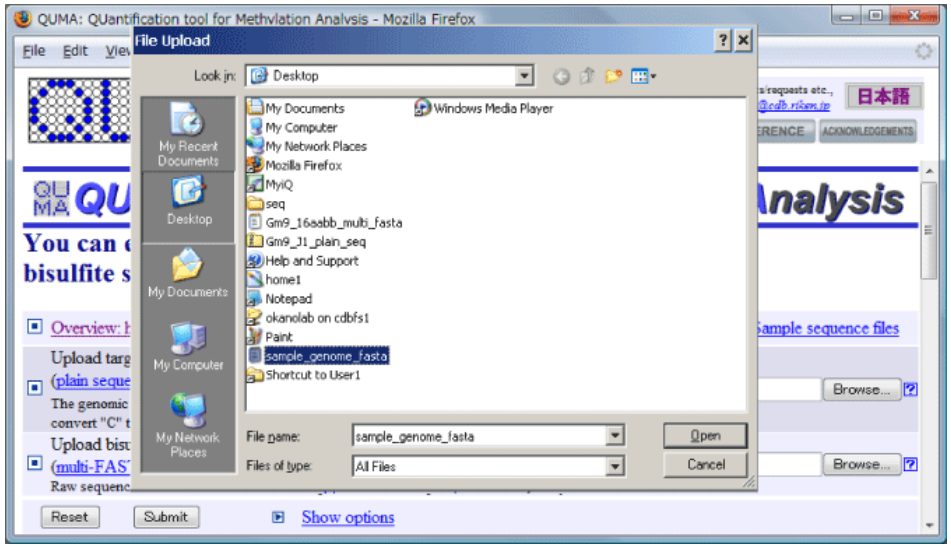
5.3.1. Genomic sequence file 1

Click the first button (in this case "Browse..." button) to upload a target genomic sequence file.



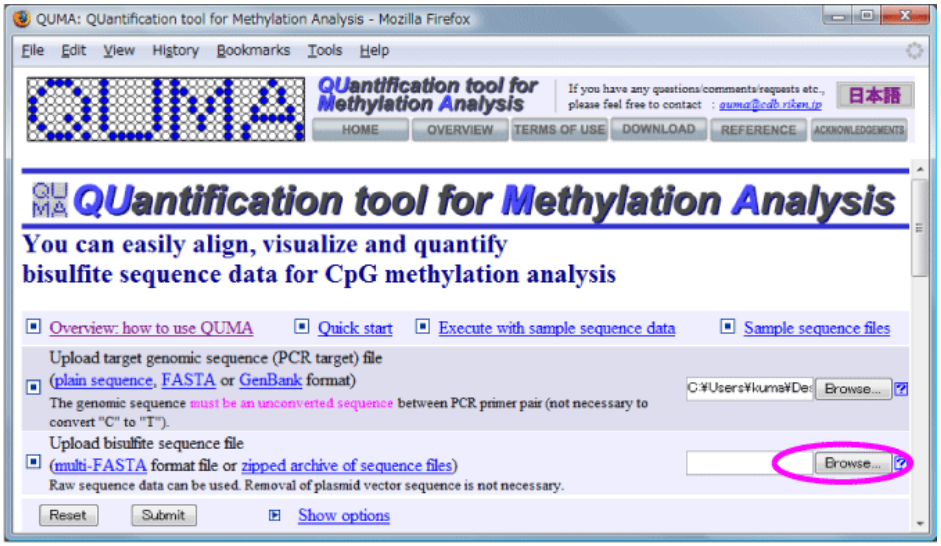
5.3.2. Genomic sequence file 2

Select a target genomic sequence file to upload. See also "7.1. Genomic sequence".



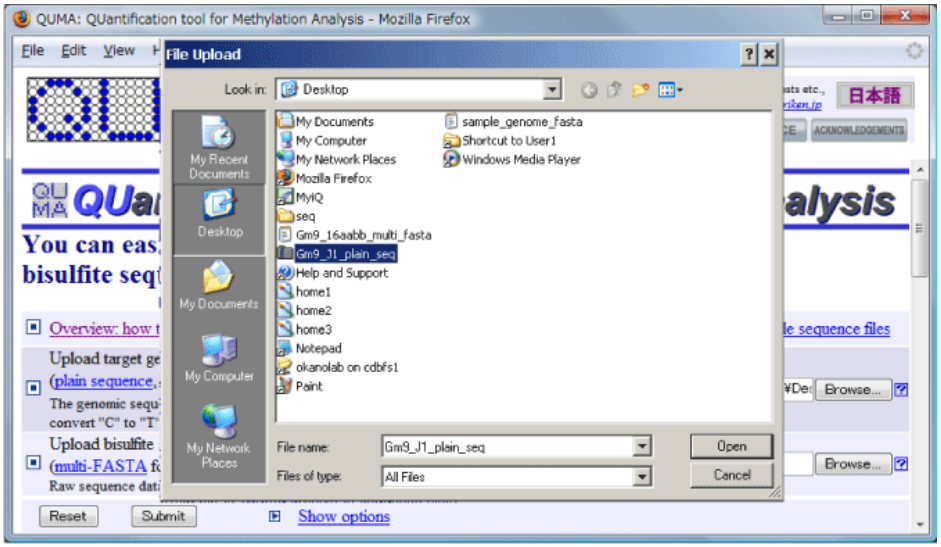
5.3.3. Bisulfite sequences file 1

Click the second button to upload a file of bisulfite sequences.



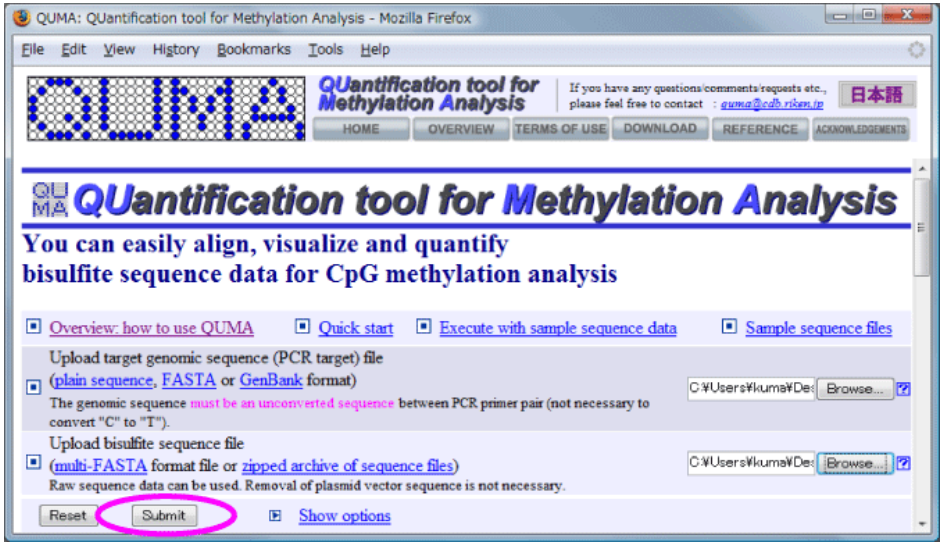
5.3.4. Bisulfite sequences file 2

Select a file of bisulfite sequences. Acceptable file formats are 8.4. Multi-FASTA or 8.5. Zipped archive of sequence files See also "7.2. Bisulfite sequences", "8.6. How to create zipped archive (Macintosh)" and "8.7. How to create zipped archive (Windows)".



5.3.5. Submit

Click the submit button to analyze. Typically, only a few seconds are necessary.

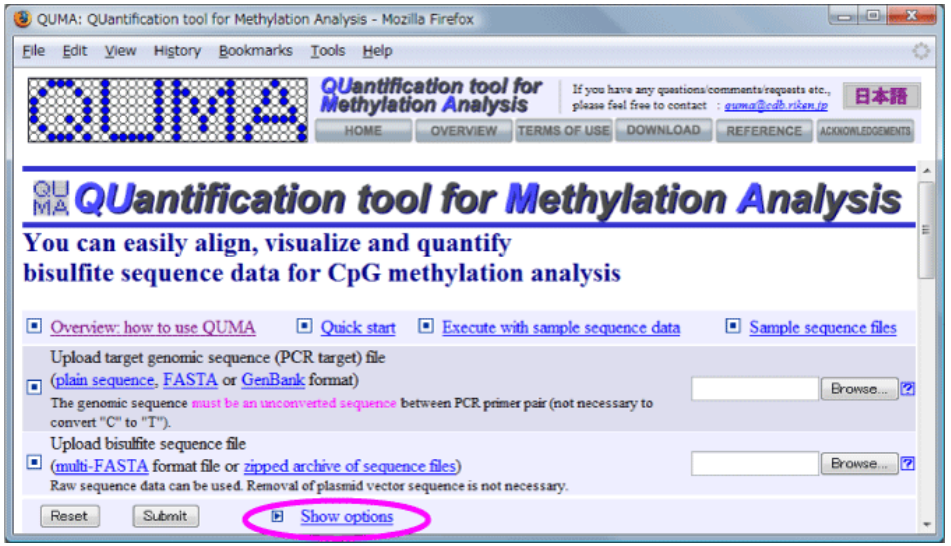


See "5.5. Analysis result page" for next step.

5.4. Top page option

5.4.1. Show options

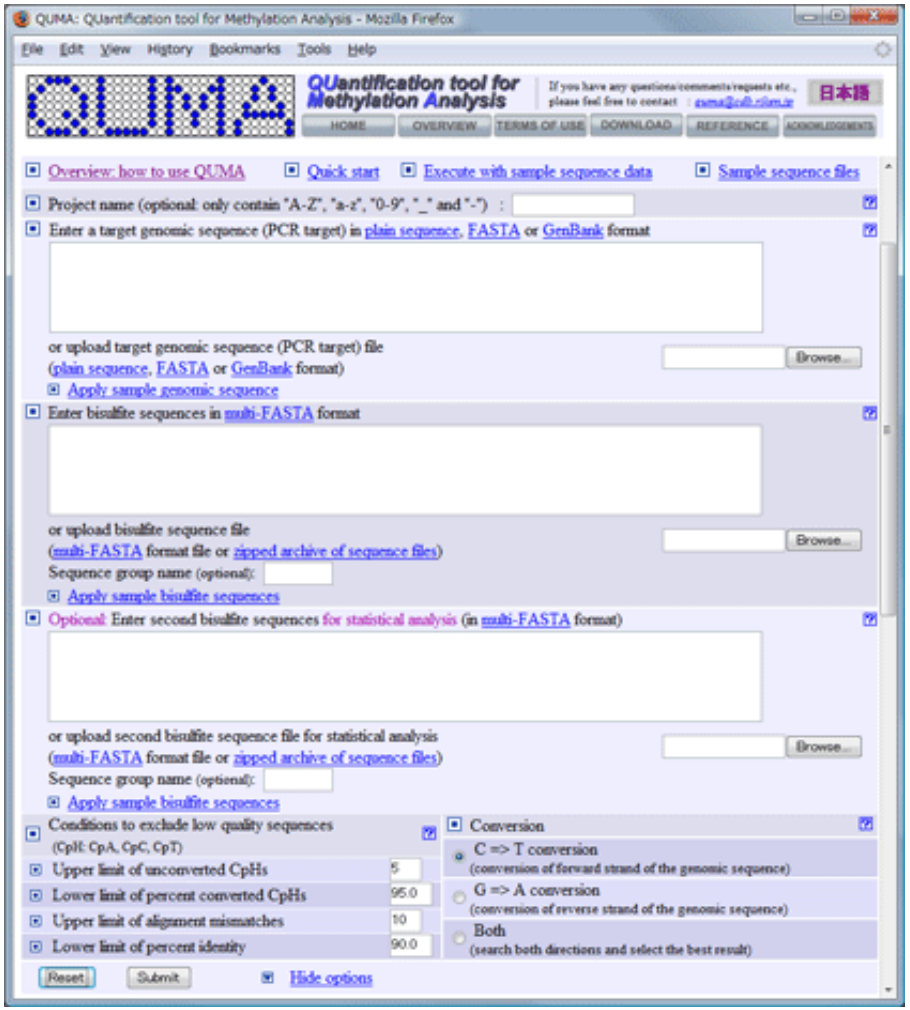
Click the "Show options" link to show optional fields.



5.4.2. Optional fields

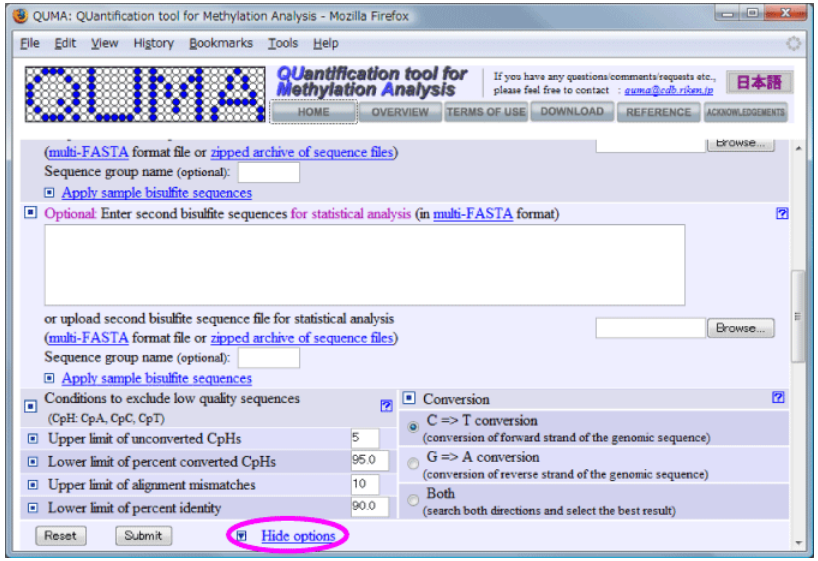
Optional fields will appear.

The third text input field is used only for the Statistical analysis mode.



5.4.3. Hide options

If you want to go back to the simple top page, click the "Hide options" link.

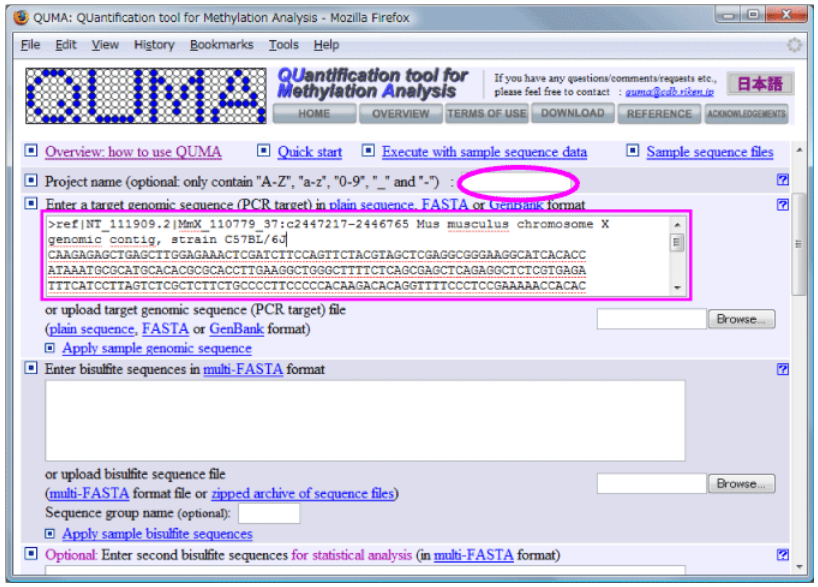


5.4.4. Genomic sequence

Input a project name (optional). When the project name is presented, it will be included in the output file name.

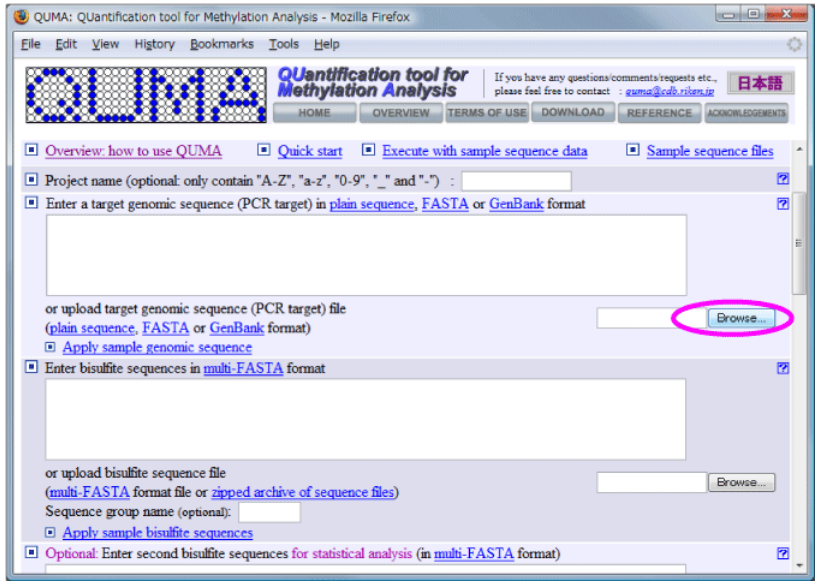
The target genomic sequence can be input by two ways of 1) direct input and 2) upload.

1) In case of direct input, paste a target genomic sequence (8.1. Plain sequence, 8.2. FASTA or 8.3. GenBank format). See also "7.1. Genomic sequence".



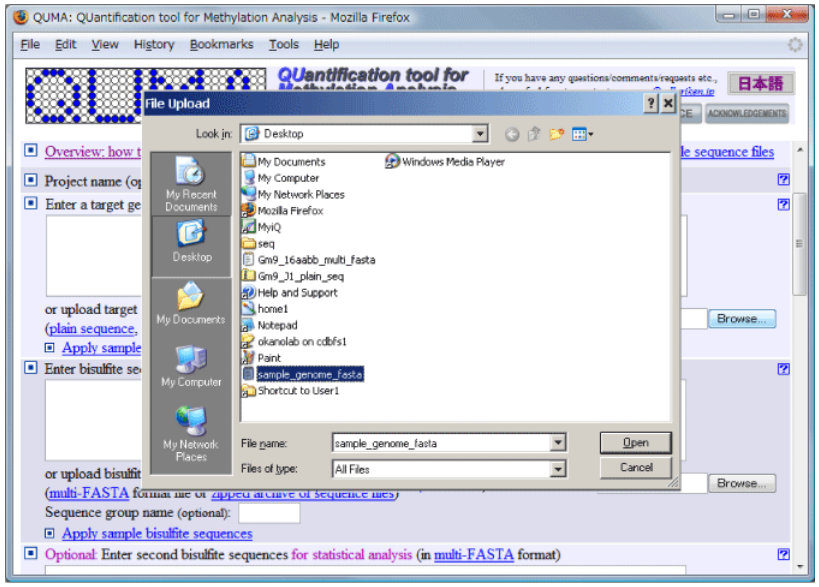
5.4.5. Genomic sequence file 1

2) Or click the first button (in this case "Browse..." button) to upload a target genomic sequence file.



5.4.6. Genomic sequence file 2

Select a target genomic sequence file to upload. See also "7.1. Genomic sequence".

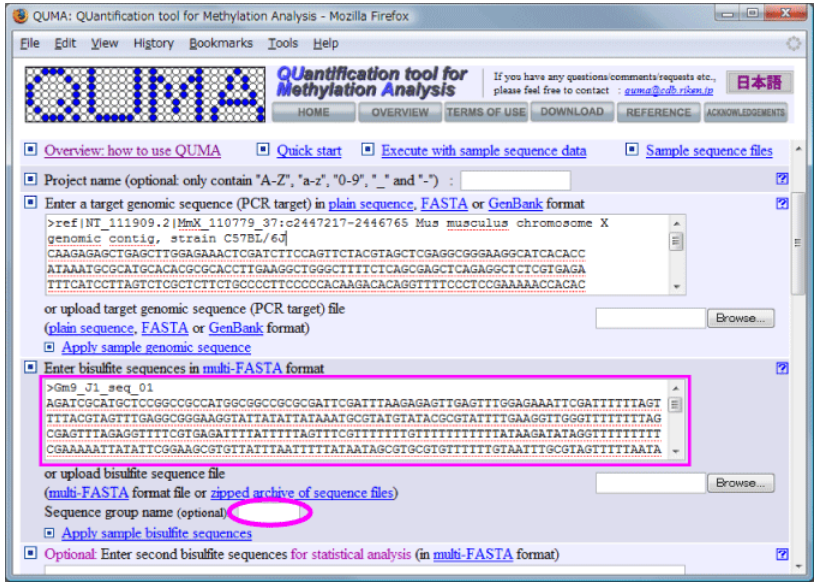


5.4.7. Bisulfite sequences

Input a group name of bisulfite sequences (optional).

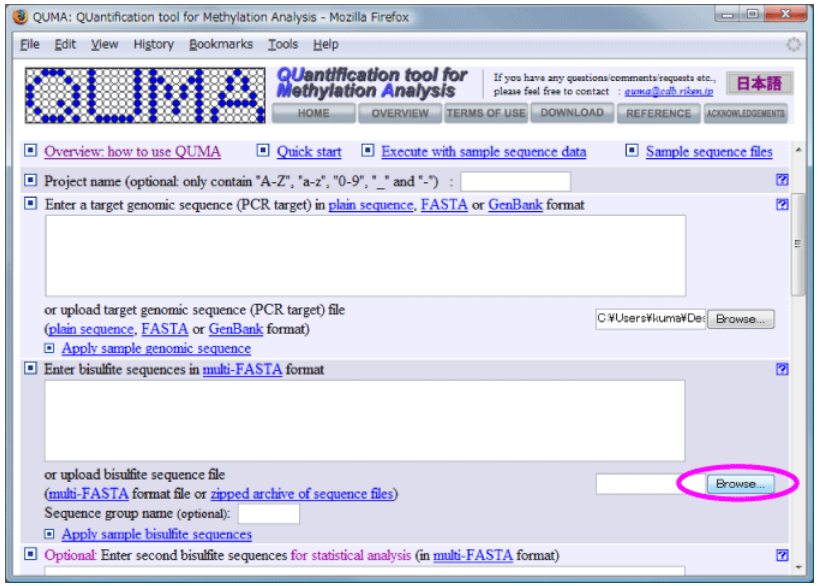
The bisulfite sequences can be input by two ways of 1) direct input and 2) upload.

1) In case of direct input, paste the bisulfite sequences (8.4. Multi-FASTA format). See also "7.2. Bisulfite sequences".



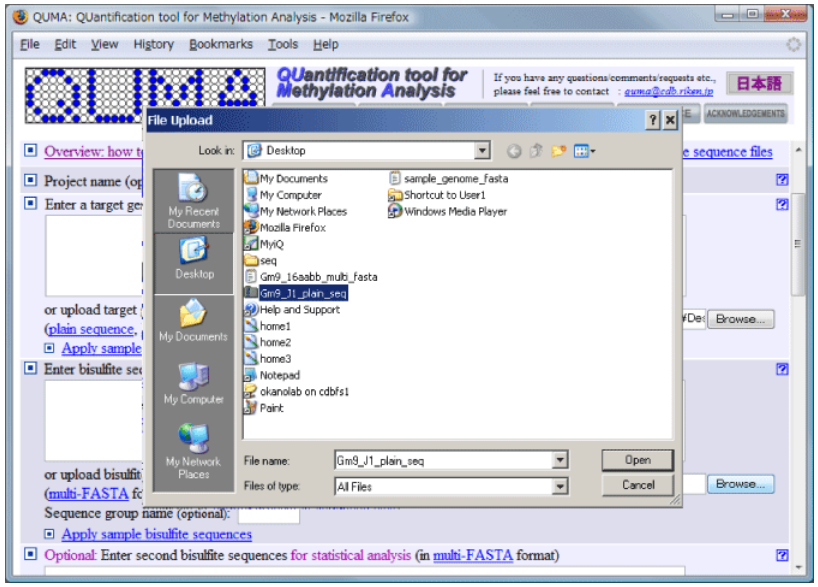
5.4.8. Bisulfite sequences file 1

2) Or click the second button to upload a file of bisulfite sequences.



5.4.9. Bisulfite sequences file 2

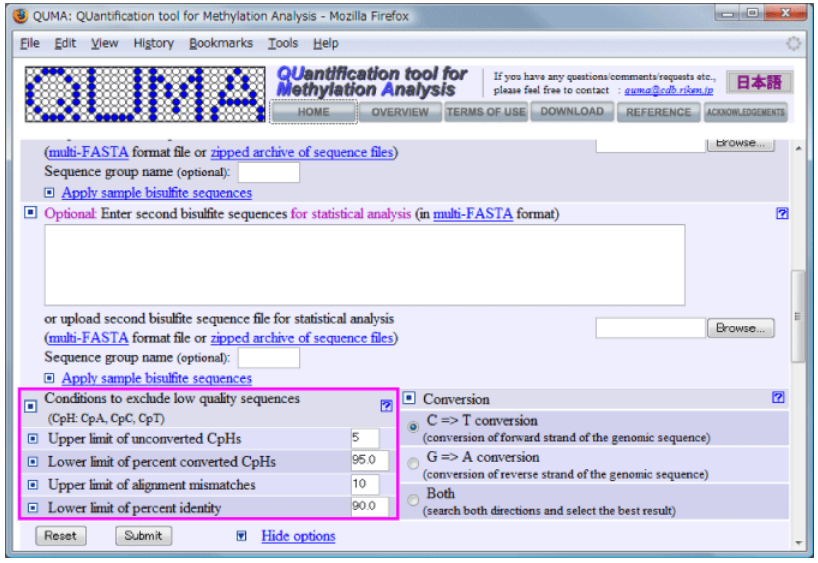
Select a file of bisulfite sequences. Acceptable file formats are 8.4. Multi-FASTA or 8.5. Zipped archive of sequence files. See also "7.2. Bisulfite sequences", "8.6. How to create zipped archive (Macintosh)" and "8.7. How to create zipped archive (Windows)".



5.4.10. Conditions to exclude bisulfite sequences

If you want, change conditions to exclude low quality bisulfite sequences.

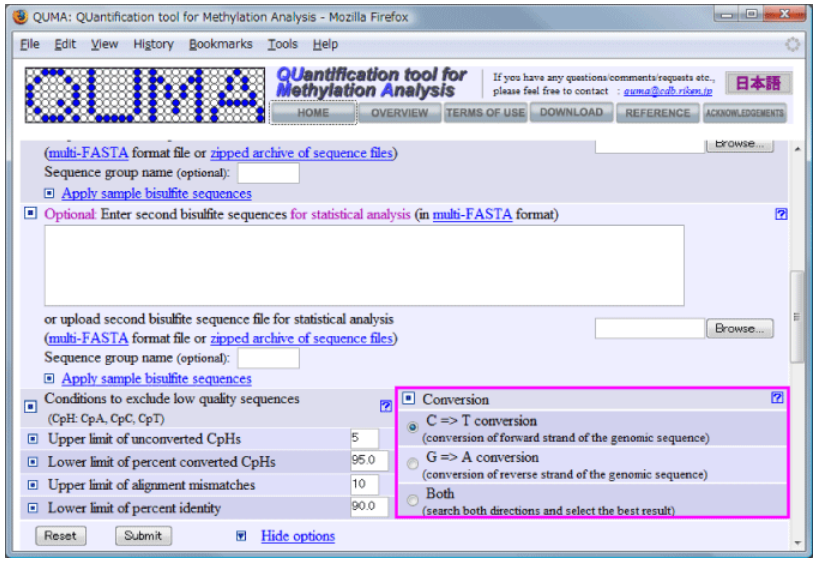
- **Upper limit of unconverted CpHs**
  - ✓ **number of unconverted CpHs (CpA, CpC and CpT)**
- **·Lower limit of percent converted CpHs**
  - ✓ **percent of "number of converted CpHs"/"number of CpHs"**
- **Upper limit of alignment mismatch**
  - ✓ **number of alignment mismatches and gaps between genomic and bisulfite sequences**
- **Lower limit of percent identity**
  - ✓ **percent of alignment identity between genomic and bisulfite sequences**

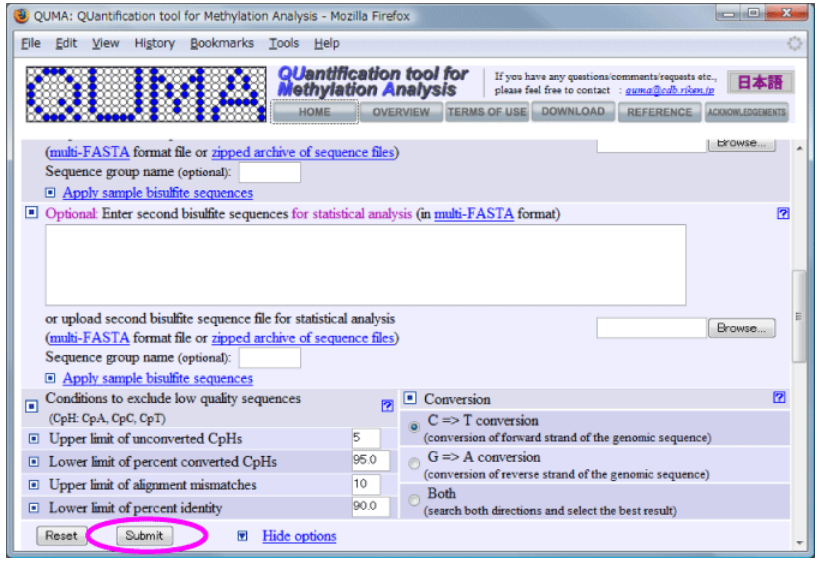


5.4.11. Strand of bisulfite conversion

Select a strand of bisulfite conversion of the target genomic sequence.

- **C=>T conversion**
  - ✓ **When bisulfite PCR primer pair was designed for forward strand of the genomic sequence (default).**
- **G=>A conversion**
  - ✓ **When bisulfite PCR primer pair was designed for reverse strand of the genomic sequence.**
- **Both**
  - ✓ **Search both direction of conversion and adopt more appropriate strand.**





5.4.12. Submit

Click the "Submit" button to analyze. Typically, only a few seconds are necessary.

5.5. Analysis result page

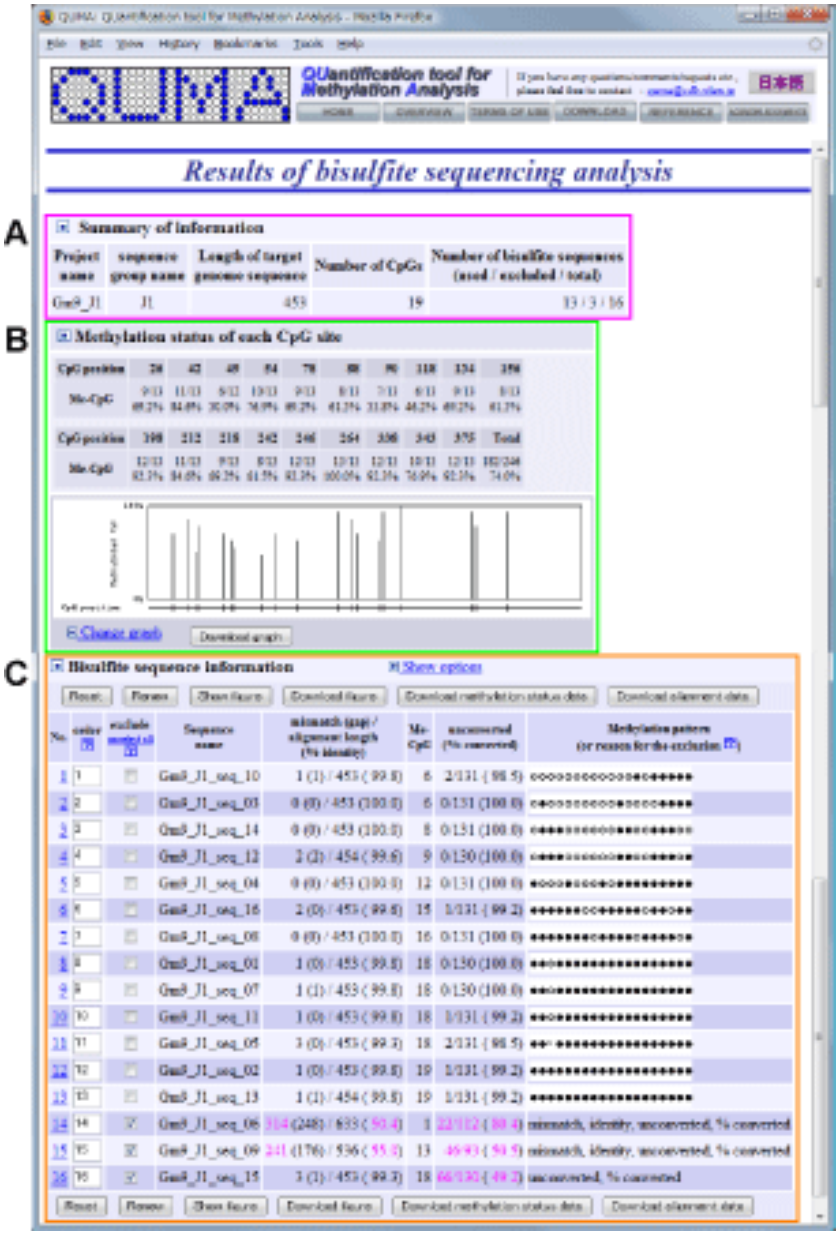
5.5.1. Overview of analysis result page 1

Analysis result page consists of three sections.

A) Summary of information

B) Methylation status of each CpG sites

C) Information and methylation pattern of each bisulfite sequences



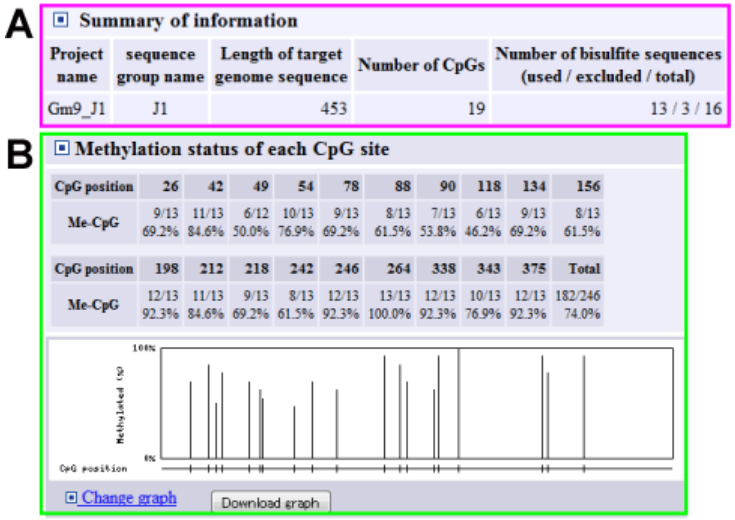
5.5.2. Overview of analysis result page 2

A) Summary of information

Length of the target genome sequence, number of CpG sites and number of bisulfite sequences are indicated.

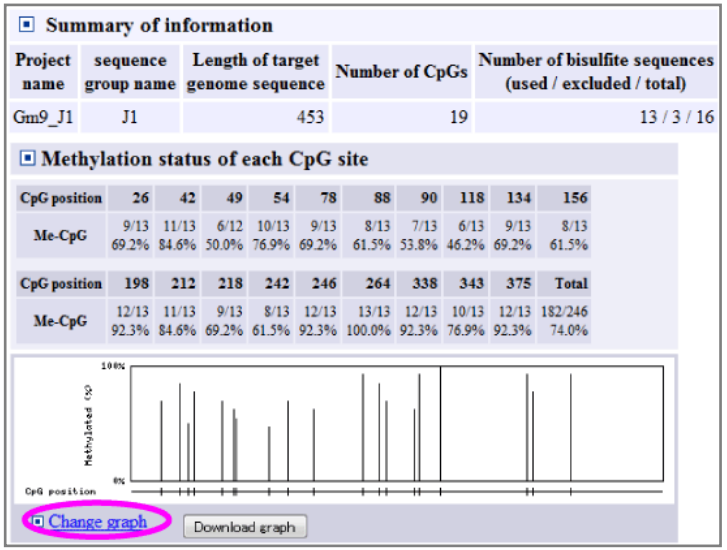
B) Methylation status of each CpG sites

Position and methylation status of each CpG sites and figure of methylation status are shown.



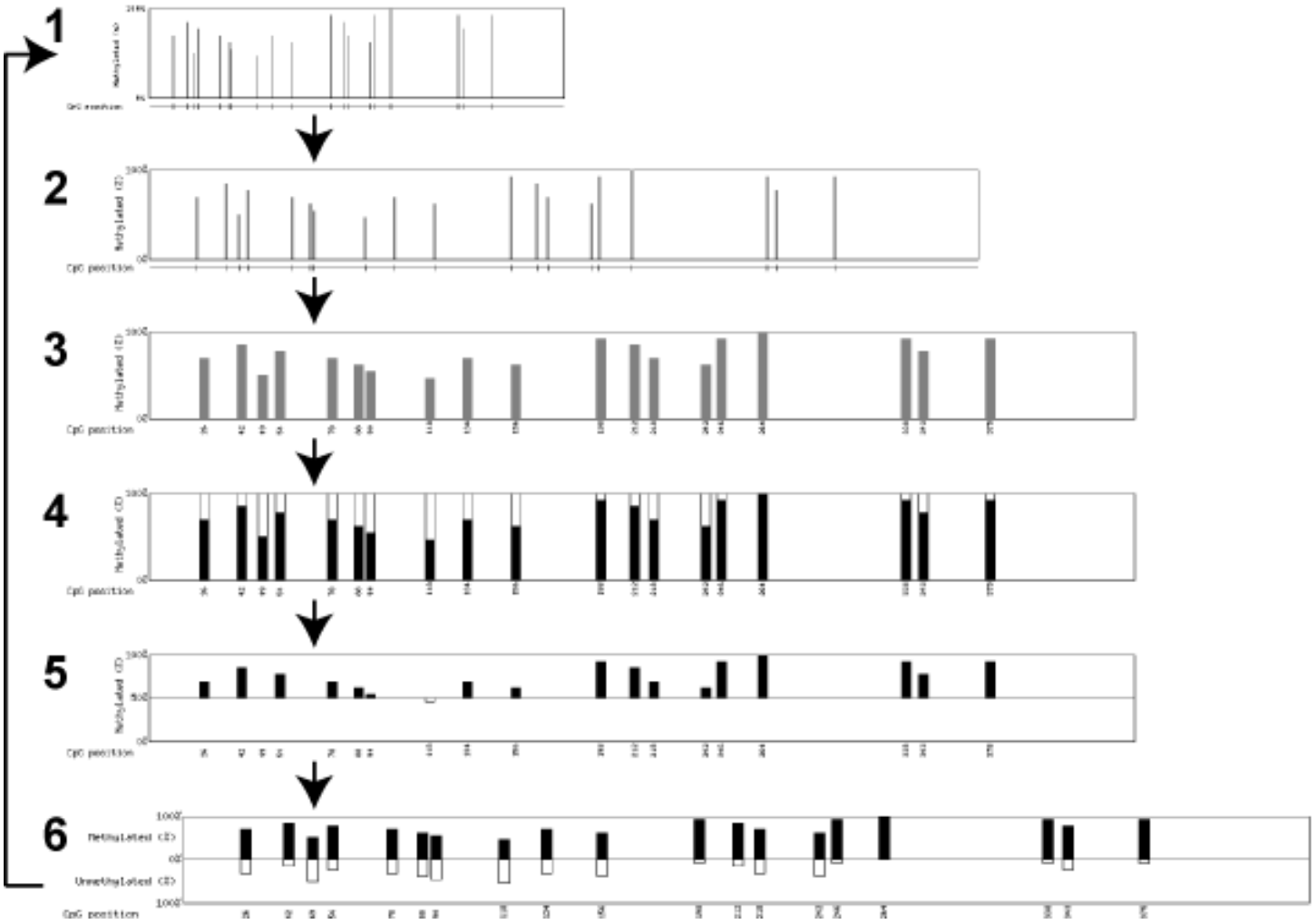
5.5.3. Change methylation status figure 1

Click "Change graph" link to switch methylation status figures.



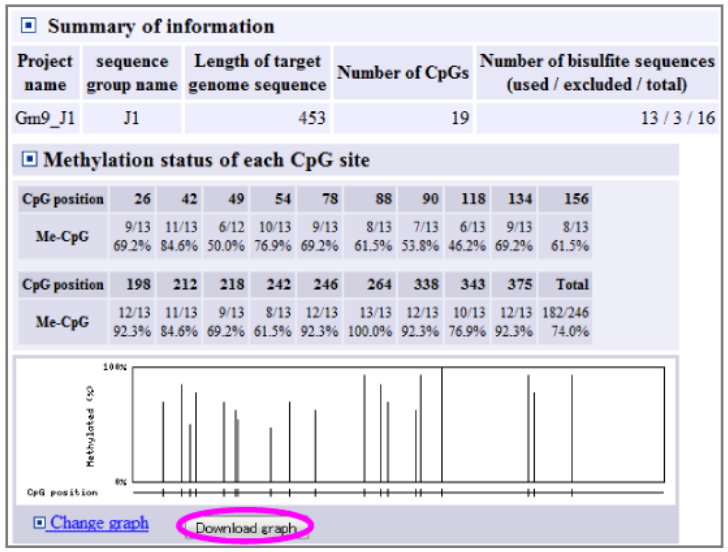
5.5.4. Change methylation status figure 2

Methylation status figures are switched one after the other by clicking "Change graph" link. Figures 1 and 2 are reflected the position of CpG sites almost accurately. Figures 3-6 are not reflected accurately.



5.5.5. Download methylation status figure

Click "Download graph" button to download the methylation status figure which displayed at that time.



Summary of information

Project name	sequence group name	Length of target genome sequence	Number of CpGs	Number of bisulfite sequences (used / excluded / total)
Gm9_J1	J1	453	19	13 / 3 / 16

Methylation status of each CpG site

CpG position	26	42	49	54	78	88	90	118	134	156
Me-CpG	9/13 69.2%	11/13 84.6%	6/12 50.0%	10/13 76.9%	9/13 69.2%	8/13 61.5%	7/13 53.8%	6/13 46.2%	9/13 69.2%	8/13 61.5%

CpG position	198	212	218	242	246	264	338	343	375	Total
Me-CpG	12/13 92.3%	11/13 84.6%	9/13 69.2%	8/13 61.5%	12/13 92.3%	13/13 100.0%	12/13 92.3%	10/13 76.9%	12/13 92.3%	182/246 74.0%

Change graph | Download graph

C

Bisulfite sequence information — Show options

Reset | Renew | Show figure | Download figure | Download methylation status data | Download alignment data

No.	order	exclude (unselect all)	Sequence name	mismatch (gap) / alignment length (% identity)	Me-CpG	unconverted (% converted)	Methylation pattern (or reason for the exclusion)
1	1		Gm9_J1_seq_10	1 (1) / 453 (99.8)	6	2/131 (98.5)	○○○○○○○○○○○○●○●●●●●
2	2		Gm9_J1_seq_03	0 (0) / 453 (100.0)	6	0/131 (100.0)	●●○○○○○○○○●○○○○●●●●
3	3		Gm9_J1_seq_14	0 (0) / 453 (100.0)	8	0/131 (100.0)	●●●●○○○○○○●●○●●●○○
4	4		Gm9_J1_seq_12	2 (2) / 454 (99.6)	9	0/130 (100.0)	●●●●○○○○○○●●○●●●○●
5	5		Gm9_J1_seq_04	0 (0) / 453 (100.0)	12	0/131 (100.0)	●●○○●○○○●○●●●●●●●●
6	6		Gm9_J1_seq_16	2 (0) / 453 (99.6)	15	1/131 (99.2)	●●●●●●○○●●●●○●●○●●
7	7		Gm9_J1_seq_08	0 (0) / 453 (100.0)	16	0/131 (100.0)	●●●●●●●○●●●●○●●●●●
8	8		Gm9_J1_seq_01	1 (0) / 453 (99.8)	18	0/130 (100.0)	●●○●●●●●●●●●●●●●●●
9	9		Gm9_J1_seq_07	1 (1) / 453 (99.8)	18	0/130 (100.0)	●●○●●●●●●●●●●●●●●●
10	10		Gm9_J1_seq_11	1 (0) / 453 (99.8)	18	1/131 (99.2)	●●○●●●●●●●●●●●●●●●
11	11		Gm9_J1_seq_05	3 (0) / 453 (99.3)	18	2/131 (98.5)	●●×●●●●●●●●●●●●●●●
12	12		Gm9_J1_seq_02	1 (0) / 453 (99.8)	19	1/131 (99.2)	●●●●●●●●●●●●●●●●●●●
13	13		Gm9_J1_seq_13	1 (1) / 454 (99.8)	19	1/131 (99.2)	●●●●●●●●●●●●●●●●●●●
14	14	☑	Gm9_J1_seq_06	314 (248) / 633 (50.4)	1	22/112 (80.4)	mismatch, identity, unconverted, % converted
15	15	☑	Gm9_J1_seq_09	241 (176) / 536 (55.0)	13	46/93 (50.5)	mismatch, identity, unconverted, % converted
16	16	☑	Gm9_J1_seq_15	3 (1) / 453 (99.3)	18	66/130 (49.2)	unconverted, % converted

Reset | Renew | Show figure | Download figure | Download methylation status data | Download alignment data

5.5.6. Overview of analysis result page 3

C) **Information and methylation pattern of each bisulfite sequences**

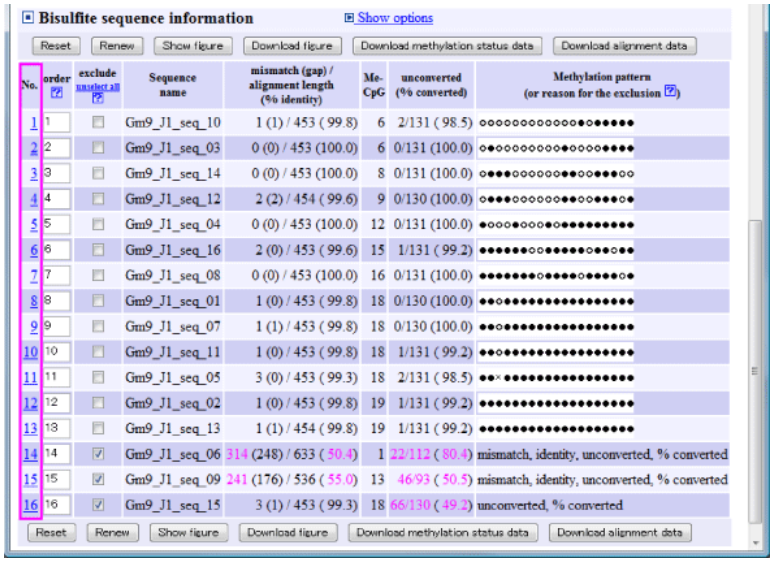
1. **Number of mismatches and percent identity of bisulfite alignment**
2. **Number of methylated CpG sites**
3. **Number of bisulfite unconverted CpHs (CpA, CpC, CpT)**
4. **Pattern of CpG methylation (Black circle: methylated, White circle: unmethylated, Cross: mismatch or gap)**

Methylation pattern (4.) is not present when quality of bisulfite sequence is low or excluded from user. Low quality value is shown as magenta. When excluded, reason(s) for the exclusion will be indicated at methylation pattern column (4.). Conditions to exclude low quality bisulfite sequences can be changed (See "5.6.1. Show options 1" for more detail).

- **mismatch:**
  - ✓ **The number of alignment mismatches (includes gaps) between genomic and bisulfite sequences exceeded the upper limit (default: 10).**
  - ✓ **This means low quality sequence read.**
- **% ident**
  - ✓ **Percent of alignment identity between genomic and bisulfite sequences exceeded the lower limit (default: 90%).**
  - ✓ **This means low quality sequence read.**
- **Unconv**
  - ✓ **The number of unconverted CpHs (CpA, CpC and CpT) exceeded the upper limit (default: 5).**
  - ✓ **This means incomplete bisulfite conversion or low quality sequence read.**
- **% conv**
  - ✓ **Percent of "number of converted CpHs" / "number of CpHs" exceeded the lower limit (default 95%).**
  - ✓ **This means incomplete bisulfite conversion or low quality sequence read.**
- **user desired**
  - ✓ **Sequence was excluded by checking on the "exclude" checkbox.**

5.5.7. Show alignment

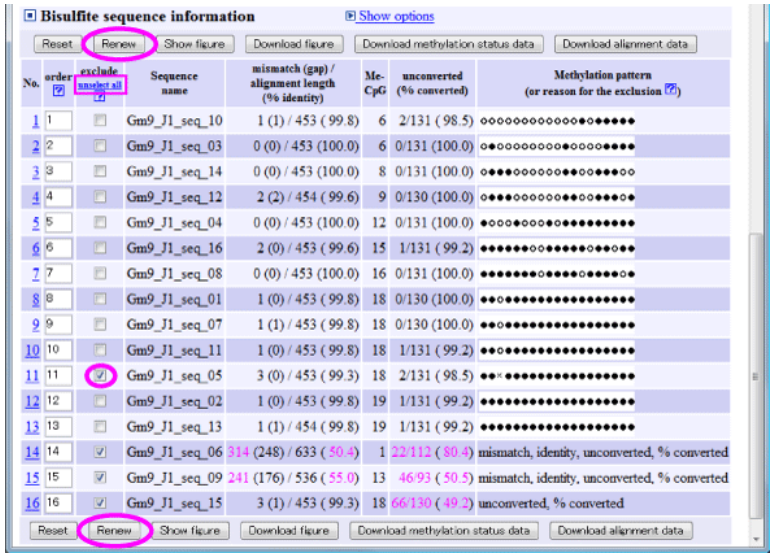
Click links to show bisulfite alignment between bisulfite sequence to genomic sequence.



See "5.7. Alignment page" for next step.

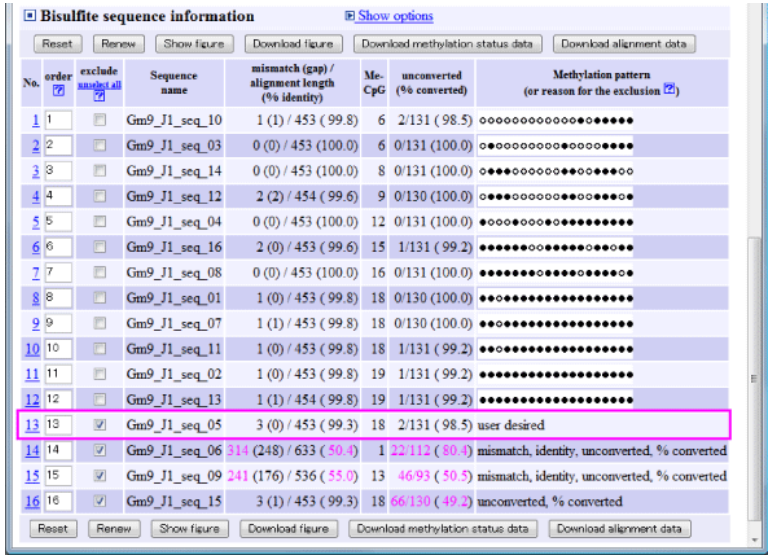
5.5.8. Include/exclude bisulfite sequence 1

To include/exclude a bisulfite sequence, check off/on "exclude" checkbox. Then click "Renew" button. To include all bisulfite sequence information, click "unselect all" link.



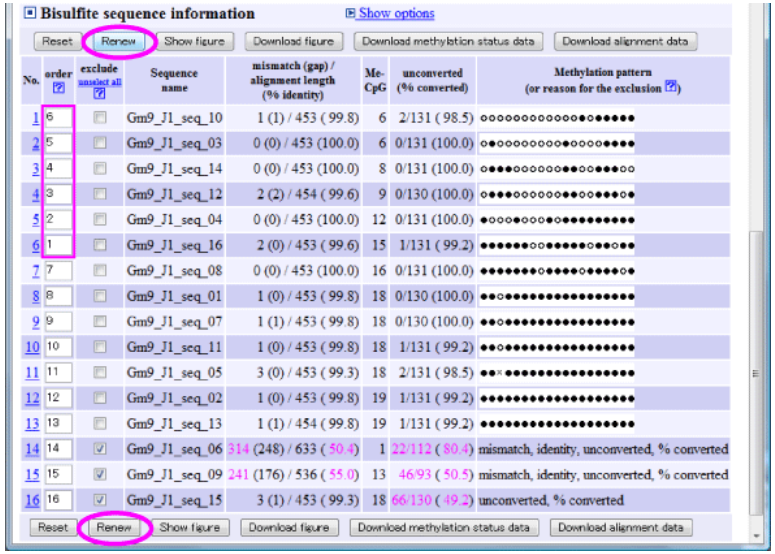
5.5.9. Include/exclude bisulfite sequence 2

The change is reflected.



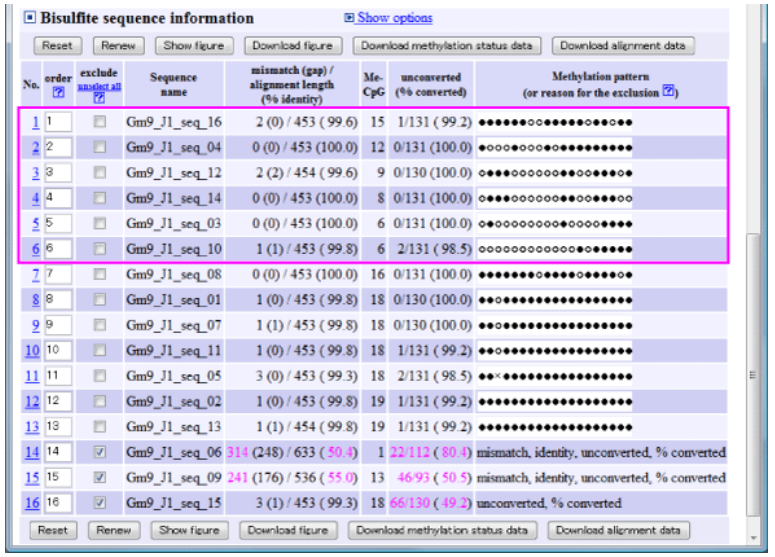
5.5.10. Change the order of bisulfite sequences 1

Change the value of "order" column to desired order. Then click "Renew" button.



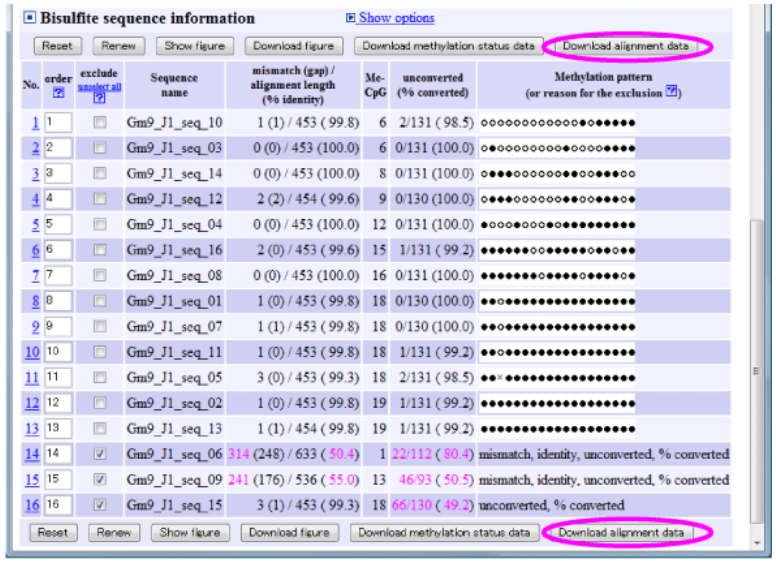
5.5.11. Change the order of bisulfite sequences 2

The change is reflected.



5.5.12. Download alignments data

Click "Download alignment data" button to download alignments data.

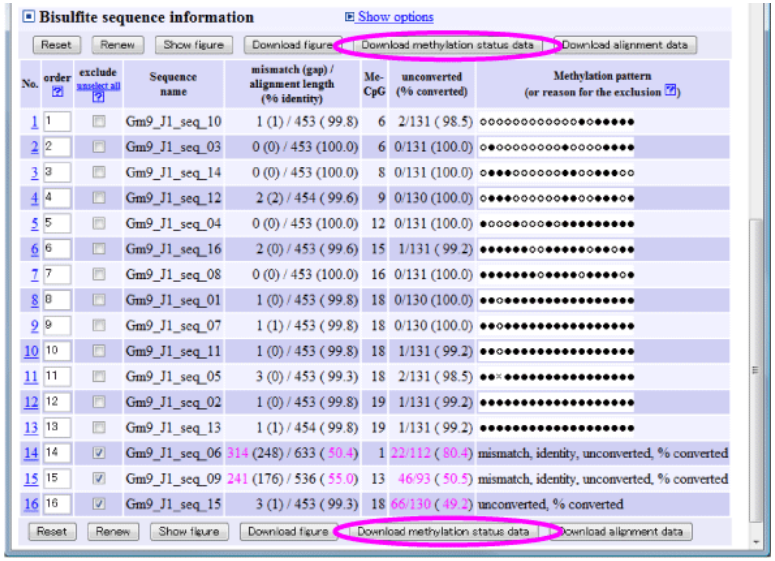


5.5.13. Alignments data

Downloaded alignments data file can be opened by TextEdit (Mac), Notepad (Win) or other text editors.

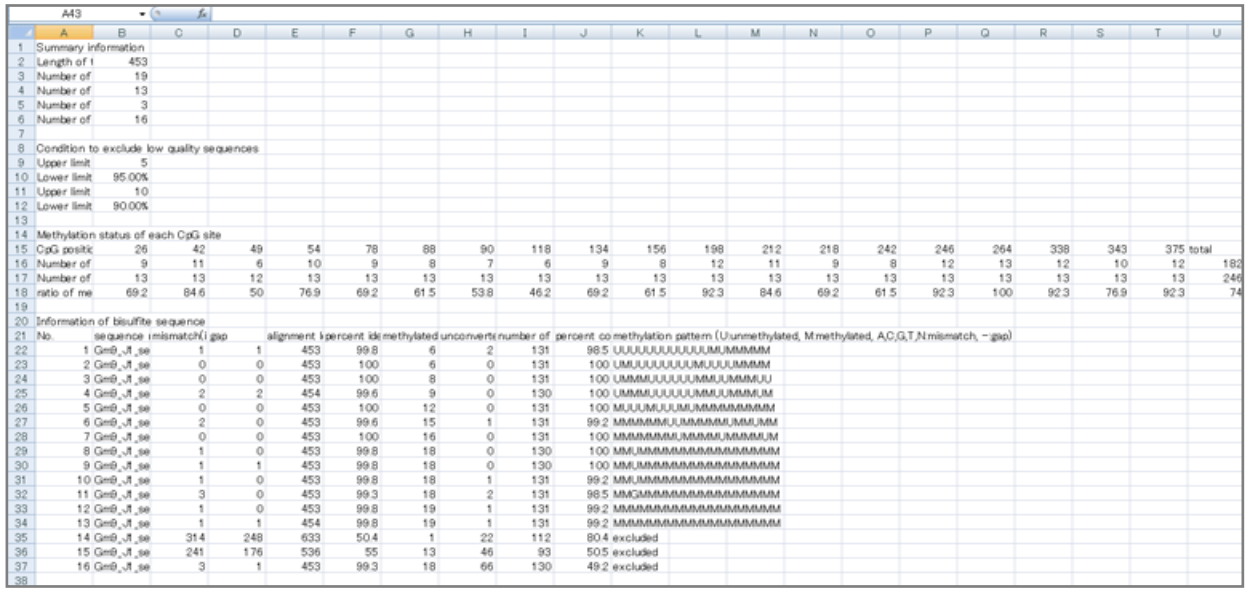
5.5.14. Download analysis data

Click "Download methylation status data" button to download analysis data.



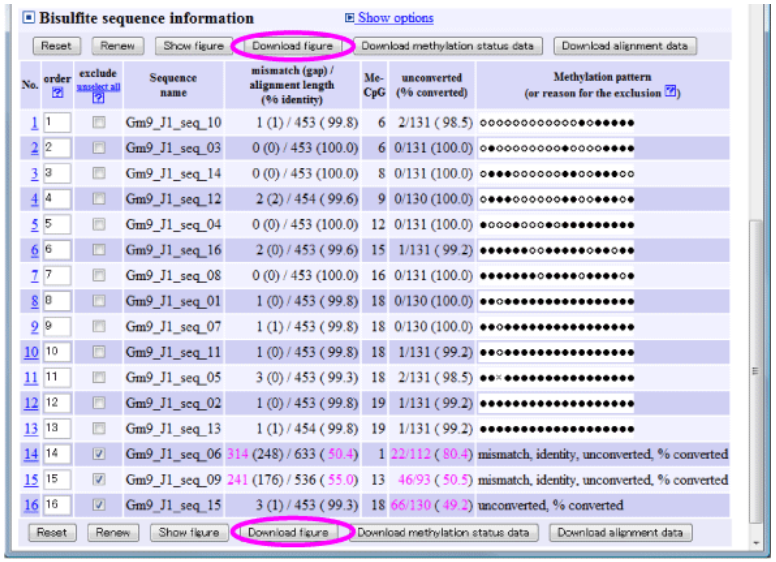
5.5.15. Analysis data

Downloaded analysis data file can be opened by Microsoft Excel, OpenOffice/StartSuite or other spreadsheet software (CSV file format). See also "10.1. How to open a CSV file".



5.5.16. Download methylation pattern figure

Click "Download figure" button to download methylation pattern figure.

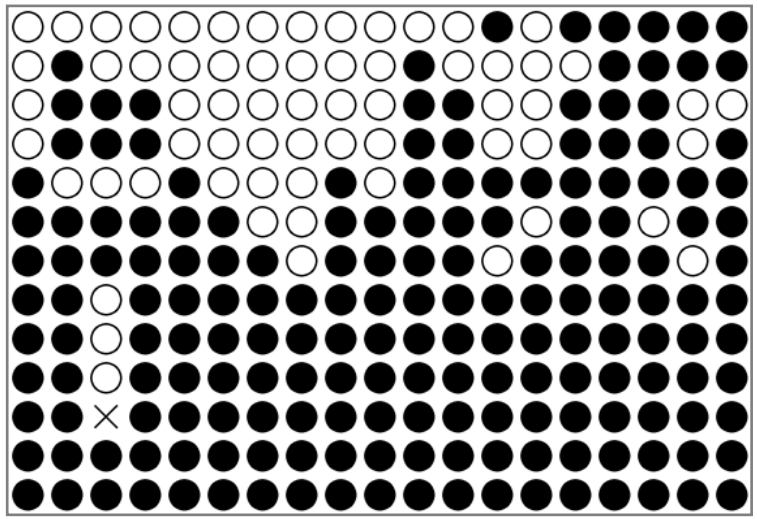


5.5.17. Methylation pattern figure

This figure reflects order and include/exclude sequences in analysis result page.

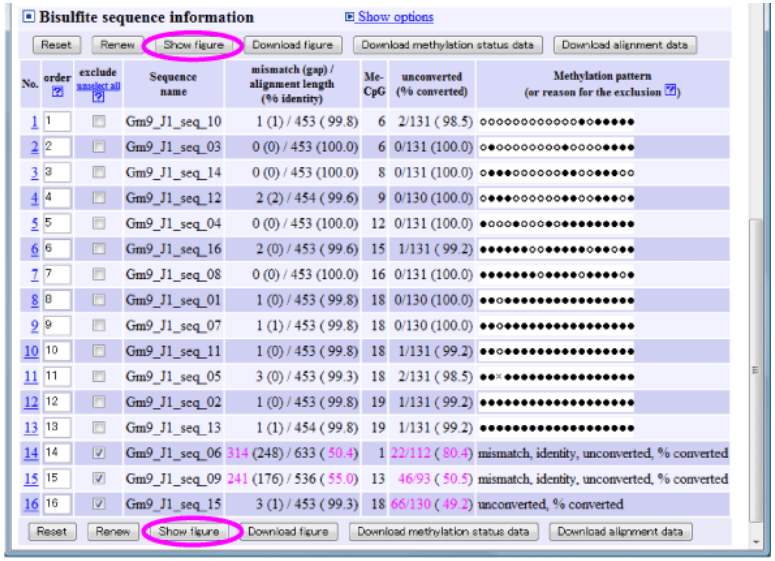
Black and white circle indicate methylated and unmethylated CpG respectively. Cross indicate mismatch or gap in the alignment.

Other types of figures can be created at 5.8. Figure page. Detailed parameters, such as line width, diameter of circle and etc., can also be changed at 5.8. Figure page.



5.5.18. Go to figure page

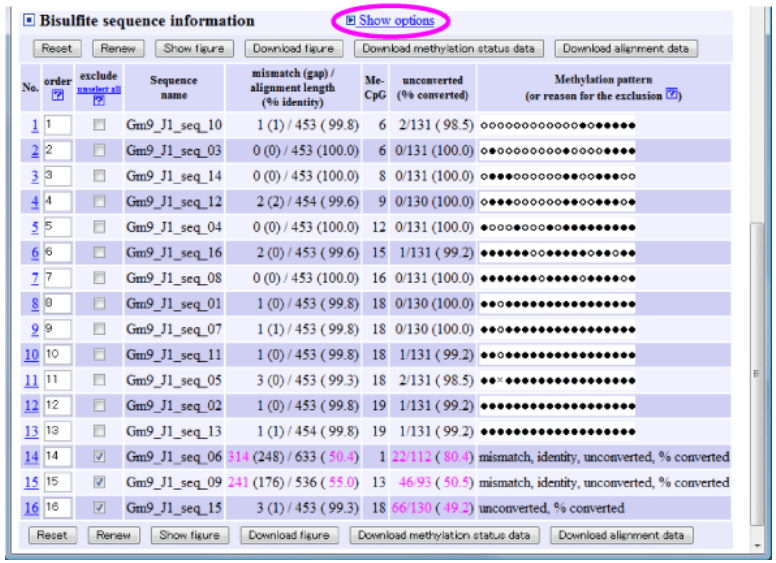
Click "Show figure" button to go to figure page where other types of figures can be created with detailed parameters.



5.6. Result page options

5.6.1. Show options 1

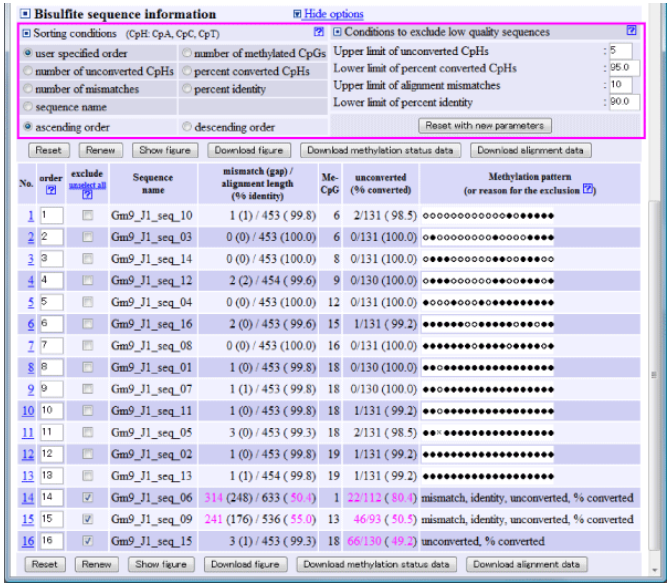
Click the "Show options" link to show optional fields.



See "5.8. Figure page" for next step.

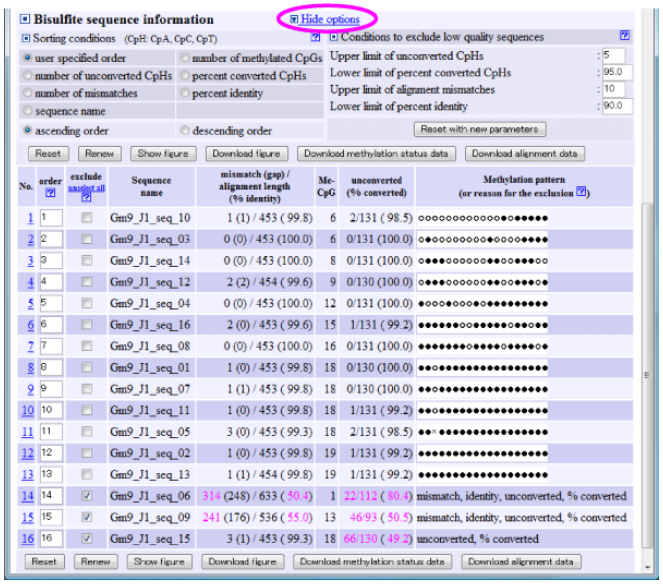
5.6.2. Show options 2

Optional fields will appear.



5.6.3. Hide options

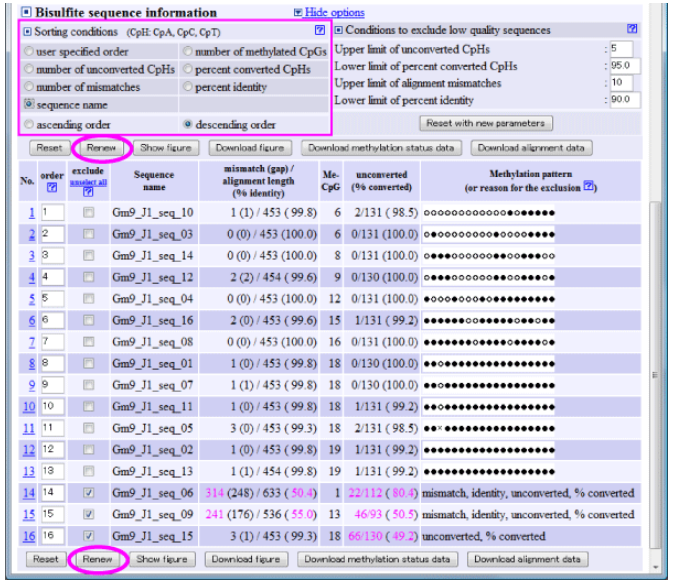
Click the "Hide options" link to hide optional fields.



5.6.4. Change the order of bisulfite sequences 1

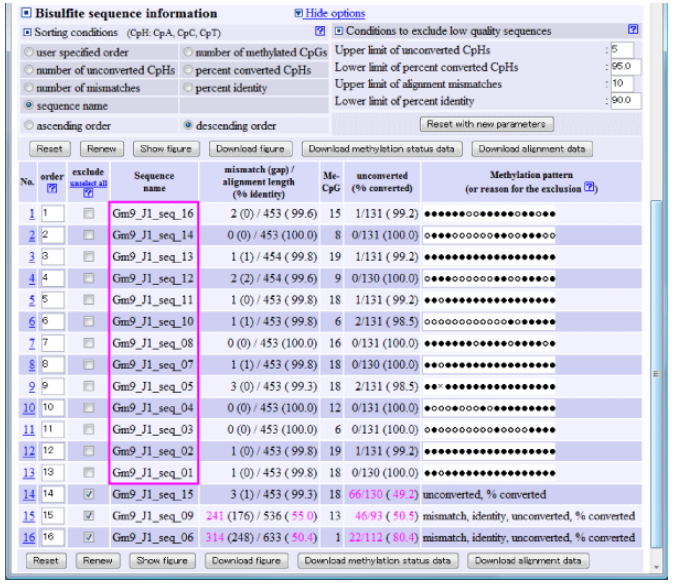
Order of bisulfite sequences can be changed by several parameters and ascending/descending order. Then click "Renew" button.

- **user specified order**
  - ✓ **The value of "order" column.**
- **number of methylated CpGs**
- **number of unconversions**
  - ✓ **unconverted CpHs (CpA, CpC, CpT)**
- **percent conversion**
  - ✓ **percent of converted CpHs / total CpHs**
- **number of mismatches**
- **percent identity**
- **sequence name**
- **ascending order**
- **descending order**



5.6.5. Change the order of bisulfite sequences 2

The change is reflected.



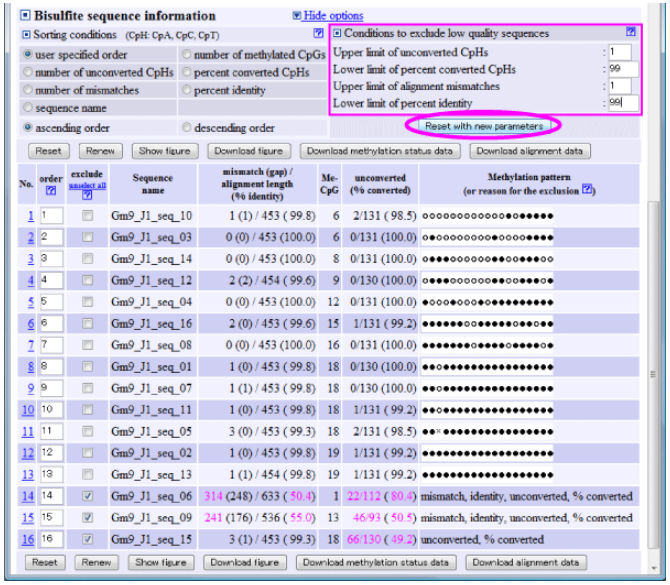
5.6.6. Conditions to exclude bisulfite sequences 1

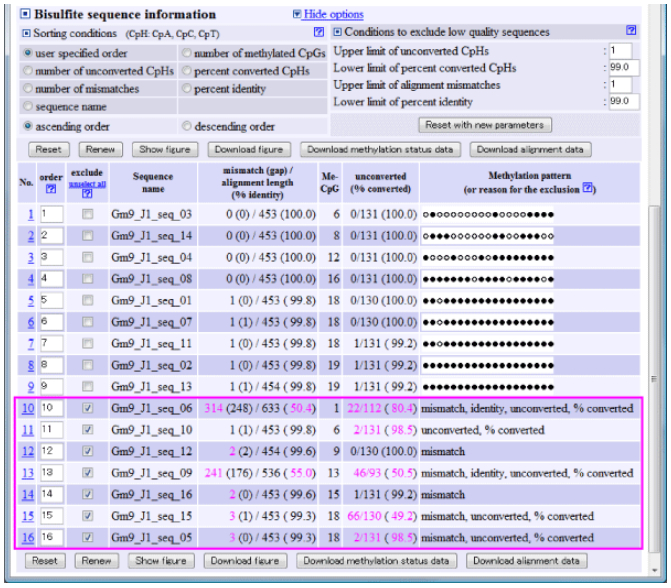
Conditions to exclude low quality bisulfite sequences can be changed. Then click "Reset with new parameter" button (order and exclusion of bisulfite sequences will be reset).

- **Upper limit of unconversion**
  - ✓ **number of unconverted CpHs (CpA, CpC and CpT)**
- **Lower limit of percent conversion**
  - ✓ **percent of "number of converted CpHs"/"number of CpHs"**
- **Upper limit of alignment mismatch**
  - ✓ **number of alignment mismatches and gaps between genomic and bisulfite sequences**
- **Lower limit of percent identity**
  - ✓ **percent of alignment identity between genomic and bisulfite sequences**

5.6.7. Conditions to exclude bisulfite sequences 2

The change is reflected.





5.7. Alignment page

5.7.1. Overview of alignment page

Alignment page consists of four sections.

A) Summary of information

Information about bisulfite alignment.

B) Genome sequence

C) Bisulfite sequence

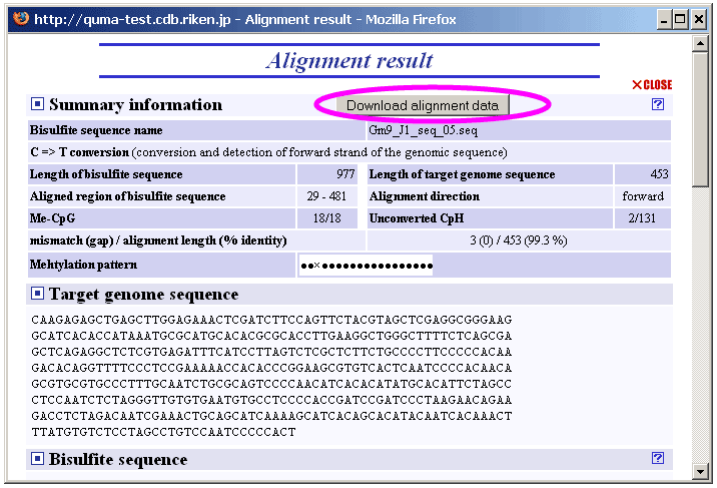
Sequence outside alignment is indicated as gray color.

D) Bisulfite alignment

Methylated C of CpG site, unmethylated C of CpG site, Unconverted C (CpA, CpC, CpT) are indicated as different colors.

5.7.2. Download alignment data

Click "Download alignment data" button to download alignment data which displayed hear.



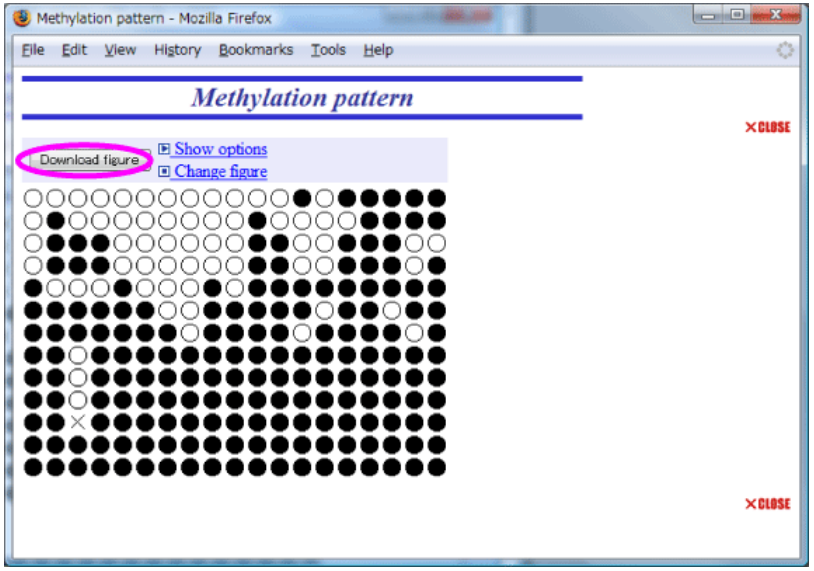
5.7.3. Alignment data

Downloaded alignment data file can be opened by TextEdit (Mac), Notepad (Win) or other text editors.

5.8. Figure page

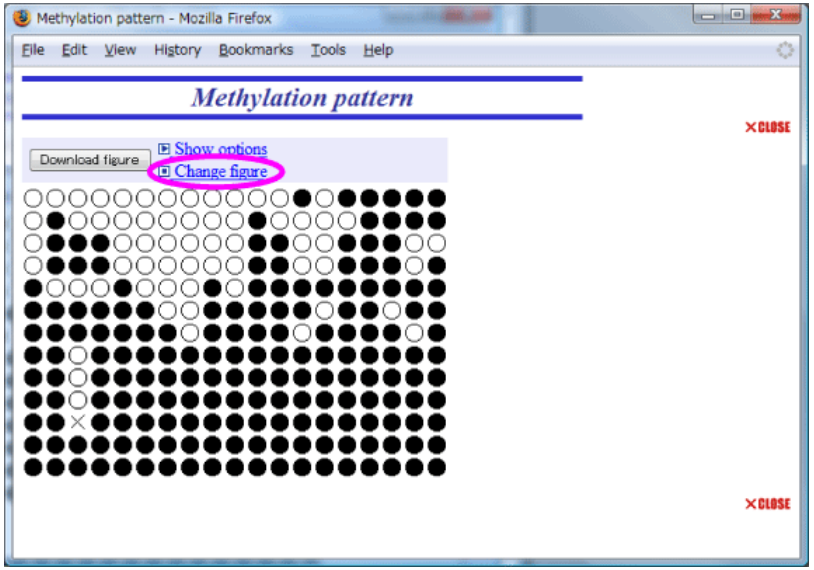
5.8.1. Download methylation pattern figure

Click "Download figure" button to download methylation pattern figure which displayed at that time.



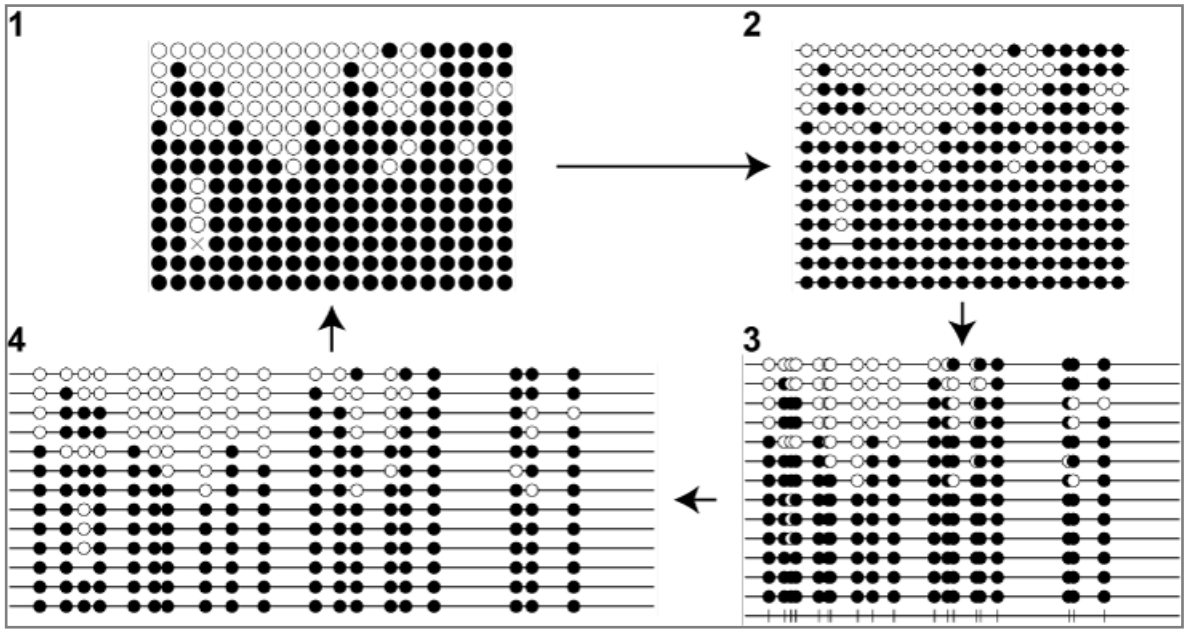
5.8.2. Change methylation pattern figure 1

Click "Change figure" link to switch methylation pattern figures.



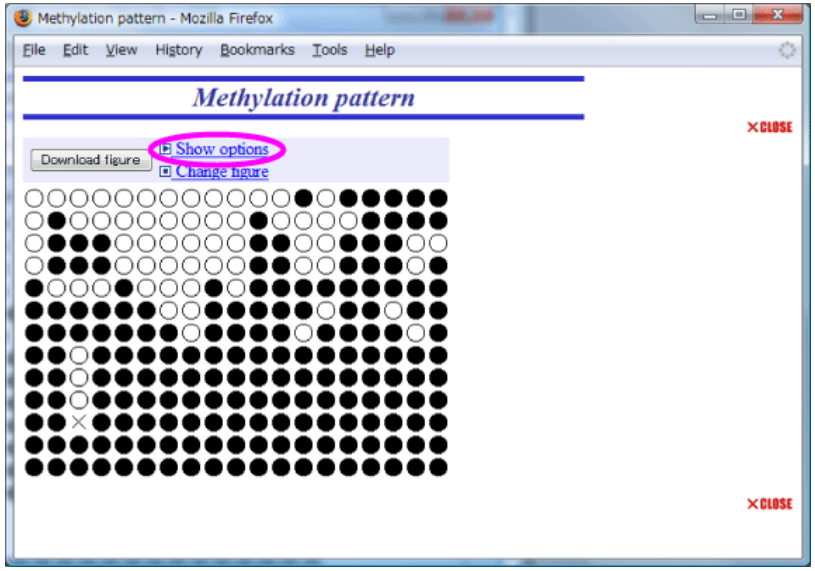
5.8.3. Change methylation pattern figure 2

Methylation pattern figures are switched one after the other.



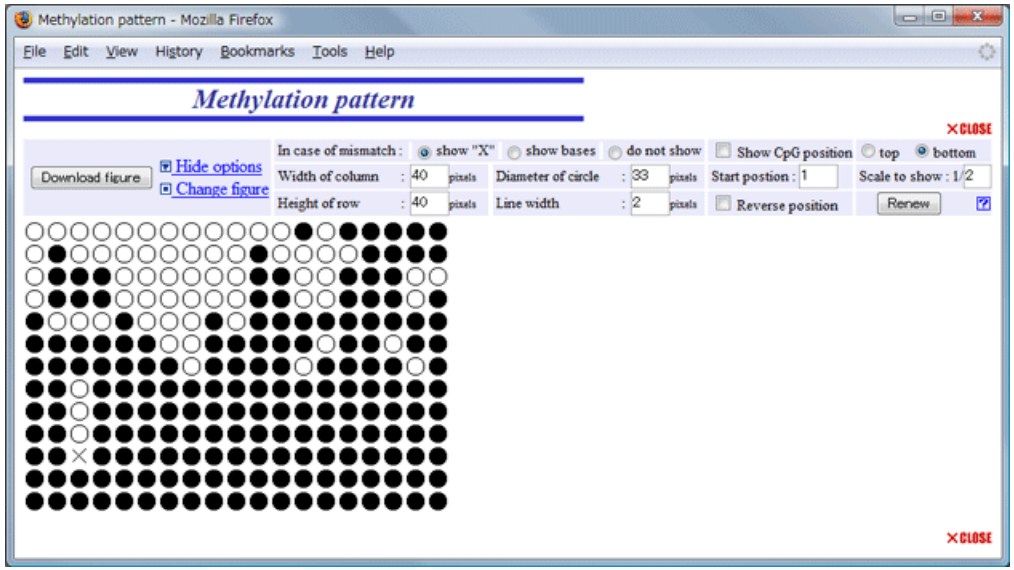
5.8.4. Show options

Click the "Show options" link to show optional fields.



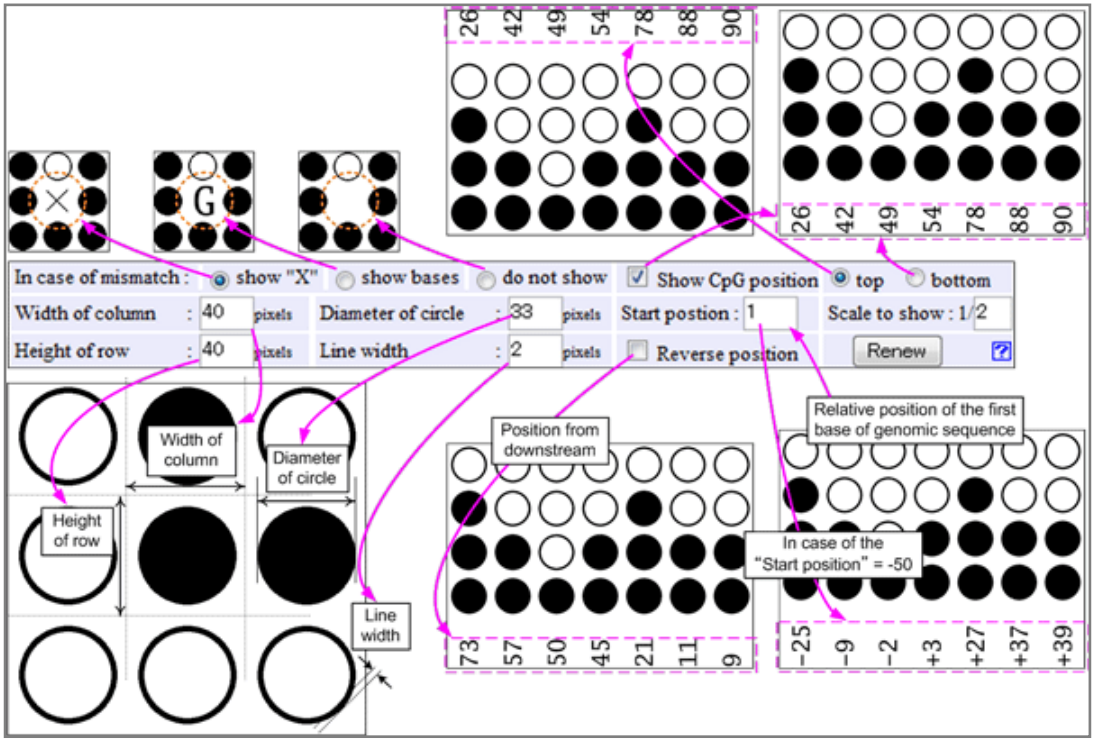
5.8.5. Figure 1

This figures is displayed circle at even intervals (not depend on CpG positions).



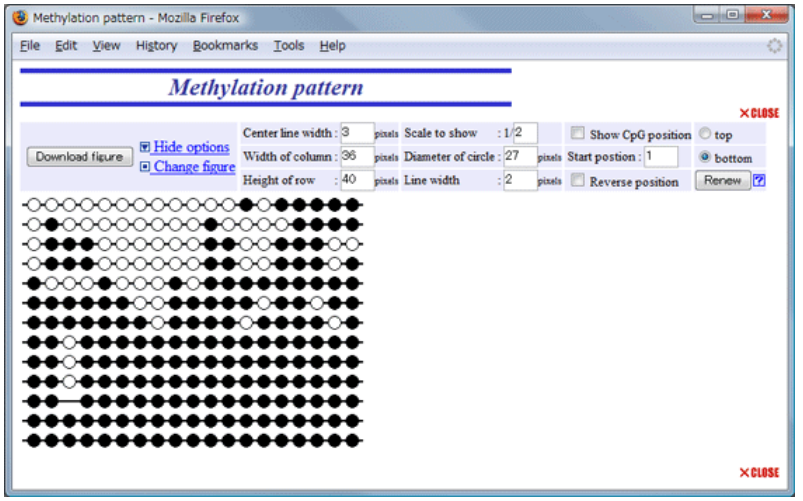
5.8.6. Option of figure 1

The meaning of the value of each option parameter is shown. "Scale to show" means size reduction rate to show in the window. Click "Renew" button to reflect parameters.



5.8.7. Figure 2

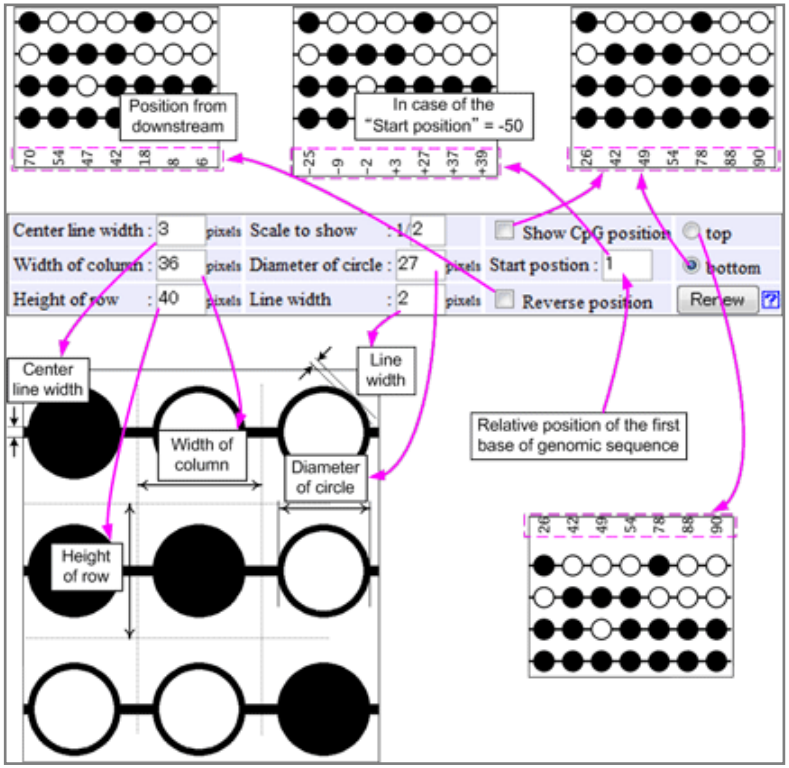
This figure is displayed circles at even intervals with the center line for each bisulfite sequences.



s

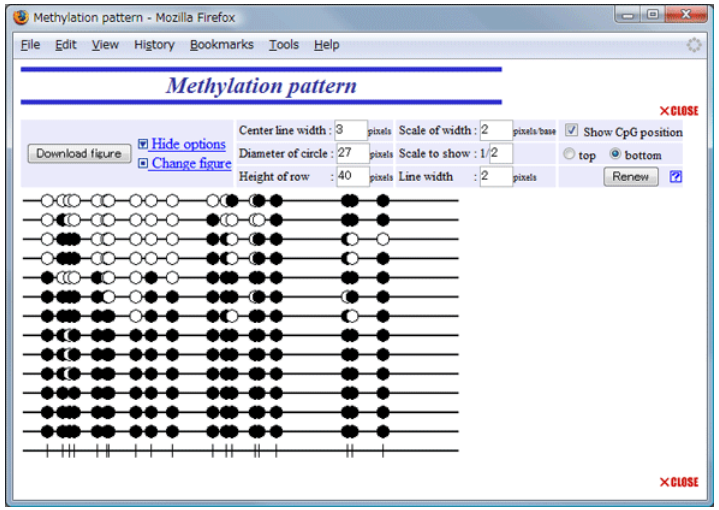
5.8.8. Option of figure 2

The meaning of the value of each option parameter is shown. "Scale to show" means size reduction rate to show in the window. Click "Renew" button to reflect parameters.



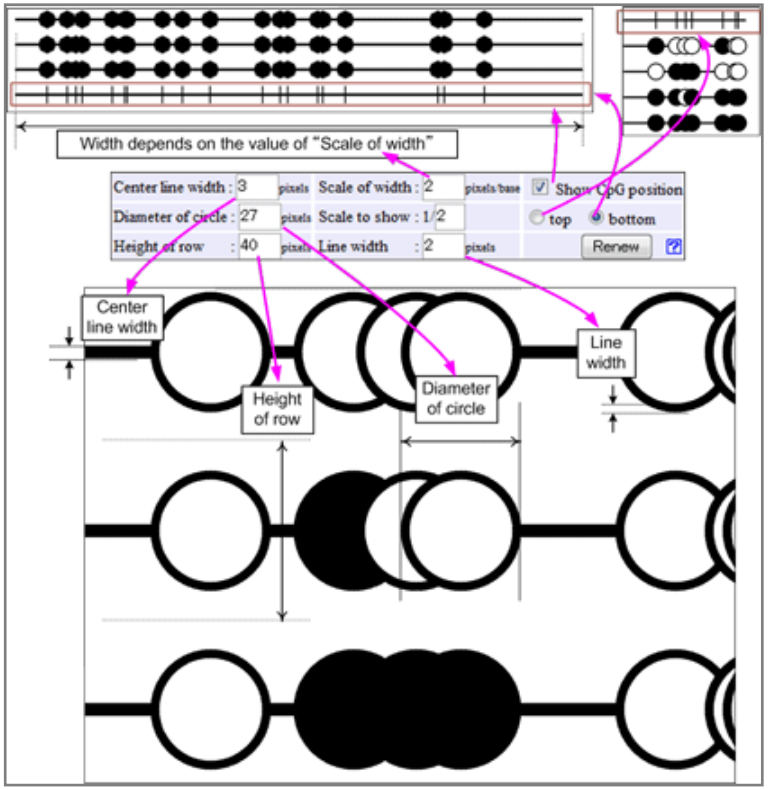
5.8.9. Figure 3

The positions of circles are reflected the position of CpG sites almost accurately. But closely positioned CpG sites are overlapped.



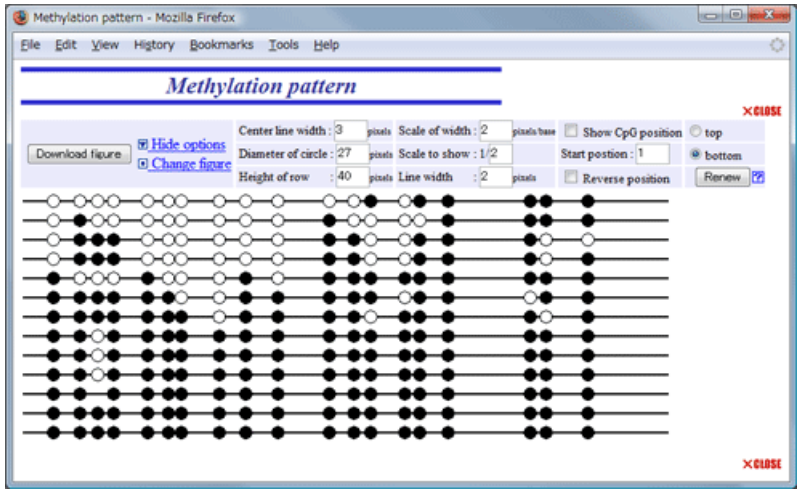
5.8.10. Option of figure 3

The meaning of the value of each option parameter is shown. "Scale to show" means size reduction rate to show in the window. Click "Renew" button to reflect parameters.



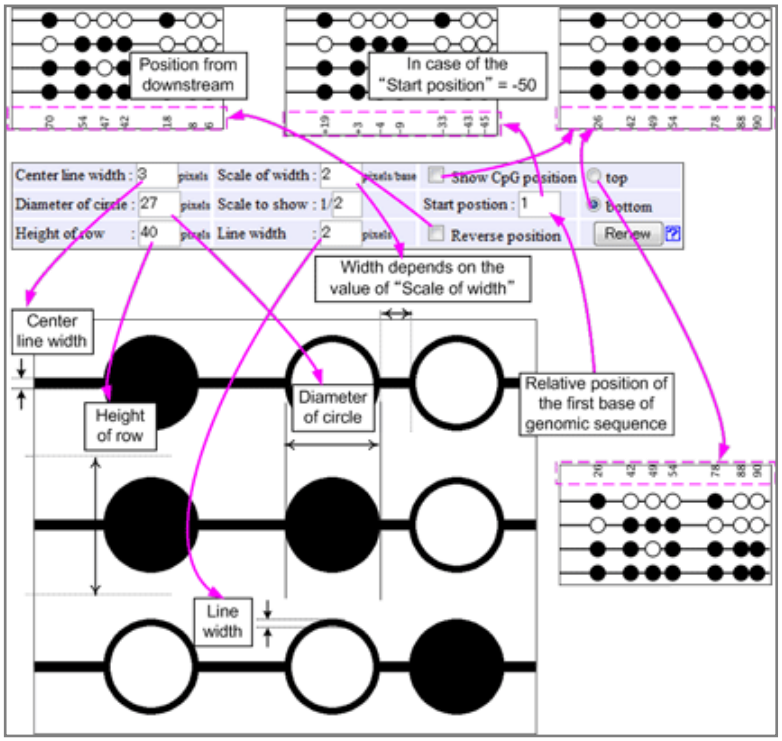
5.8.11. Figure 4

The positions of circles depend on the position of CpG sites, but not accurately. The circles are placed as not to overlap.



5.8.12. Option of figure 4

The meaning of the value of each option parameter is shown. "Scale to show" means size reduction rate to show in the window. Click "Renew" button to reflect parameters.

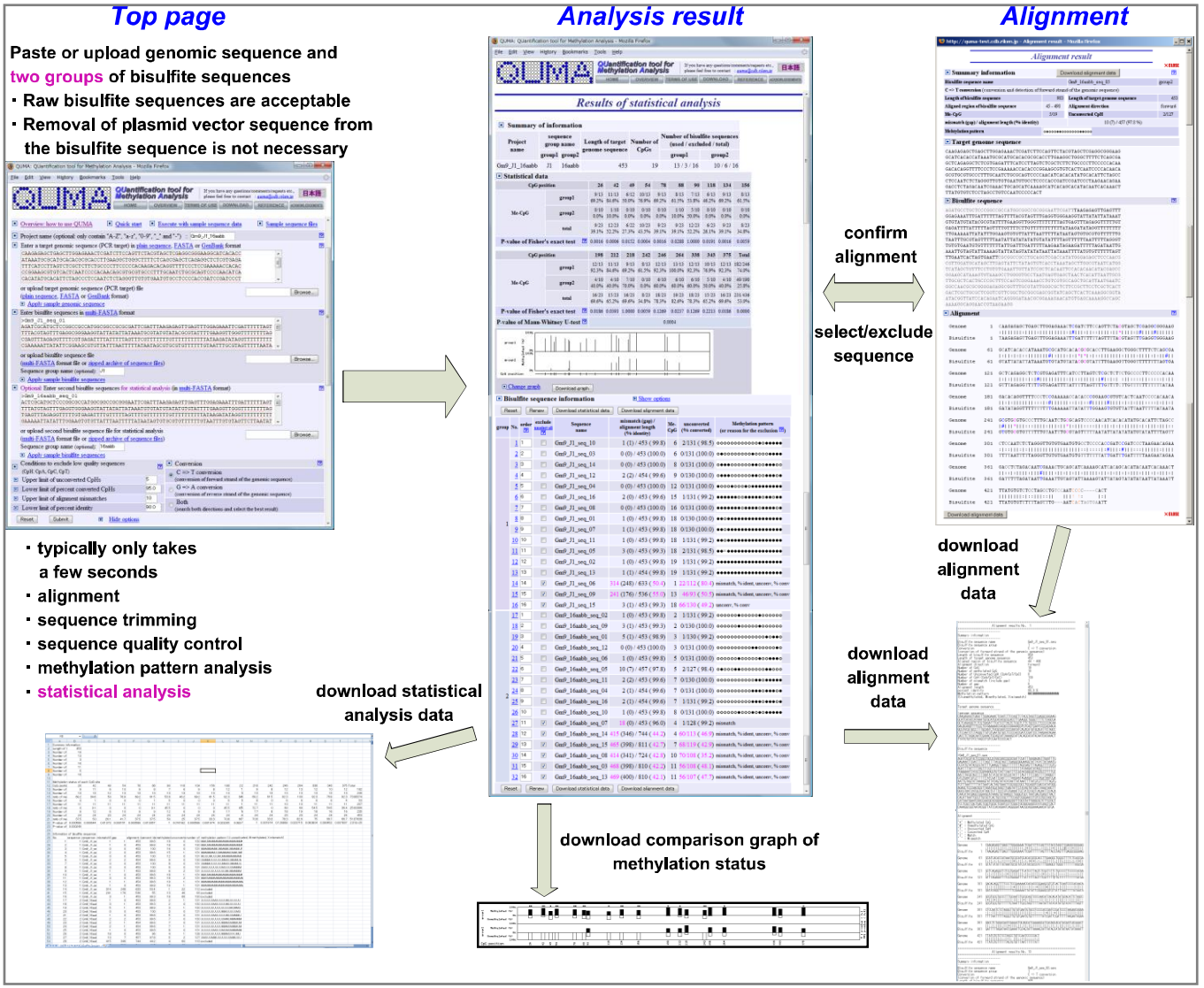


6. Statistical analysis mode

6.1. Main features

Differences from Methylation status analysis mode are listed below.

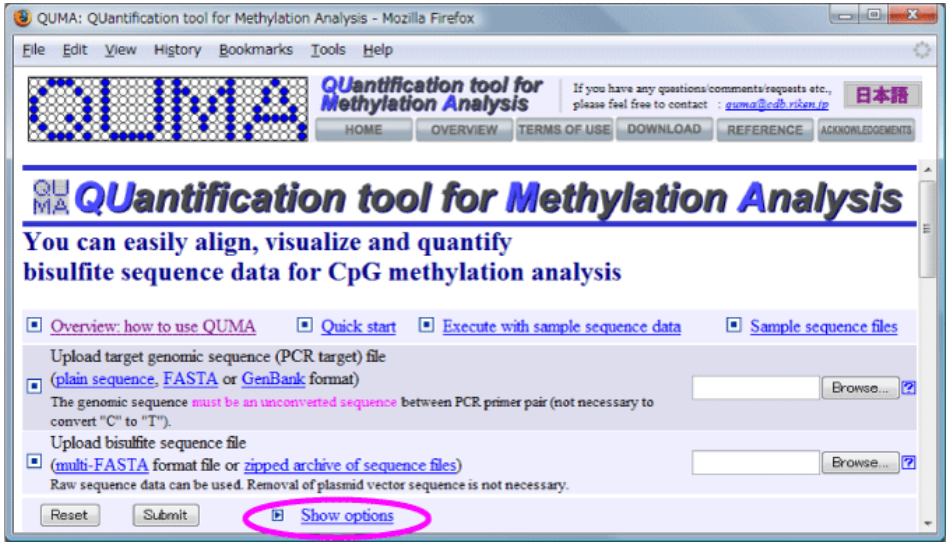
- **The target genomic sequence and two groups of bisulfite sequences are necessary for input data.**
- **Figure of comparative methylation status is shown.**
- **The statistical significance of the difference between two bisulfite sequence groups at each CpG site is evaluated with 9.1. Fisher's exact test.**
- **The statistical significance between two groups of the entire set of CpG sites is evaluated with 9.2. Mann-Whitney U-test.**



6.2. Top page

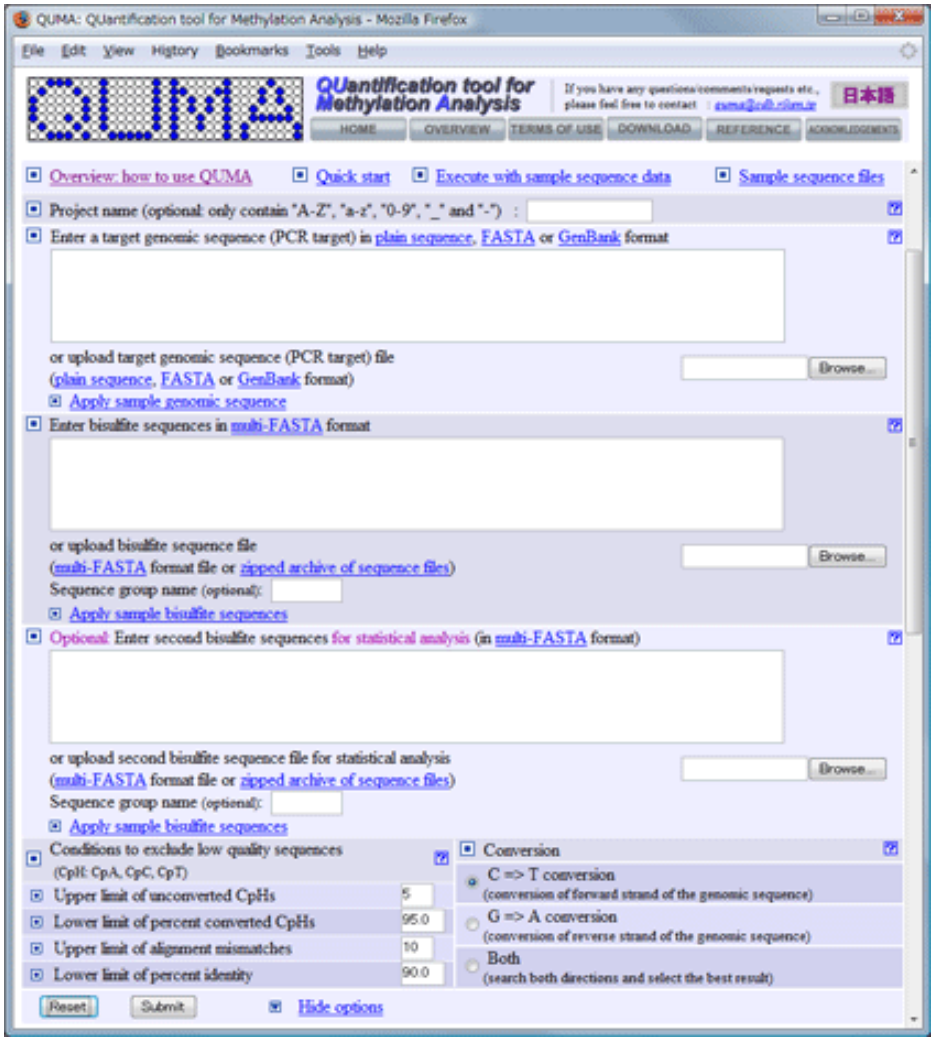
6.2.1. Show options

Click the "Show options" link to show optional fields.



6.2.2. Optional fields

Optional fields will appear.

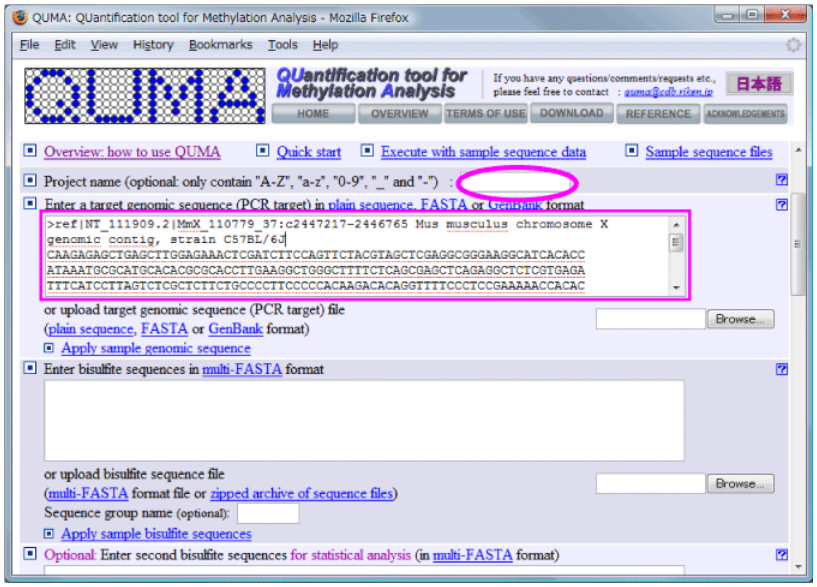


6.2.3. Genomic sequence

Input a project name (optional). When the project name is presented, it will be included in the output file name.

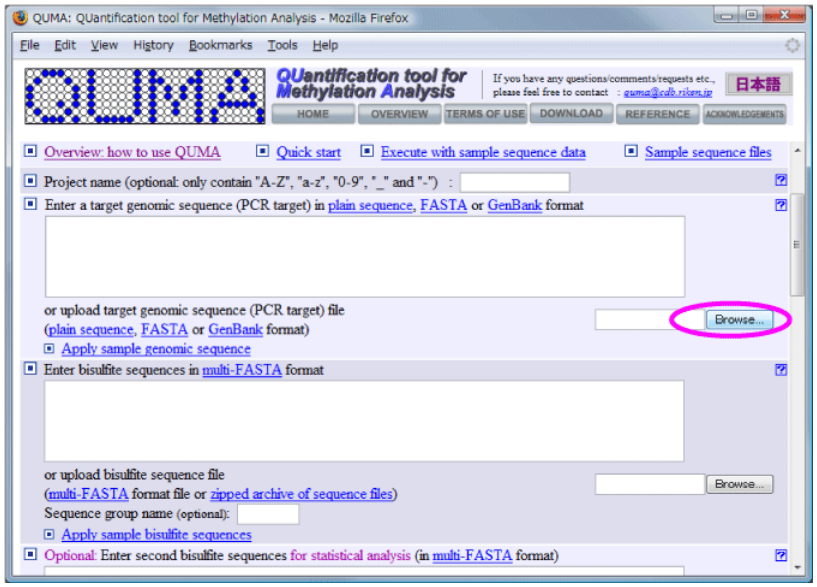
The target genomic sequence can be input by two ways of 1) direct input and 2) upload.

1) In case of direct input, paste a target genomic sequence (8.1. Plain sequence, 8.2. FASTA or 8.3. GenBank format). See also "7.1. Genomic sequence".



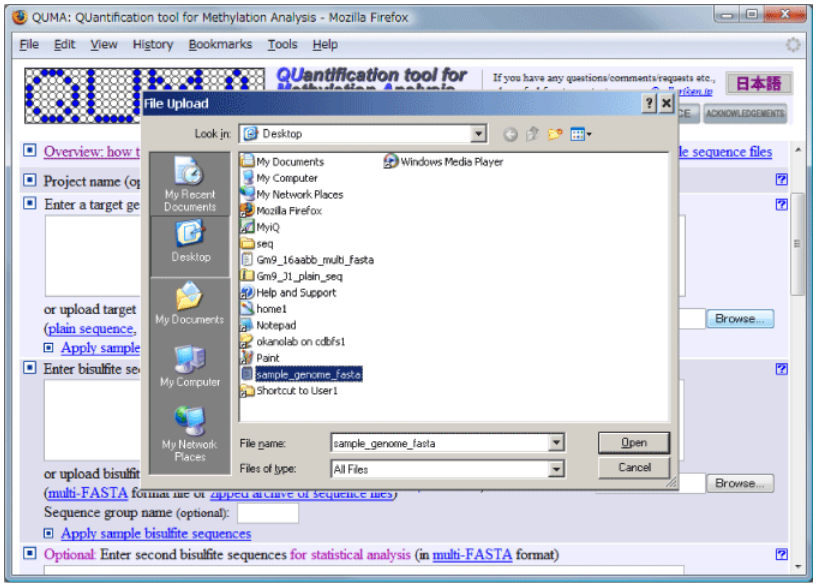
6.2.4. Genomic sequence file 1

2) Or click the first button (in this case "Browse..." button) to upload a target genomic sequence file.



6.2.5. Genomic sequence file 2

Select a target genomic sequence file to upload. See also "7.1. Genomic sequence".

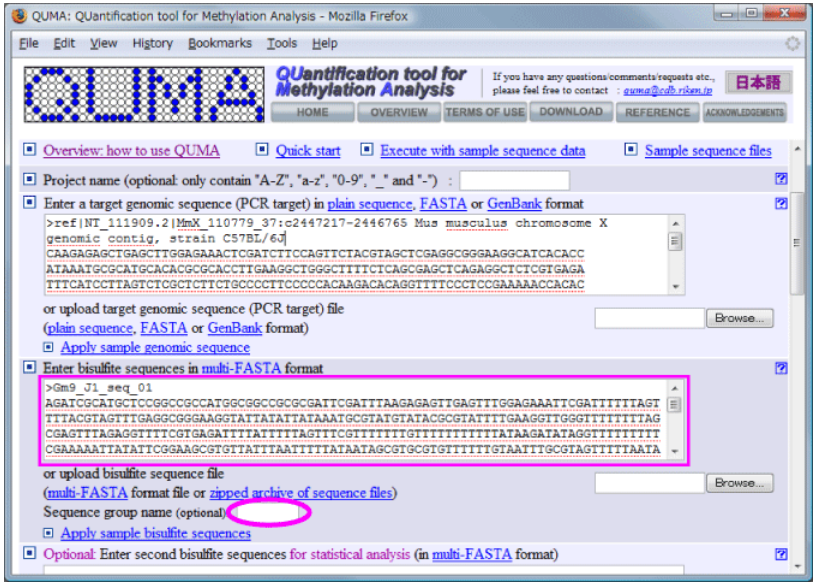


6.2.6. First bisulfite sequence group

Input a group name of first bisulfite sequence group (optional).

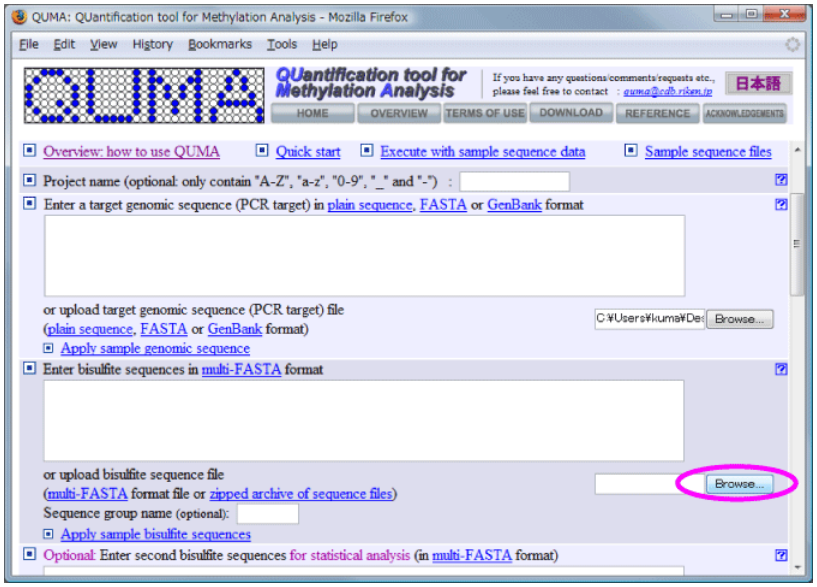
The bisulfite sequences can be input by two ways of 1) direct input and 2) upload.

1) In case of direct input, paste the bisulfite sequences (8.4. Multi-FASTA format). See also "7.2. Bisulfite sequences".



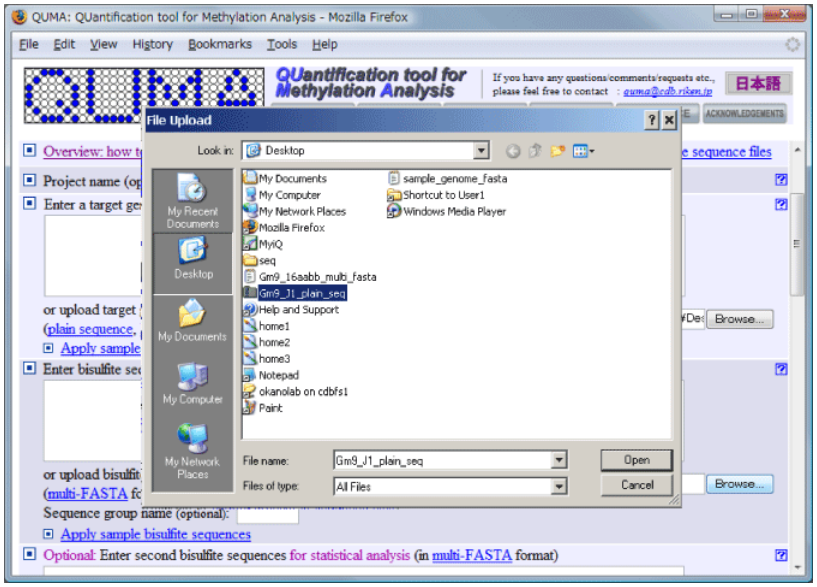
6.2.7. File of first bisulfite sequence group 1

2) Or click the second button to upload a file of bisulfite sequences of first group.



6.2.8. File of first bisulfite sequence group 2

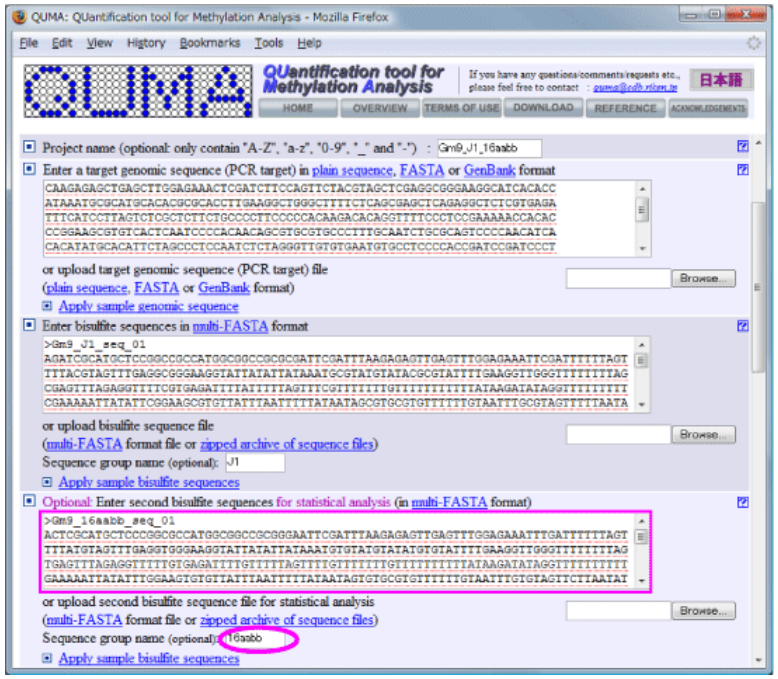
Select a file of bisulfite sequences of first group. Acceptable file formats are 8.4. Multi-FASTA or 8.5. Zipped archive of sequence files. See also "7.2. Bisulfite sequences", "8.6. How to create zipped archive (Macintosh)" and "8.7. How to create zipped archive (Windows)".



6.2.9. Second bisulfite sequence group

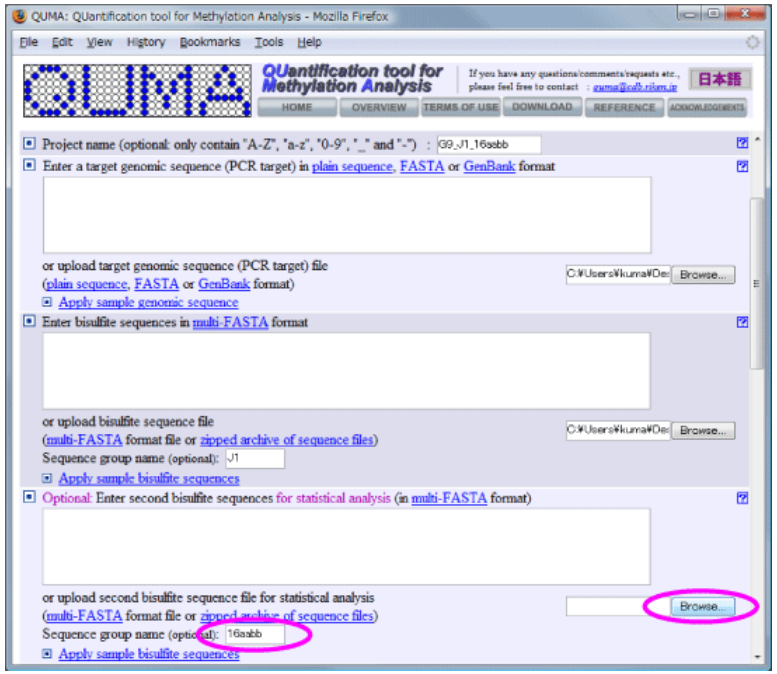
Input a group name of second bisulfite sequence group (optional).
Then, input the bisulfite sequences of second group.

1) In case of direct input, paste the bisulfite sequences of second group. The sequence format of the second group is same as the first group.



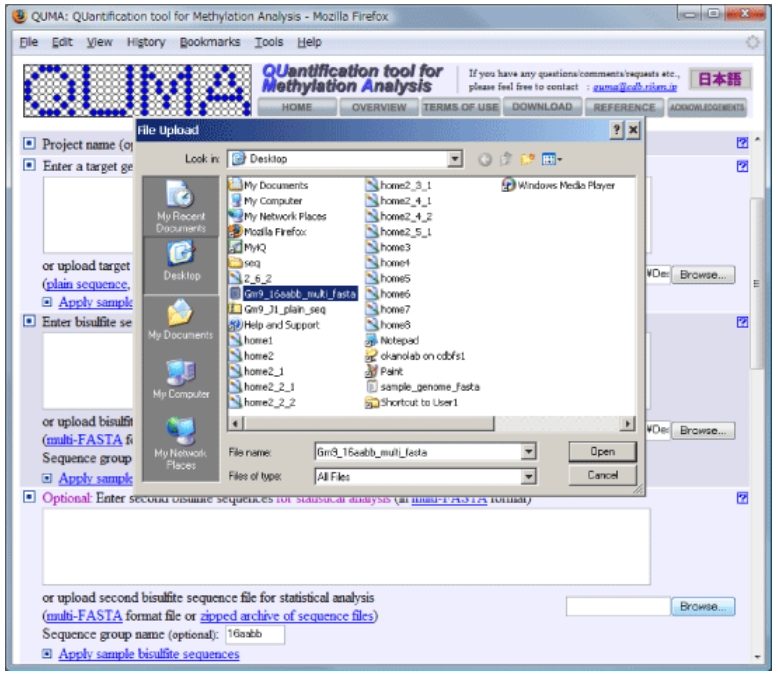
6.2.10. File of second bisulfite sequence group 1

2) Or click the third button to upload a file of bisulfite sequences of second group.



6.2.11. File of second bisulfite sequence group 2

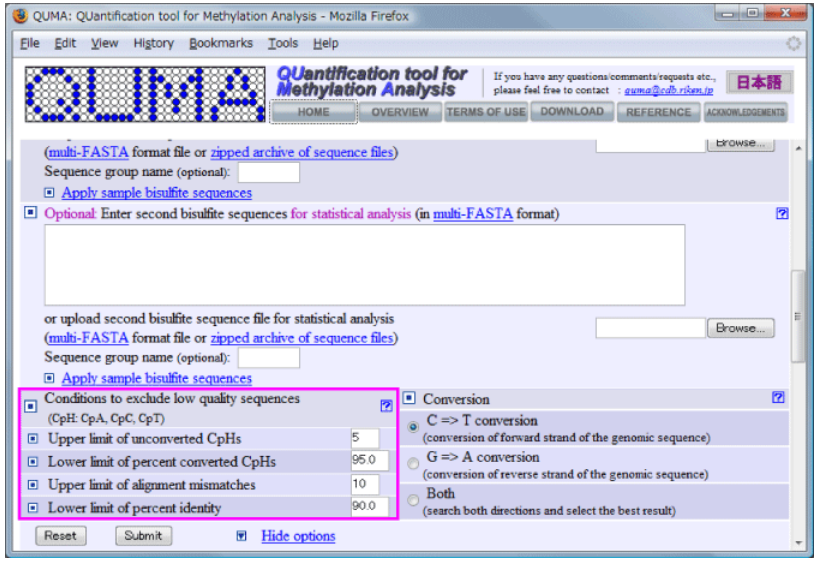
Select a file of bisulfite sequences of second group. The sequence file format of the second group is same as the first group.



6.2.12. Conditions to exclude bisulfite sequences

If you want, change conditions to exclude low quality bisulfite sequences.

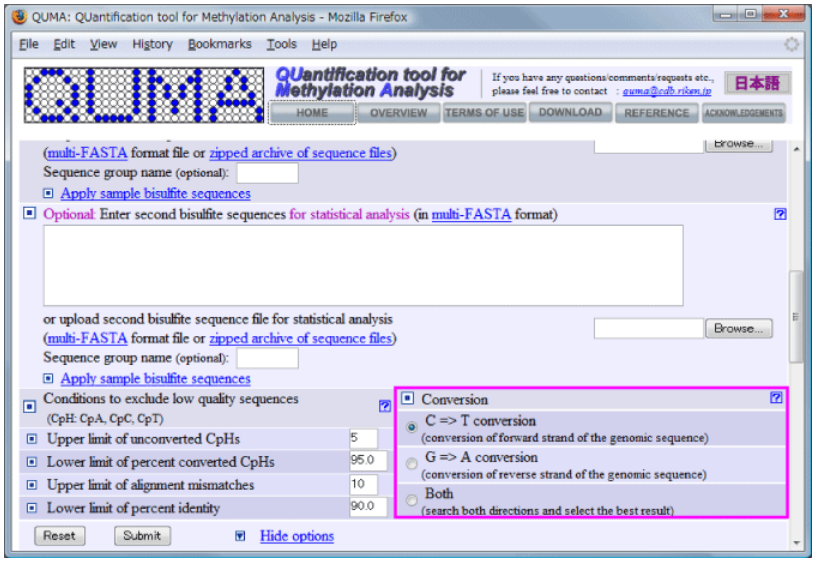
- **Upper limit of unconverted CpHs**
  - ✓ **number of unconverted CpHs (CpA, CpC and CpT)**
- **·Lower limit of percent converted CpHs**
  - ✓ **percent of "number of converted CpHs"/"number of CpHs"**
- **Upper limit of alignment mismatch**
  - ✓ **number of alignment mismatches and gaps between genomic and bisulfite sequences**
- **Lower limit of percent identity**
- **percent of alignment identity between genomic and bisulfite sequences**

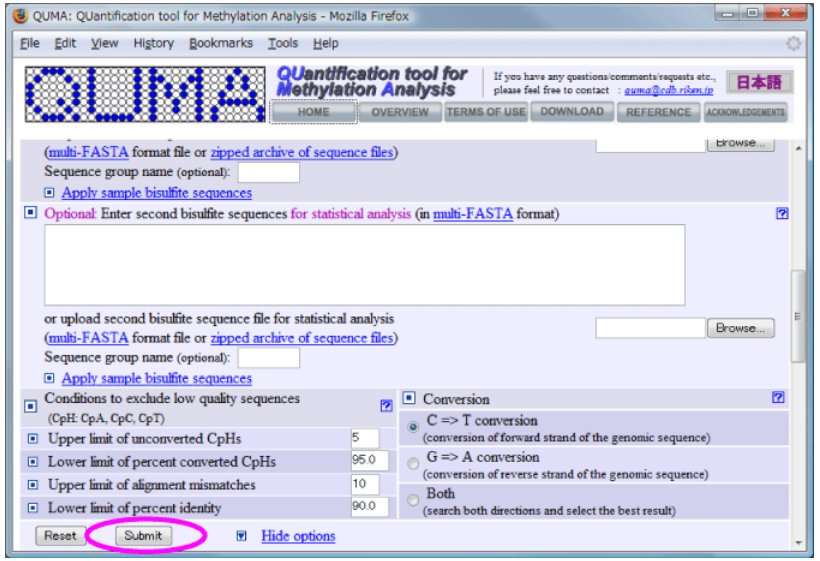


6.2.13. Strand of bisulfite conversion

Select a strand of bisulfite conversion of the target genomic sequence.

- **C=>T conversion:**
  - ✓ **When bisulfite PCR primer pair was designed for forward strand of the genomic sequence (default).**
- **G=>A conversion**
  - ✓ **When bisulfite PCR primer pair was designed for reverse strand of the genomic sequence.**
- **Both**
  - ✓ **Search both direction of conversion and adopt more appropriate strand.**





6.2.14. Submit

Click the "Submit" button to analyze. Typically, only a few seconds are necessary.

6.3. Statistical analysis result page

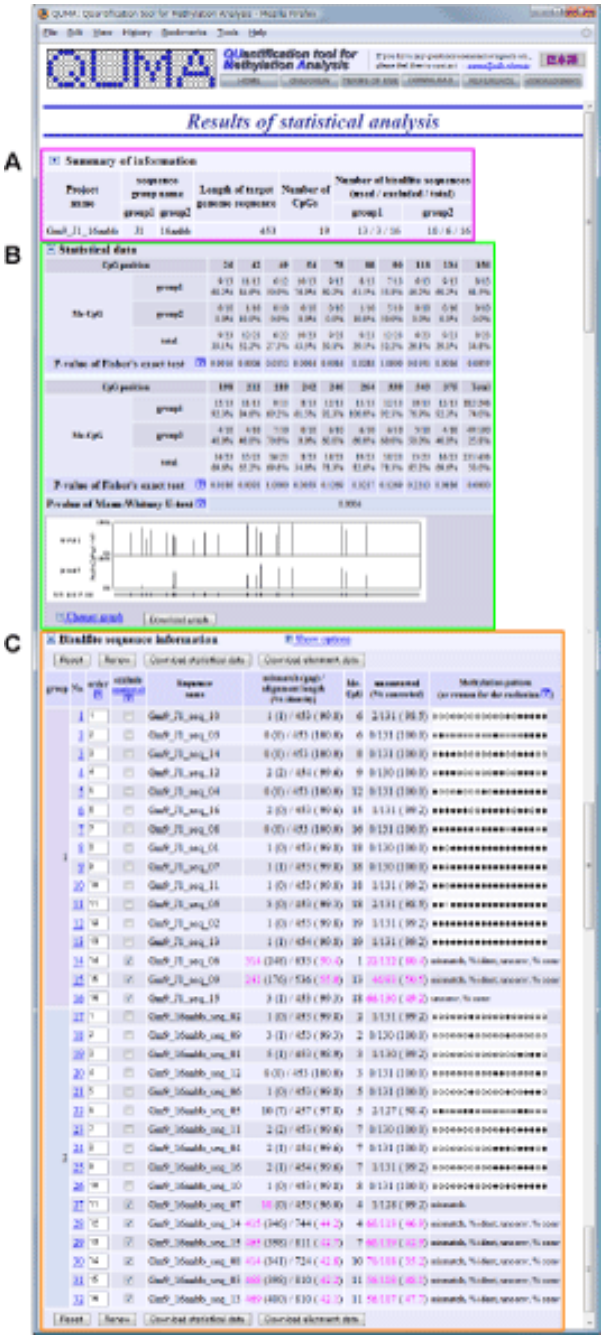
6.3.1. Overview of statistical analysis result page 1

Statistical analysis result page consists of three sections.

A) Summary of information

B) Statistical data

C) Information and methylation pattern of each bisulfite sequences



6.3.2. Overview of statistical analysis result page 2

A) **Summary of information**

Length of the target genome sequence, number of CpG sites and number of bisulfite sequences are indicated.

B) **Statistical data**

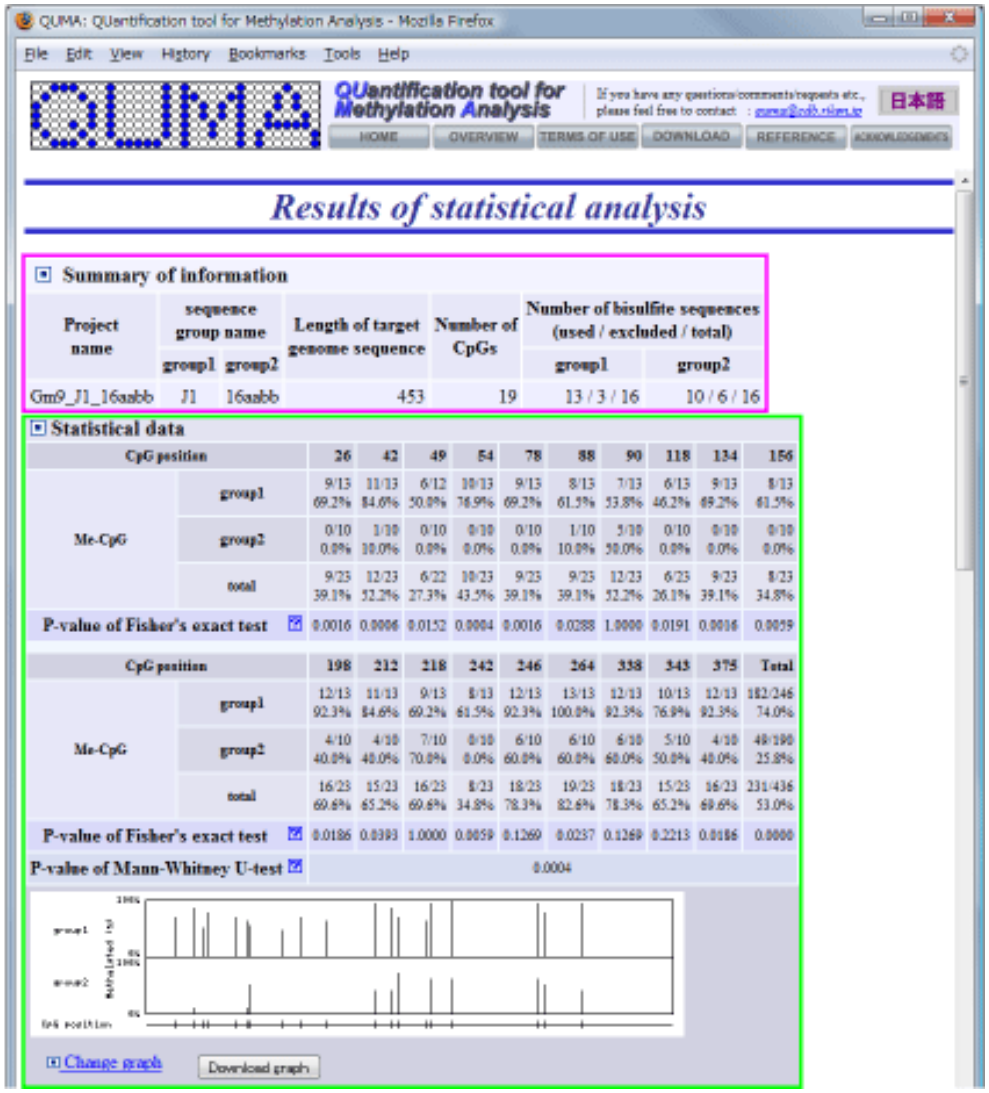
Position of CpG sites, methylation status of each CpG sites and statistical significances (P-value) of difference between two bisulfite sequence groups are shown.

Fisher's exact test: The statistical significance of the difference between two bisulfite sequence groups at each CpG site is evaluated with Fisher's exact test that is non-parametric statistical significance test to determine if there are nonrandom associations between two categorical data. See "9.1. Fisher's exact test" for more detail.

Mann-Whitney U-test: The statistical significance between two groups of the entire set of CpG sites is evaluated with the Mann-Whitney U-test (also called the Wilcoxon rank-sum test) that is non-parametric statistical significance test for two distributed samples. See "9.2. Mann-Whitney U-test" for more detail.

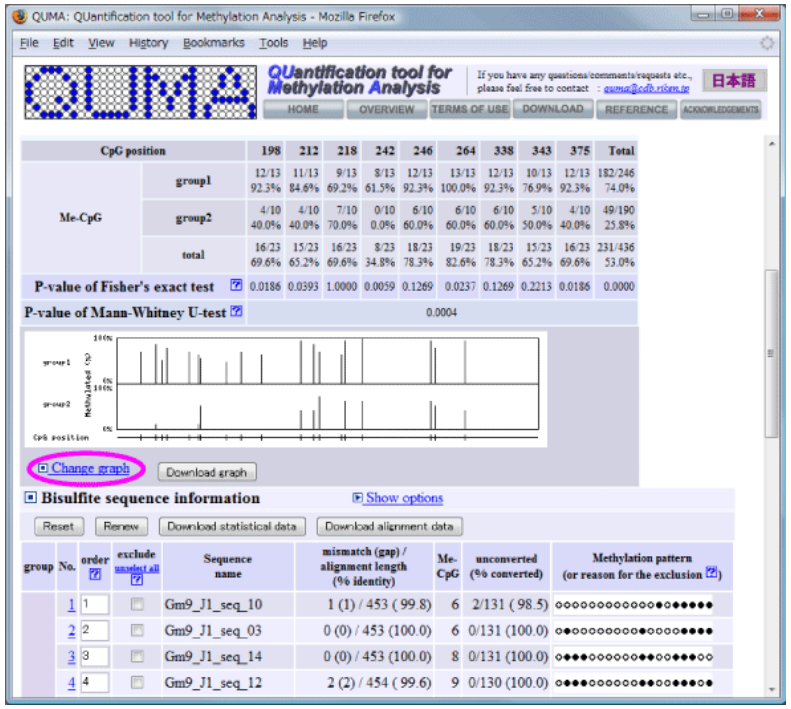
As a limitation of both tests, CpG methylation pattern is not considered and allele specific CpG methylation pattern, especially for imprinting locus, is not detectable.

Figure of comparative methylation status is also shown.



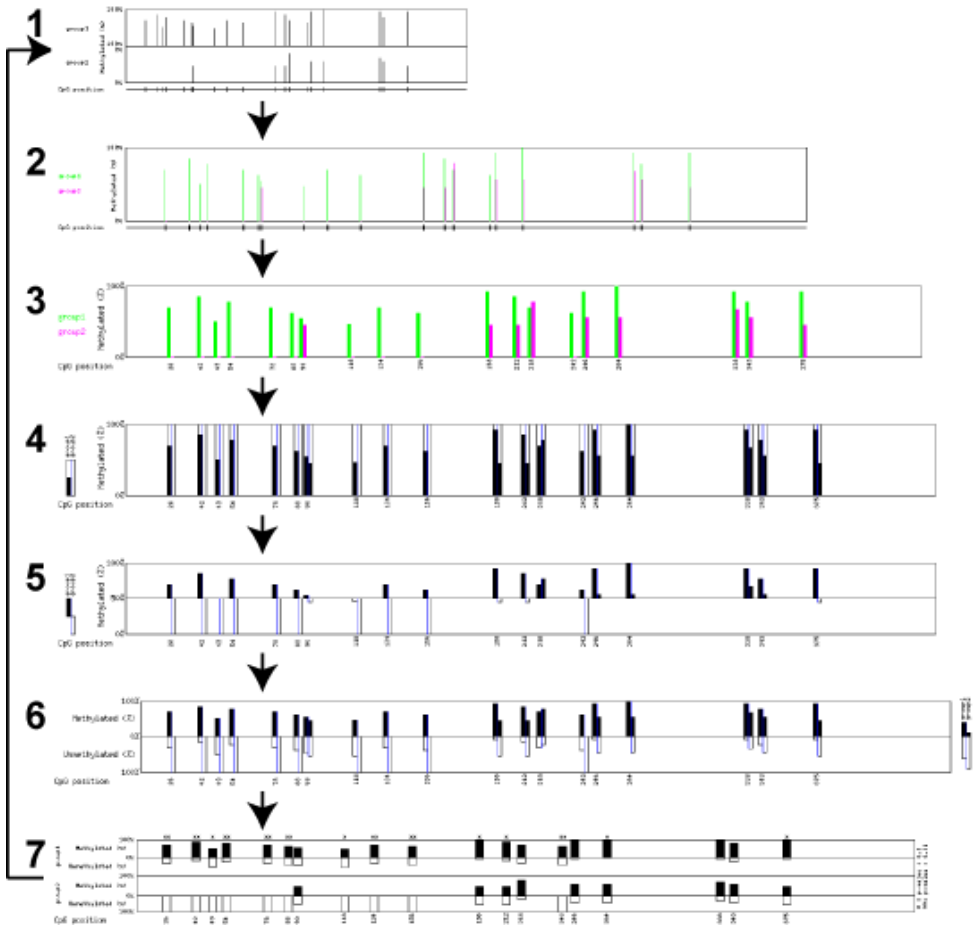
6.3.3. Change methylation status figure 1

Click "Change graph" link to switch comparative methylation status figures.



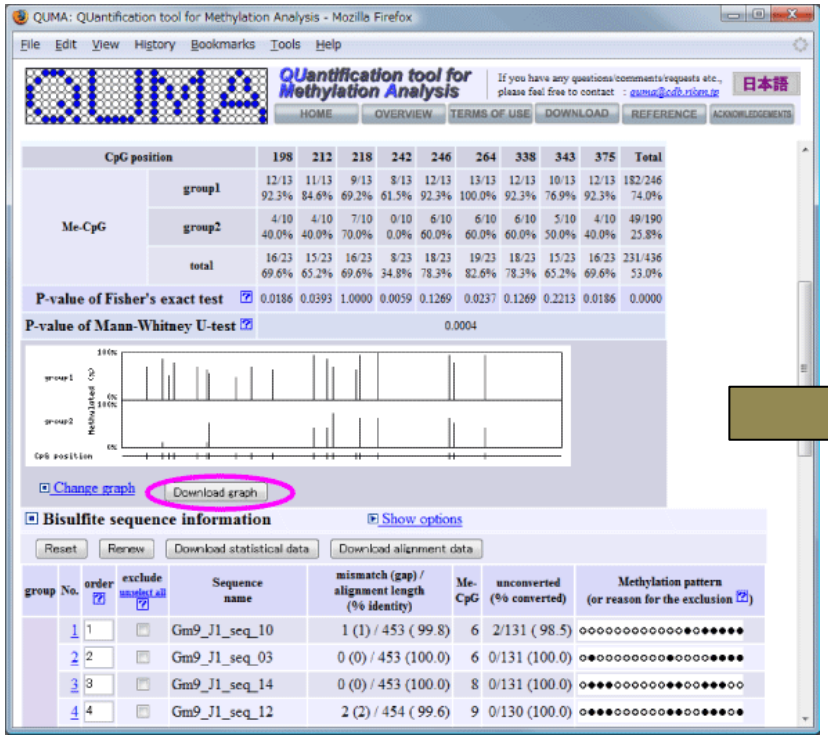
6.3.4. Change methylation status figure 2

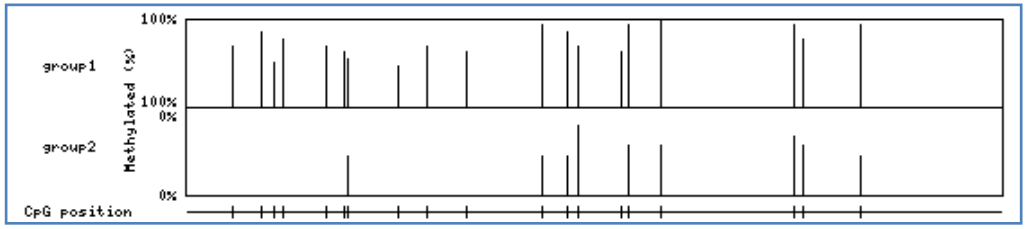
Comparative methylation status figures are switched one after the other by clicking "Change graph" link. Figures 1 and 2 are reflected the position of CpG sites almost accurately. Figures 3-7 are not reflected accurately.



6.3.5. Download comparative methylation status figure

Click "Download graph" button to download the comparative methylation status figure which displayed at that time.



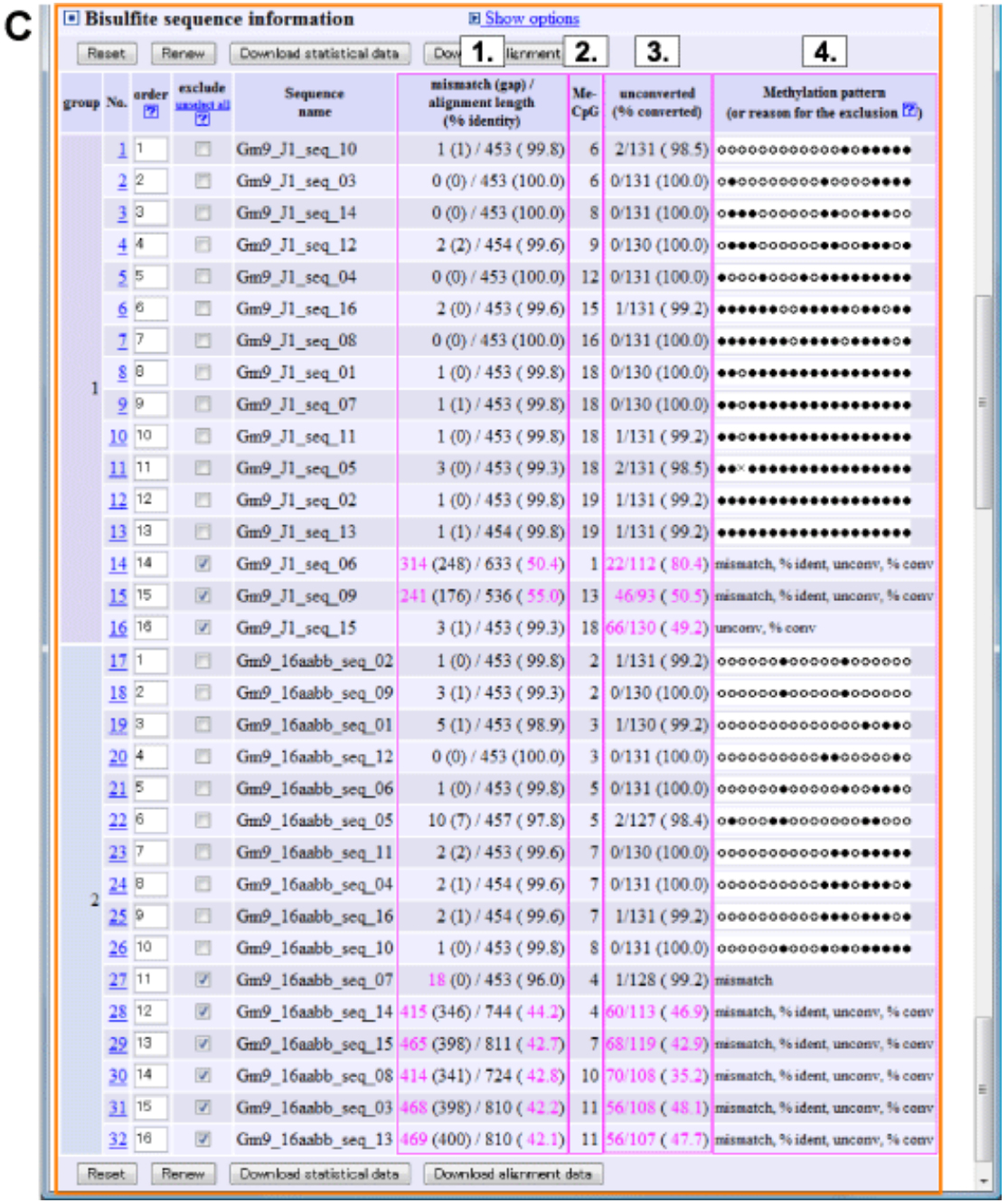


6.3.6. Overview of statistical analysis result page 3

C) . Information and methylation pattern of each bisulfite sequences.
Two sequence groups are indicated separately.

1. Number of mismatches and percent identity of bisulfite alignment
2. Number of methylated CpG sites
3. Number of bisulfite unconverted CpHs (CpA, CpC, CpT)
4. Pattern of CpG methylation (Black circle: methylated, White circle: unmethylated, Cross: mismatch or gap)

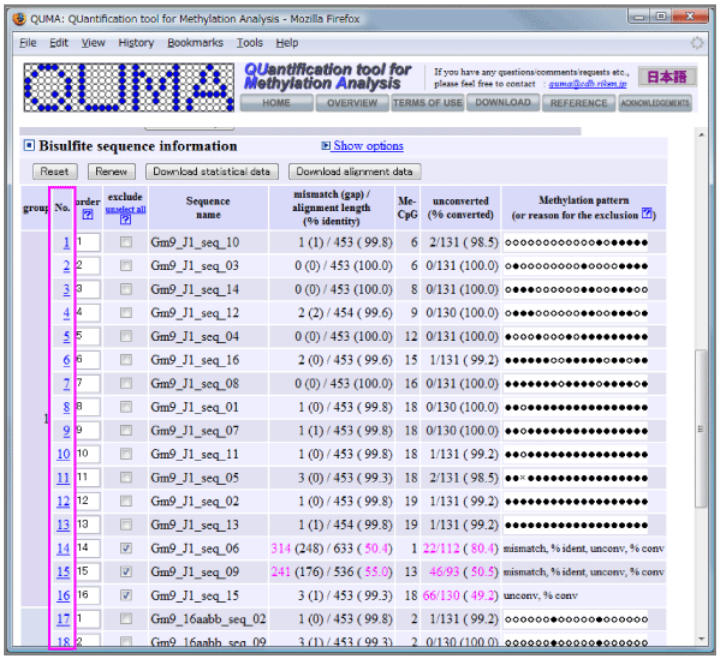
Methylation pattern (4.) is not present when quality of bisulfite sequence is low or excluded from user. Low quality value is shown as magenta. When excluded, reason(s) for the exclusion will be indicated at methylation pattern column (4.). Conditions to exclude low quality bisulfite sequences can be changed (See "5.6.1. Show options 1" for more detail).



- **mismatch:**
  - ✓ **The number of alignment mismatches (includes gaps) between genomic and bisulfite sequences exceeded the upper limit (default: 10).**
  - ✓ **This means low quality sequence read.**
- **% ident**
  - ✓ **Percent of alignment identity between genomic and bisulfite sequences exceeded the lower limit (default: 90%).**
  - ✓ **This means low quality sequence read.**
- **Unconv**
  - ✓ **The number of unconverted CpHs (CpA, CpC and CpT) exceeded the upper limit (default: 5).**
  - ✓ **This means incomplete bisulfite conversion or low quality sequence read.**
- **% conv**
  - ✓ **Percent of "number of converted CpHs" / "number of CpHs" exceeded the lower limit (default 95%).**
  - ✓ **This means incomplete bisulfite conversion or low quality sequence read.**
- **user desired**
  - ✓ **Sequence was excluded by checking on the "exclude" checkbox.**

6.3.7. Show alignment

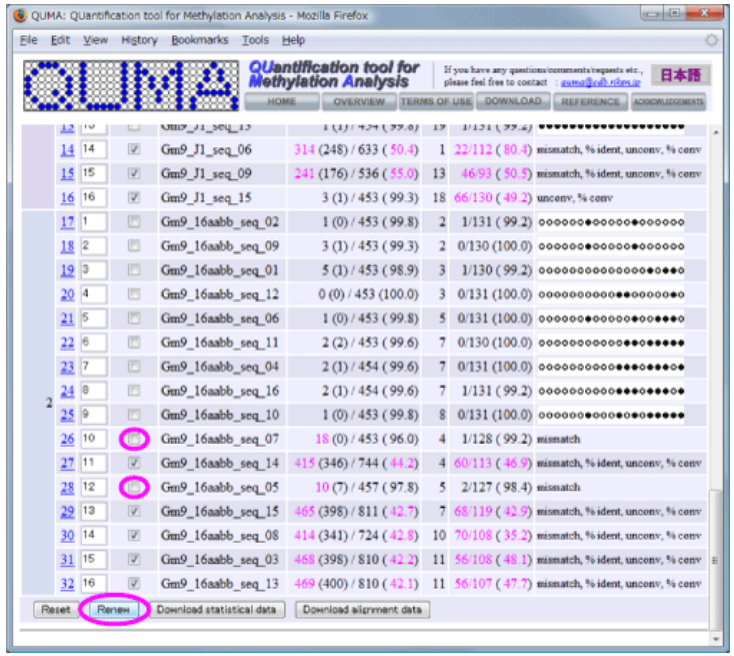
Click links to show bisulfite alignment between bisulfite sequence to genomic sequence.



See "6.5. Alignment page" for next step.

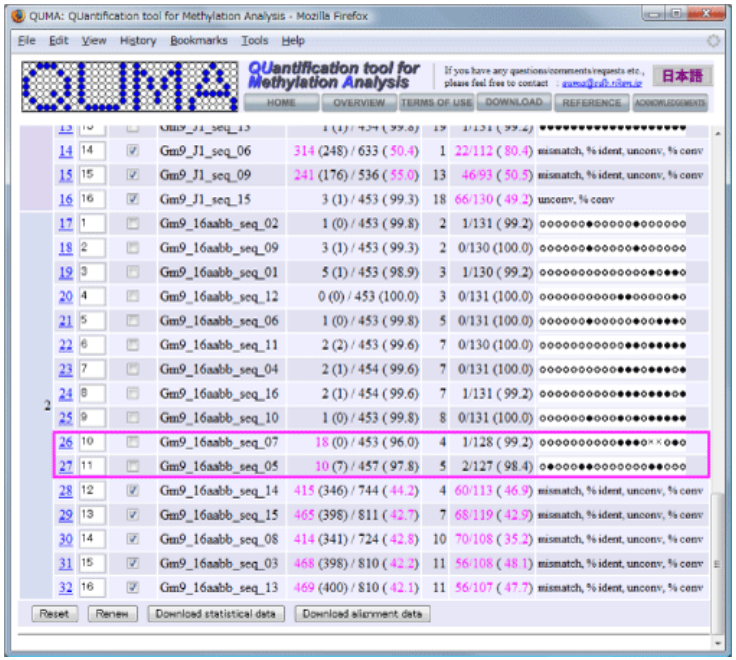
6.3.8. Include/exclude bisulfite sequence 1

To include/exclude a bisulfite sequence, check off/on "exclude" checkbox. Then click "Renew" button. To include all bisulfite sequence information, click "unselect all" link.



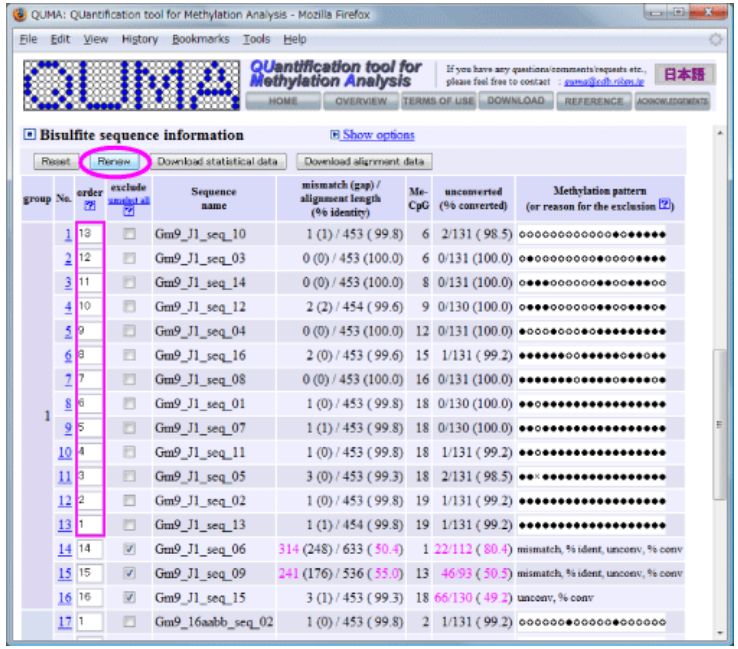
6.3.9. Include/exclude bisulfite sequence 2

The change is reflected.



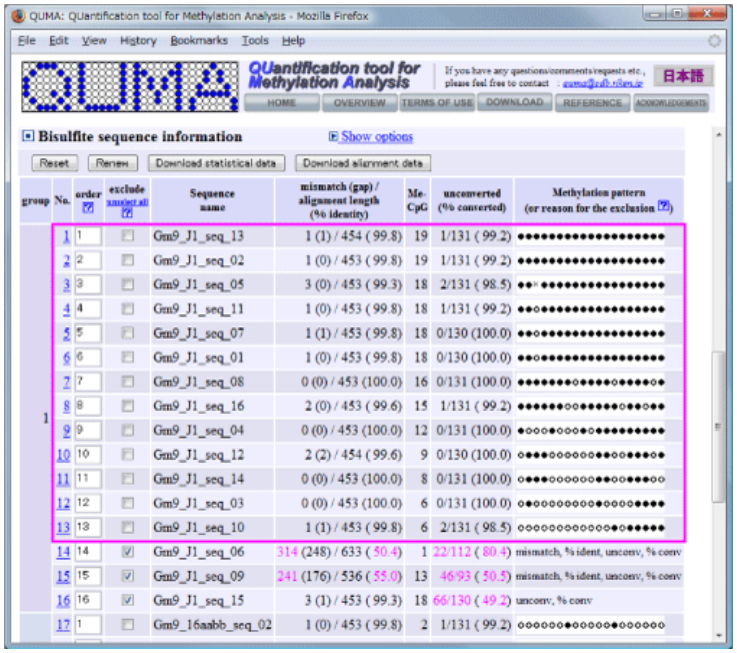
6.3.10. Change the order of bisulfite sequences 1

Change the value of "order" column to desired order. Then click "Renew" button.



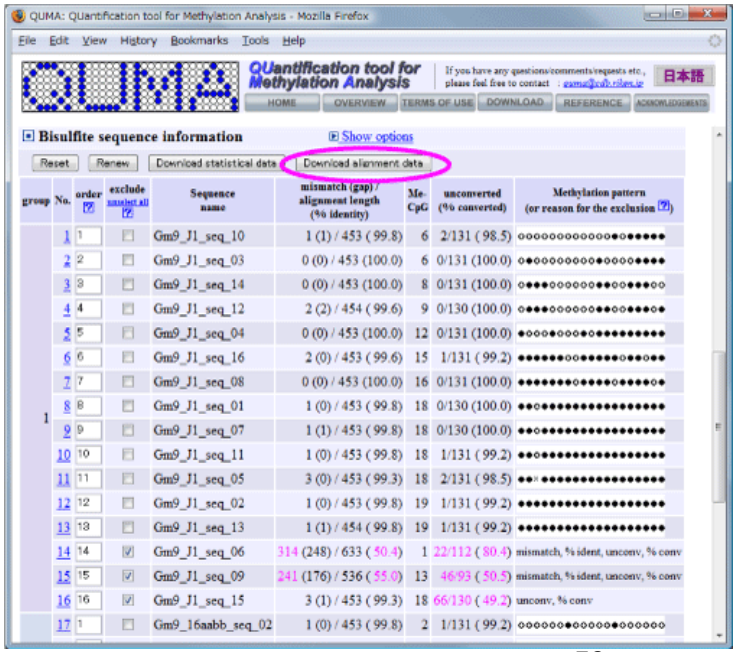
6.3.11. Change the order of bisulfite sequences 2

The change is reflected. Two sequence groups are ordered separately.



6.3.12. Download alignments data

Click "Download alignment data" button to download alignments data.

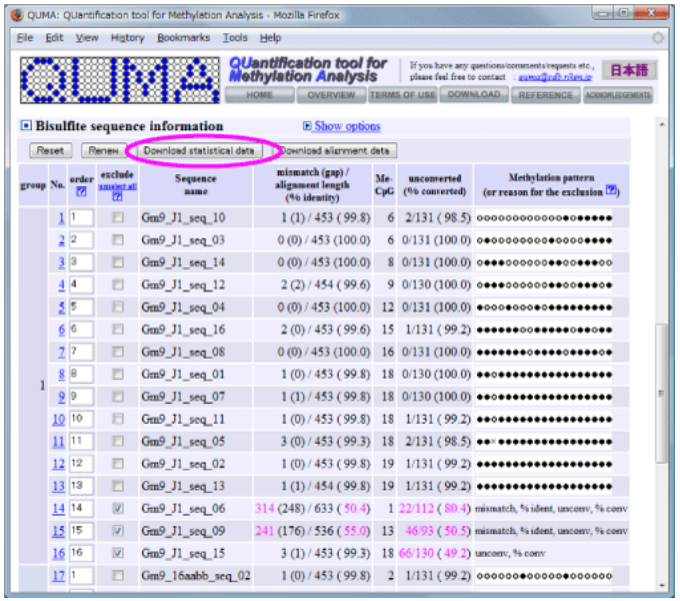


6.3.13. Alignments data

Downloaded alignments data file can be opened by TextEdit (Mac), Notepad (Win) or other text editors.

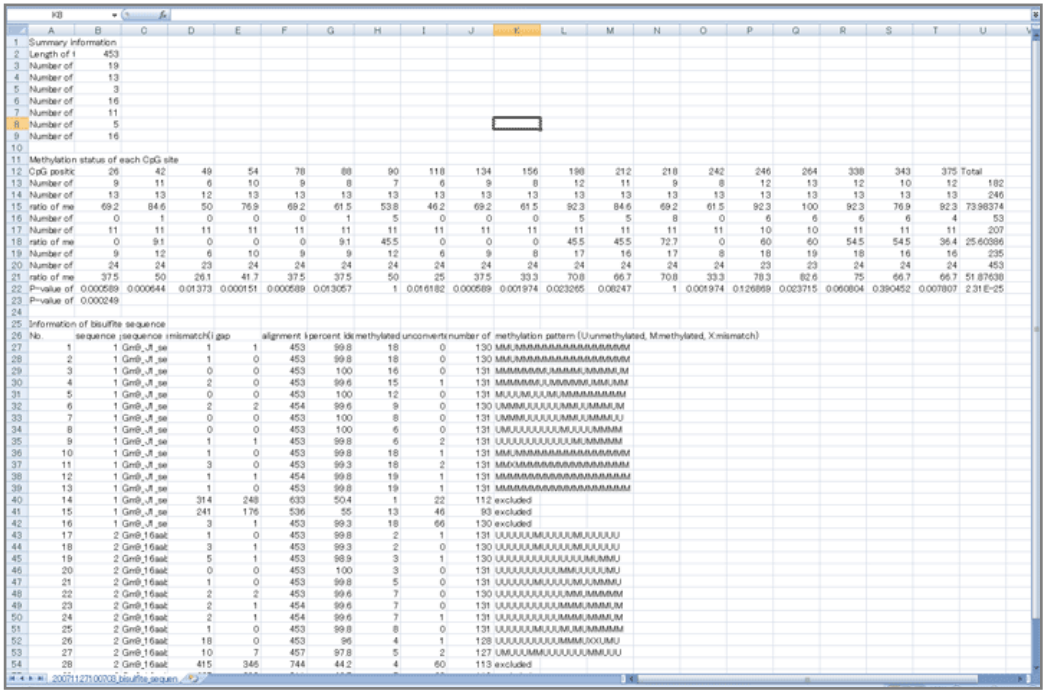
6.3.14. Download statistical analysis data

Click "Download statistical data" button to download statistical analysis data.



6.3.15. Statistical analysis data

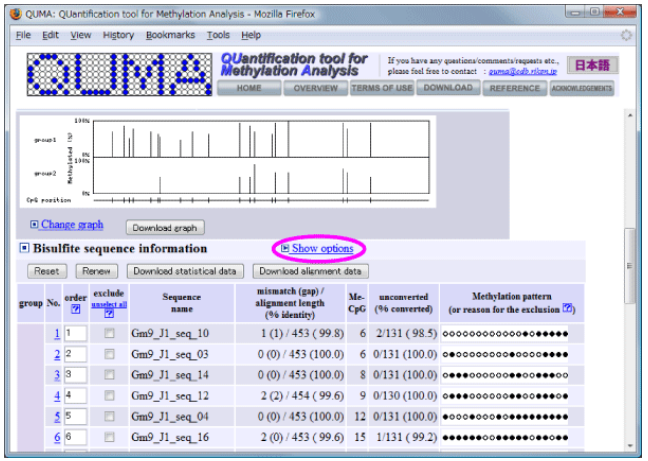
Downloaded statistical analysis data file can be opened by Microsoft Excel, OpenOffice/StartSuite or other spreadsheet software (CSV file format). See also "10.1. How to open a CSV file".



6.4. Statistical analysis result page options

6.4.1. Show options 1

Click the "Show options" link to show optional fields (right top figure).

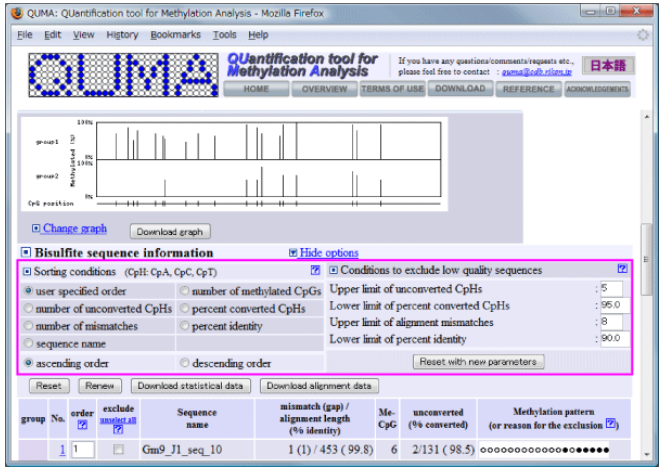


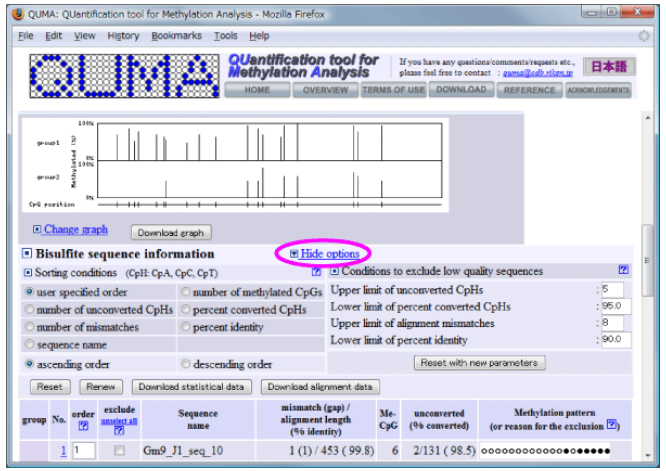
6.4.2. Show options 2

Optional fields will appear (left bottom figure).

6.4.3. Hide options

Click the "Hide options" link to hide optional fields (right bottom figure).

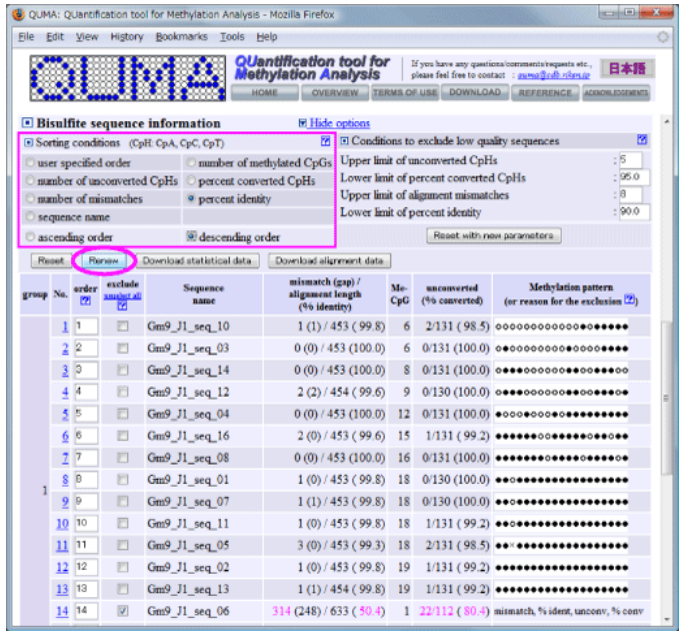


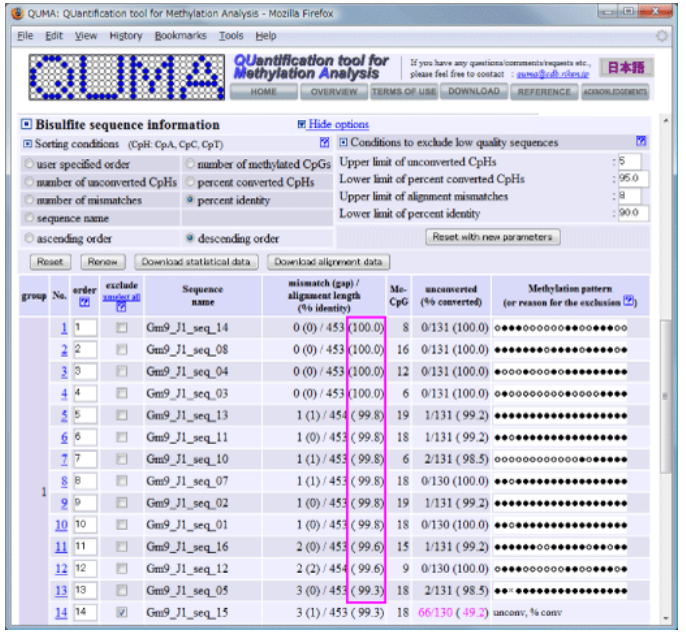


6.4.4. Change the order of bisulfite sequences 1

Order of bisulfite sequences can be changed by several parameters and ascending/descending order. Then click "Renew" button.

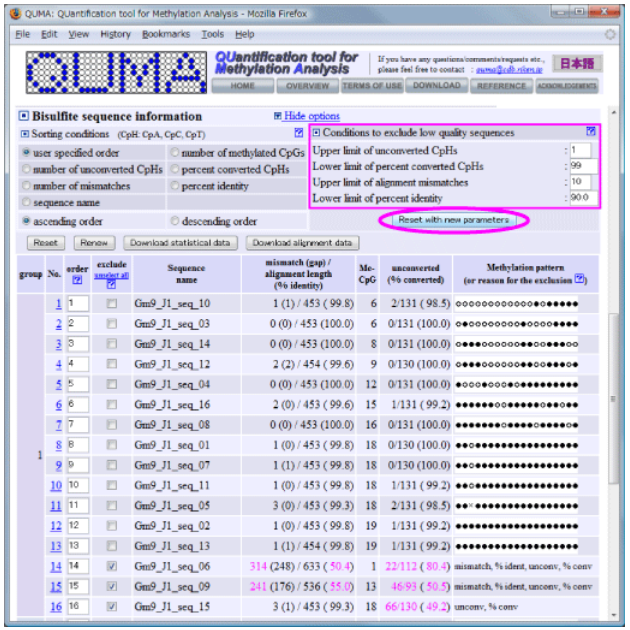
- **user specified order**
  - ✓ **The value of "order" column.**
- **number of methylated CpGs**
- **number of unconversions**
  - ✓ **unconverted CpHs (CpA, CpC, CpT)**
- **percent conversion**
  - ✓ **percent of converted CpHs / total CpHs**
- **number of mismatches**
- **percent identity**
- **sequence name**
- **ascending order**
- **descending order**

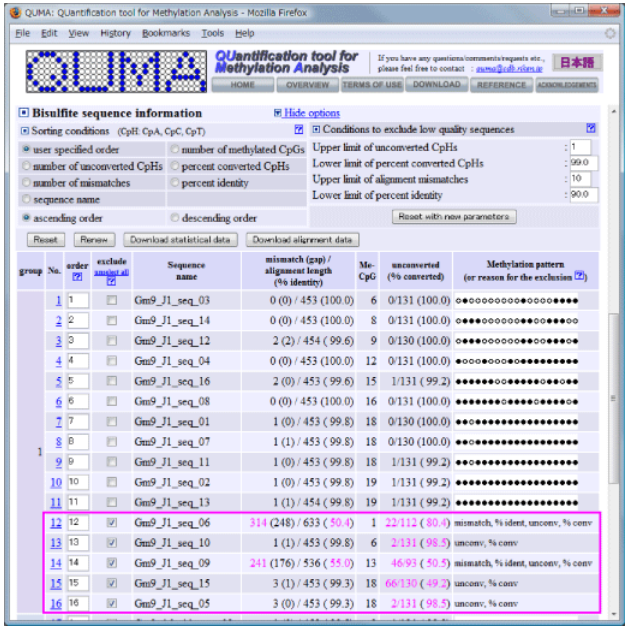




6.4.5. Change the order of bisulfite sequences 2

The change is reflected. Two sequence groups are ordered separately.





6.4.6. Conditions to exclude bisulfite sequences 1

Conditions to exclude low quality bisulfite sequences can be changed. Then click "Reset with new parameter" button (order and exclusion of bisulfite sequences will be reset).

- **Upper limit of unconversion**
  - ✓ **number of unconverted CpHs (CpA, CpC and CpT)**
- **Lower limit of percent conversion**
  - ✓ **percent of "number of converted CpHs"/"number of CpHs"**
- **Upper limit of alignment mismatch**
  - ✓ **number of alignment mismatches and gaps between genomic and bisulfite sequences**
- **Lower limit of percent identity**
  - ✓ **percent of alignment identity between genomic and bisulfite sequences**

6.4.7. Conditions to exclude bisulfite sequences 2

The change is reflected.

6.5. Alignment page

6.5.1. Overview of alignment page

Alignment page consists of four sections.

A) Summary of information

Information about bisulfite alignment.

B) Genome sequence

C) Bisulfite sequence

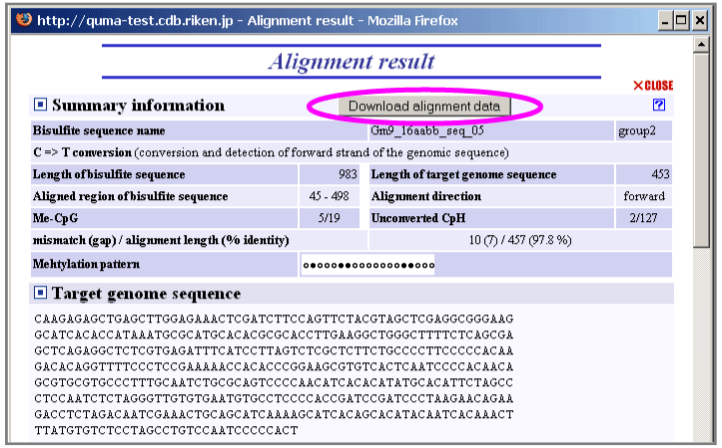
Sequence outside alignment is indicated as gray color.

D) Bisulfite alignment

Methylated C of CpG site, unmethylated C of CpG site, Unconverted C (CpA, CpC, CpT) are indicated as different colors.

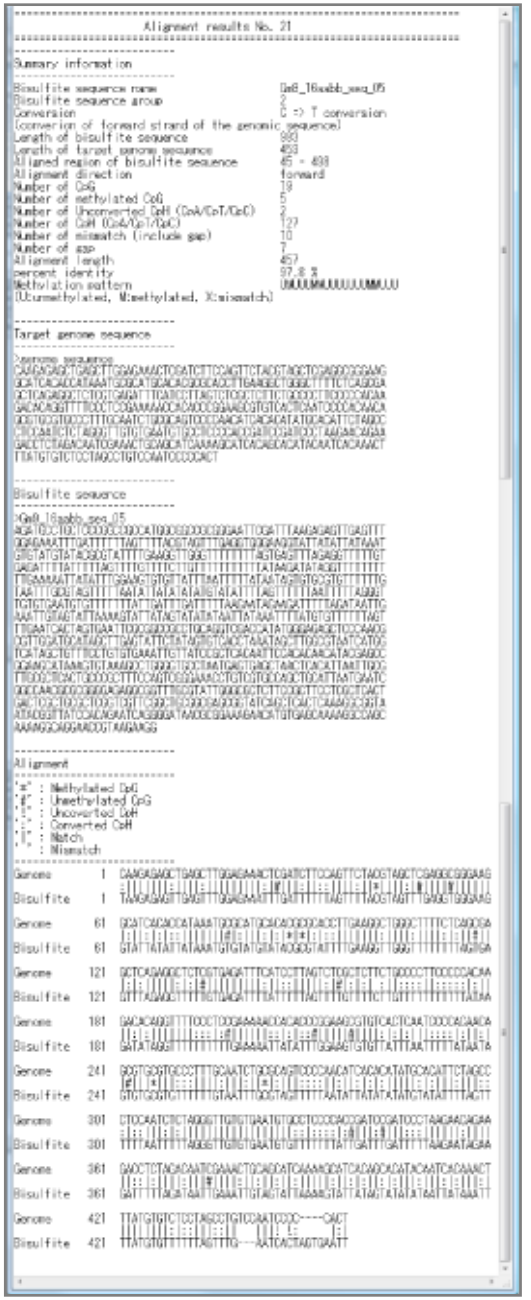
6.5.2. Download alignment data

Click "Download alignment data" button to download alignment data which displayed hear.



6.5.3. Alignment data

Downloaded alignment data file can be opened by TextEdit (Mac), Notepad (Win) or other text editors.



7. Input data

7.1. Genomic sequence

Select the genomic sequence file of target region to upload. Or paste the target genomic sequence into the text box (only for "5.4. Top page option"). The genomic sequence must be unconverted (not necessary to convert "C" to "T") and use sequence between PCR primer pair.

Sequence of 8.1. Plain sequence, 8.2. FASTA or 8.3. GenBank format is acceptable. Only rich text format (with ".rtf" file extension) or plain text format text file is acceptable for upload file. Binalry file (such as Microsoft Word file) is unacceptable.

Rich text format file can be created with TextEdit (Macintosh), WordPad (Windows) or many word processors. Plain text file can be created with TextEdit (Macintosh), NotePad (Windows), many word processors or text editors.

7.2. Bisulfite sequences

Select the file of bisulfite sequences to upload (8.4. Multi-FASTA format file or 8.5. Zipped archive of sequence files). Or paste the 8.4. Multi-FASTA format bisulfite sequences into the text box (only for "5.4. Top page option"). The bisulfite sequences outputted from DNA sequencer can be used as input sequences. No need to remove plasmid vector sequence.

Only rich text format (with ".rtf" file extension) or plain text format text file is acceptable for multi-FASTA upload file.

Rich text format file can be created with TextEdit (Macintosh), WordPad (Windows) or many word processors. Plain text file can be created with TextEdit (Macintosh), NotePad (Windows), many word processors or text editors.

8. Sequence format

8.1. Plain sequence format

Plain sequence contains only sequence characters and line feed. (Only one sequence can contain in one file.)

ex.

```
CAGTCCGGCAGCGCCGGGGTTAAGCGGCCCAAGTAAACGTAGCGCAGCGA
TCGGCGCCGGAGATTCGCGAACCCGACACTCCGCGCCGCCCGCCGGCCAG
GACCCGCGGCGCGATCGCGGCGCCGCGCTACAGCCAGCCTCACTGGCGCG
CGGGCGAGCGCACGGGCGCTC
```

8.2. FASTA format

Sequence of FASTA format is started from single comment line and followed by lines of sequence. A greater-than (">") symbol is used at the first character of comment line to distinguish from sequence lines.

See more detail about FASTA format (Wikipedia)

ex.

```
>Dnmt3a partial sequence
ACTCCCCGTGCGCGCCCGGCCCGTAGCGTCCTCGTCGCCGCCCCTCGTCT
CGCAGCCGCAGCCCGCGTGGACGCTCTCGCCTGAGCGCCGCGGACTAGCC
CGGGTGGCCCACTGGCGCGCGGGCGAGCGCACGGGCGCTCCAGTCCGGCA
GCGCCGGGGTTAAGCGGCCCAAGTAAACGTAGCGCAGCGATCGGCGCCGG
AGATTCGCGAACCCGACACTCCGCGCCGCCCGCCGGCCAGGACCCGCGGC
GCGATCGCGGCGCCGCGCTACAGCCAGCCTCACGACAGGCCCGCTGAGGC
TTGTGCCAGACCTTGGAAACCTCAGGTATATACCTTTCCAGACGCGGGAT
CTCCCCTCCCCCATCCATAGTGCCTTGGGACCAAATCCAGGGCCTTCTTT
CAGGAAACAATGAAGGGAGACAGCAGACATCTGAATGAAGAAGAGGGTGC
CAGCGGGTATGAGGAGTGCATTATCGTTAATGGGAACTTCAGTGACCAGT
CCTCAGACACGAAGGATGCTCCCTCACCCCCAGTCTTGGAGGCAATCTGC
ACAGAGCCAGTCTGCACACC
```

8.3. GenBank format

GenBank format (GenBank Flat File Format) consists of annotation section and sequence section. The start of annotation section is marked by a line beginning the word "LOCUS". The start of sequence section is marked by a line beginning the word "ORIGIN" and the end of the section is marked by line only contains "//".

See more detail about GenBank format (NCBI)

ex.

```
LOCUS       AF068625               200 bp    mRNA    linear   ROD 06-DEC-1999
DEFINITION  Mus musculus DNA cytosine-5 methyltransferase 3A (Dnmt3a) mRNA,
            complete cds.
ACCESSION   AF068625 REGION: 1..200
VERSION     AF068625.2  GI:6449467
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 200)
  AUTHORS   Okano,M., Xie,S. and Li,E.
  TITLE     Cloning and characterization of a family of novel mammalian DNA
            (cytosine-5) methyltransferases
  JOURNAL   Nat. Genet. 19 (3), 219-220 (1998)
   PUBMED   9662389
REFERENCE   2  (bases 1 to 200)
  AUTHORS   Xie,S., Okano,M. and Li,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-MAY-1998) CVRC, Mass. Gen. Hospital, 149 13th Street,
            Charlestown, MA 02129, USA
REFERENCE   3  (bases 1 to 200)
  AUTHORS   Okano,M., Chijiwa,T., Sasaki,H. and Li,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-NOV-1999) CVRC, Mass. Gen. Hospital, 149 13th Street,
            Charlestown, MA 02129, USA
  REMARK    Sequence update by submitter
COMMENT     On Nov 18, 1999 this sequence version replaced gi:3327977.
FEATURES             Location/Qualifiers
     source          1..200
                     /organism="Mus musculus"
                     /mol_type="mRNA"
                     /db_xref="taxon:10090"
                     /chromosome="12"
                     /map="4.0 cM"
     gene            1..>200
                     /gene="Dnmt3a"
ORIGIN
        1 gaattccggc ctgctgccgg gccgcccgac ccgccgggcc acacggcaga gccgcctgaa
       61 gcccagcgct gaggctgcac ttttccgagg gcttgacatc agggtctatg tttaagtctt
      121 agctcttgct tacaaagacc acggcaattc cttctctgaa gccctcgcag ccccacagcg
      181 ccctcgcagc cccagcctgc
//
```

8.4. Multi-FASTA format

Multi-FASTA format consists of multiple sequences of 8.2. FASTA format.

ex.

```
>sequence1
ACTCCCCGTGCGCGCCCGGCCCGTAGCGTCCTCGTCGCCGCCCCTCGTCTCGCAGCCGCA
GCCCGCGTGGACGCTCTCGCCTGAGCGCCGCGGACTAGCCCGGGTGGCC
>sequence2
CAGTCCGGCAGCGCCGGGGTTAAGCGGCCCAAGTAAACGTAGCGCAGCGATCGGCGCCGG
AGATTCGCGAACCCGACACTCCGCGCCGCCCGCCGGCCAGGACCCGCGGCGCGATCGCGG
CGCCGCGCTACAGCCAGCCTCACTGGCGCGCGGGCGAGCGCACGGGCGCTC
>sequence3
CACGACAGGCCCGCTGAGGCTTGTGCCAGACCTTGGAAACCTCAGGTATATACCTTTCCA
GACGCGGGATCTCCCCTCCCC
>sequence4
CAGCAGACATCTGAATGAAGAAGAGGGTGCCAGCGGGTATGAGGAGTGCATTATCGTTAA
TGGGAACTTCAGTGACCAGTCCTCAGACACGAAGGATGCTCCCTCACCCCCAGTCTTGGA
GGCAATCTGCACAGAGCCAGTCTGCACACC
```

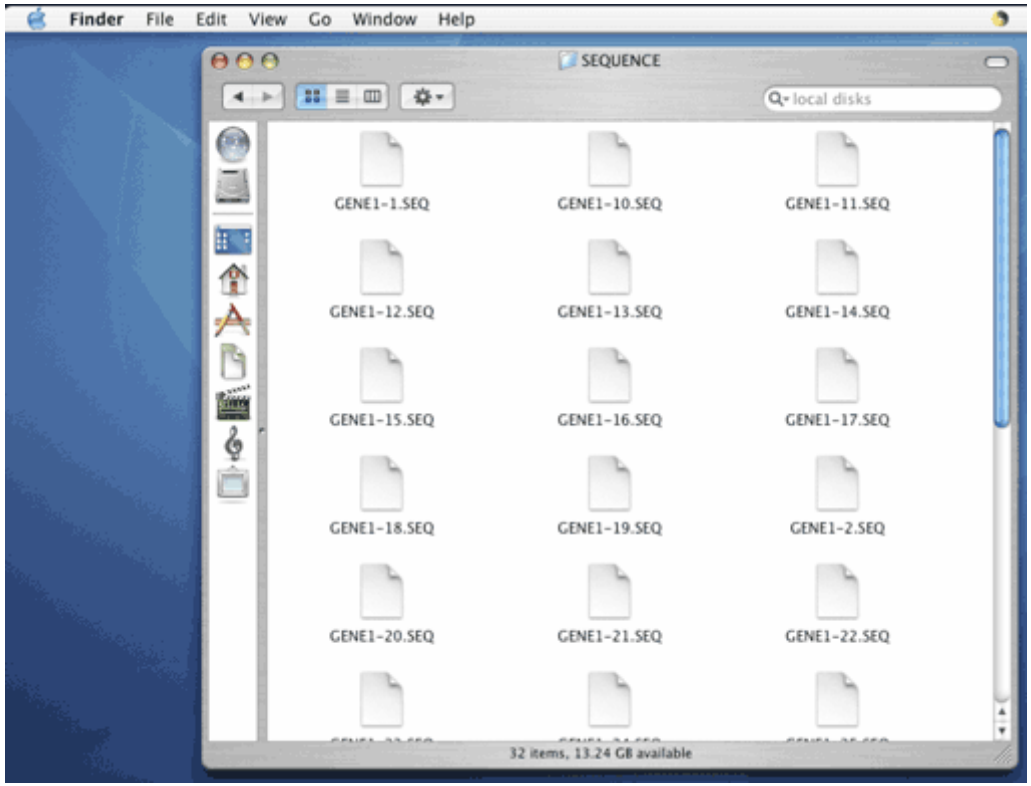
8.5. Zipped archive of sequence files

Zipped archive, which consists single folder and includes bisulfite sequence files of 8.2. FASTA or 8.1. Plain sequence format, is uploadable. Acceptable file extension of sequence file is ".seq", ".fa", ".fas", ".fasta" or ".txt".

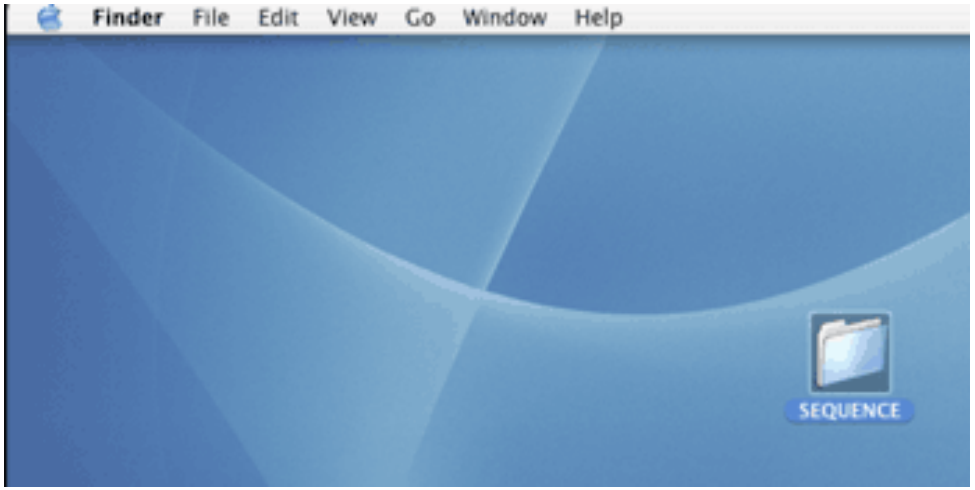
8.6. How to create zipped archive (Macintosh)

8.6.1. Mac OS X 10.3 and later

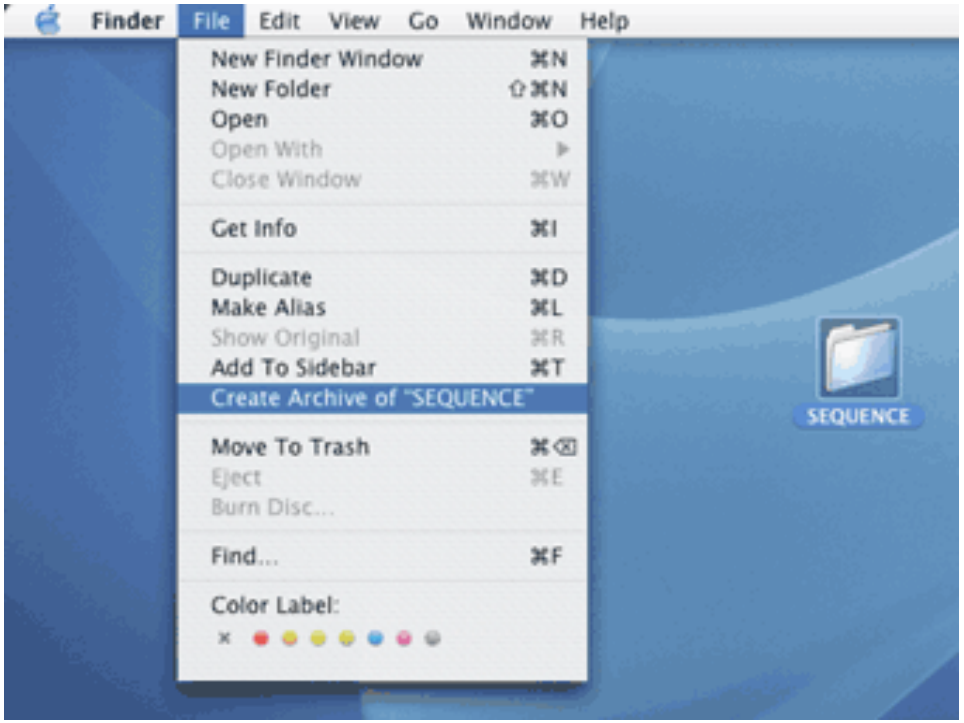
1. **Put bisulfite sequence files of 8.2. FASTA or 8.1. Plain sequence format into a folder. (Acceptable file extension of sequence file is ".seq", ".fa", ".fas", ".fasta" or ".txt".)**



2. **Click to select the folder.**



3. **Select 'Create Archive of "FOLDER NAME"' from "File" menu in the Finder toolbar.**



4. **The zipped archive automatically appears with extension ".zip" at the same location as the folder you selected.**

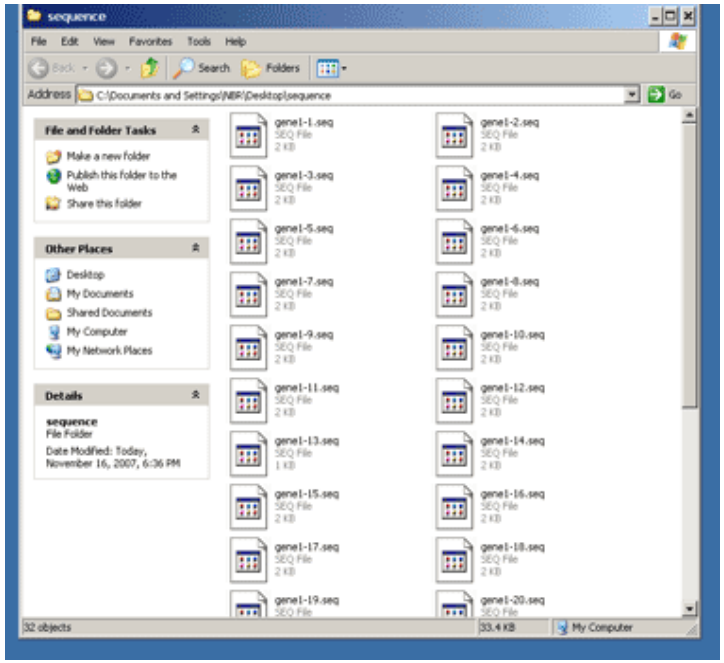
8.6.2. Other Mac OS

Please use ZipIT!, CleanArchiver, MacZip, STUFFIT or other program to create zipped archive.

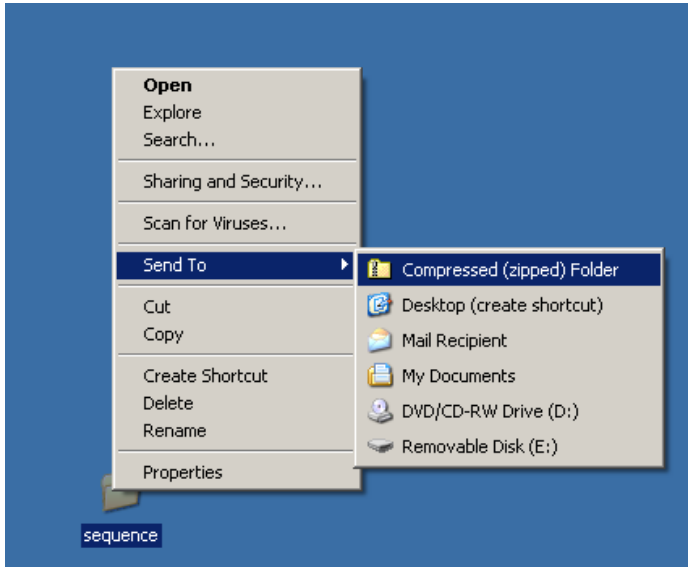
8.7. How to create zipped archive (Windows)

8.7.1. Windows Me/XP/Vista

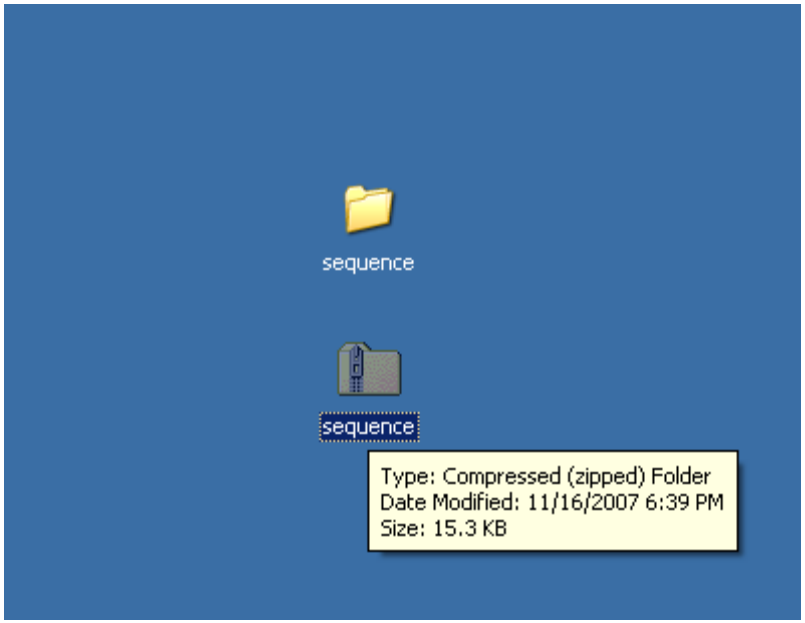
1. **Put bisulfite sequence files of 8.2. FASTA or 8.1. Plain sequence format into a folder. (Acceptable file extension of sequence file is ".seq", ".fa", ".fas", ".fasta" or ".txt".)**



2. **Right-click on the folder. Slide the mouse up to "Send To" and then click on "Compressed (zipped) Folder".**



3. **The zipped archive automatically appears as a folder icon with a zipper at the same location as the folder you selected.**



8.7.2. Other Windows

Please use 7-Zip, WinZip or other program to create zipped archive.

9. Statistical test

9.1. Fisher's exact test

The statistical significance of the difference between two bisulfite sequence groups at each CpG site is evaluated with Fisher's exact test that is non-parametric statistical significance test to determine if there are nonrandom associations between two categorical data. Fisher's exact test can use the same way as the Chi-square test for independence and more exact for small number of methylated CpGs or unmethylated CpGs, that is usually detected in CpG methylation analysis. Two-tailed p-value of Fisher's exact test is calculated from the 2 x 2 tables (exampled below) at each CpG site. This p-value is used to show the independence of CpG methylation between two groups at the CpG site.

▣ Example 2 x 2 table for CpG methylation status

a: number of methylated CpGs of group1 at the CpG site

b: number of unmethylated CpGs of group1 at the CpG site

c: number of methylated CpGs of group2 at the CpG site

d: number of unmethylated CpGs of group2 at the CpG site

	methylated CpG	unmethylated CpG
group1	a	b
group2	c	d

In case of sample data show in table1, this data can be transformed as table2.

Table 1

CpG position		375
Me-CpG	group1	12/13 (92.3%)
	group2	4/10 (40.0%)
	total	16/23 (69.6%)

Table2

	methylated CpG	unmethylated CpG	total
group1	12	1	13
group2	4	6	10
total	16	7	23

The probability *p* of this table can be determined by following formula:

$p = {}_{a+b}C_a * {}_{c+d}C_c / {}_{a+b+c+d}C_{a+c} = {}_{13}C_{12}\ {}_{10}C_4 / {}_{23}C_{16}$ = (13! 10! 16! 7!) / (12! 1! 4! 6! 23!) = 0.0111357212

where the symbol ! indicates the factorial operator.

When the marginal totals are fixed, there are 9 cases indicated below.

a	b	c	d	|ad - bc|	probability
6	7	10	0	70	0.0069995962
7	6	9	1	47	0.0699959618
8	5	8	2	24	0.2362363710
9	4	7	3	1	0.3499798089
10	3	6	4	22	0.2449858662
11	2	5	5	45	0.0801771926
12	1	4	6	68	0.0111357212
13	0	3	7	91	0.0004894823

To determine a two-tailed p-value of the significance, make a sum of probabilities of the case when the absolute value of "ad - bc" is not less than the absolute value of "ad - bc" of the sample.
In this data, the cases of a = 6, 12 and 13 are used. Then, the two-tailed p-value = 0.0069995962 + 0.0111357212 + 0.0004894823 = 0.0186257997

9.2. Mann-Whitney U-test

The statistical significance between two groups of the entire set of CpG sites is evaluated with the Mann-Whitney U-test (also called the Wilcoxon rank-sum test) that is non-parametric statistical significance test for two distributed samples. Although, Student's t-test is useful in the same situations as Mann-Whitney U-test, we adopt not the parametric Student's t-test but the non-parametric Mann-Whitney U-test, because methylation status does not distribute as a normal distribution, especially in case of hyper- or hypo-methylation. Two-tailed p-value of the Mann-Whitney U-test is determined from ranks of ratio of CpG methylation to all CpG at each bisulfite sequence (exampled below). This p-value indicates the independence of distribution of the ratio of CpG methylation to all CpG. Importantly, this test dose not detect differences in the some situations, especially CpG methylation of imprinting regions, because this test only check the difference of the average of two groups. Additionally, the patterns of CpG methylation are not considered.

▣ **Example**

The sample data sets are:

	Me-CpGs/CpGs of each sequence (number of methylated CpGs / number of CpGs)	average ratio of methylation	number of sequences
group1	6/19, 6/19, 8/19, 9/19 12/19, 15/19, 16/19, 18/19, 18/19, 18/19, 18/18, 19/19, 19/19	0.7409	13 (= n_1)
group2	2/19, 2/19, 3/19, 3/19 5/19, 5/19, 7/19, 7/19, 7/19, 8/19	0.2579	10 (= n_2)

(This is the analyzed data of the QUMA sample sequence files.)

Is this difference between the average ratio of methylation (0.7409 vs. 0.2579) significant?

First, make ranking of the values (methylation ratio) and determine a rank. When two or more values are share the same rank, take an average of the rank values. In the sample data, two sequences are Me-CpGs/CpGs = 3/19 and the rank values are 3 and 4. Then use 3.5 (average of 3 and 4) as the rank.
Second, calculate sum of the rank (Rank sum): R_1 and R_2.

Position i		1	2	3	4	5	6	7	8	9	10	11	12	**Rank sum**
Me-CpGs/CpGs		2/19	3/19	5/19	6/19	7/19	8/19	9/19	12/19	15/19	16/19	18/19	1	
rank		1,2	3,4	5,6	7,8	9-11	12,13	14	15	16	17	18-20	21-23	
rank (average)		1.5	3.5	5.5	7.5	10	12.5	14	15	16	17	19	22	
number of sequences	**group1**	0	0	0	2	0	1	1	1	1	1	3	3	212.5 (=R_1)
	group2	2	2	2	0	3	1	0	0	0	0	0	0	63.5 (=R_2)
	total	2	2	2	2	3	2	1	1	1	1	3	3	

Third, determine temporary U-value, U1 and U2, as below.
$U1 = n_1 * n_2 + n_1 * (n_1 + 1) / 2 - R_1 = 8.5$
$U2 = n_1 * n_2 + n_2 * (n_2 + 1) / 2 - R_2 = 121.5$
Take the smaller value of U1 and U2 as the U-value. In this case, U = 8.5
Then determine a two-tailed p-value from the U-value. To determine the p-value, we take the approximation using the normal distribution for the number of sequences above 20. In the case of small sequences (20 and below), we determine the p-value from exact probabilities (Mann Whitney U exact test).

The normal approximation is performed as:

$$z = |U - E(U)| / \sqrt{V(U)}$$

where z is a standard normal deviate, E(U) is the mean of U and V(U) is the variance of U:

$$E(U) = n_1 n_2 / 2$$

$$V(U) = \frac{n_1 n_2}{12(n^2 - n)} \left\{ n^3 - n - \sum_{i=1}^{m} (t_i^3 - t_i) \right\}$$

where t_i is the number of tied ranks of the position i.
At the sample, E(U) = 65, V(U) = 257.812 and z = 3.51879. Then, the two-tailed p-value = 0.0004 is determined from the standard normal distribution (double value for two-tail).

Another sample data sets for Mann Whitne U exact test are:

Table 1

	Me-CpGs/CpGs of each sequence (number of methylated CpGs / number of CpGs)	average ratio of methylation	number of sequences
group1	6/19, 6/19, 9/19 12/19, 15/19, 18/19	0.5789	6 (= n_1)
group2	3/19, 5/19, 5/19, 7/19, 7/19	0.2842	5 (= n_2)

Table 2

Position i		1	2	3	4	5	6	7	8	**number of sequences**	**Rank sum**
Me-CpGs/CpGs		3/19	5/19	6/19	7/19	9/19	12/19	15/19	18/19		
rank		1	2,3	4,5	6,7	8	9	10	11		
rank (average)		1	2.5	4.5	6.5	8	9	10	11		
number of sequences	**group1**	0	0	2	0	1	1	1	1	6	47 (=R_1)
	group2	1	2	0	2	0	0	0	0	5	19 (=R_2)
	total	1	2	2	2	1	1	1	1	11	

$U1 = n_1 * n_2 + n_1 * (n_1 + 1) / 2 - R_1 = 4$
$U2 = n_1 * n_2 + n_2 * (n_2 + 1) / 2 - R_2 = 26$
$U = \min(U1, U2) = 4$

When the marginal totals are fixed, there are 179 cases and 11 cases indicated below have U-value not more than the U-value of the sample.

Position i	1	2	3	4	5	6	7	8	Rank sum	U-value	Probability
Me-CpGs/CpGs	3/19	5/19	6/19	7/19	9/19	12/19	15/19	18/19			
rank	1	2,3	4,5	6,7	8	9	10	11			
rank (average)	1	2.5	4.5	6.5	8	9	10	11			
group1/group2	1/0	2/0	2/0	1/1	0/1	0/1	0/1	0/1	21.5/44.5	0.5	0.00433
group1/group2	1/0	2/0	2/0	0/2	1/0	0/1	0/1	0/1	23/43	2	0.00216
group1/group2	1/0	2/0	2/0	0/2	0/1	1/0	0/1	0/1	24/42	3	0.00216
group1/group2	1/0	2/0	2/0	0/2	0/1	0/1	1/0	0/1	25/41	4	0.00216
group1/group2	1/0	2/0	1/1	2/0	0/1	0/1	0/1	0/1	23.5/42.5	2.5	0.00433
group1/group2	1/0	2/0	1/1	1/1	1/0	0/1	0/1	0/1	25/41	4	0.00866
group1/group2	0/1	1/1	0/2	1/1	1/0	1/0	1/0	1/0	47/19	4	0.00866
group1/group2	0/1	0/2	2/0	0/2	1/0	1/0	1/0	1/0	47/19	4	0.00216
group1/group2	0/1	0/2	1/1	2/0	0/1	1/0	1/0	1/0	47.5/18.5	3.5	0.00433
group1/group2	0/1	0/2	1/1	1/1	1/0	1/0	1/0	1/0	49/17	2	0.00866
group1/group2	0/1	0/2	0/2	2/0	1/0	1/0	1/0	1/0	51/15	0	0.00216

To determine a two-tailed p-value of the significance, make a sum of probabilities of these 11 cases. Then, the two-tailed p-value = 0.0498

10. Other

10.1. How to open a CSV file

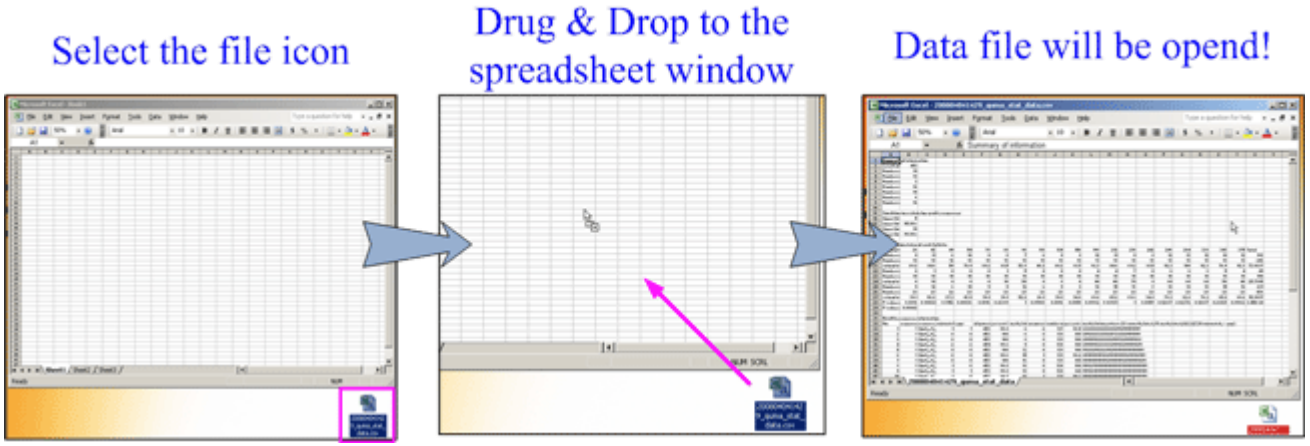
In many case, CSV formatted analysis data file can be opened from Microsoft Excel by double-clicking the file icon. If not, try the "drug & drop" procedure indicated below.

10.1.1. Mac OS

Drug & drop the data file icon to the software icon of the Microsoft Excel or OpenOffice.

10.1.2. Windows

Open a blank window of the Microsoft Excel or OpenOffice/StartSuite. Then drug & drop the data file icon to the window.



Alternatively, open the data file from the "File" menu -> "Open" sub-menu (change "Files of type" tab to "All" or "Text files").