diagenode

Innovating Epigenetics Solutions

Premium RRBS kit

Reduced Representation Bisulfite Sequencing

Cat. No. C02030032 (24 rxns)
C02030033 (96 rxns)

USER GUIDE

Version 6 02_2019

Please read this manual carefully
before starting your experiment

Contents

Introduction

DNA methylation is a key epigenetic mechanism with important regulatory functions in development and disease. Bisulfite sequencing enables the detection of cytosine methylation at single-base resolution. Nowadays, this method is widely used for targeted gene analysis due to a relatively low price per reaction. However very few researchers perform genome-wide studies, discouraged by the enormous sequencing efforts and corresponding costs. Reduced Representation Bisulfite Sequencing (RRBS) is the best alternative to increase the scale of the analysis cost efficiently. In the initial step of the RRBS protocol, DNA is digested with the restriction enzyme MspI wich recognizes CCGG sites. Thus, all resulting genomic fragments will start and end with a CpG dinucleotide. Due to the biased distribution of CpGs throughout the genome (depicted in Figure 1), MspI digestion followed by size selection enriches for the most CpG-rich regions, including CpG islands. DNA methylation at these regulating regions, which are often located at gene promoters, is most likely to influence gene expression. Thus, RRBS provides a cost-effective method to analyze DNA methylation in the most relevant genomic regions.

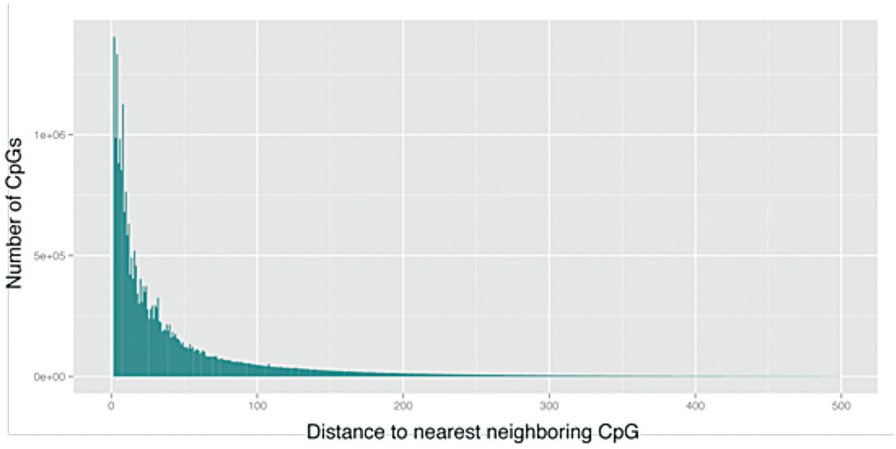


***Figure 1**: Distance between neighboring CpGs*

Diagenode's Premium RRBS kit offers a complete solution, including reagents for enzymatic digestion, library preparation, bisulfite conversion and amplification. Moreover this protocol requires only very little starting material (100 ng of gDNA is recommended) and is optimized for high throughput and sequencing on Illumina platforms. Since six samples can be pooled together and processed simultaneously, an experiment starting with 96 samples will require handling only 16 tubes for the bisulfite conversion and only 16 sequencing lanes. Handling time, amount of reagents, and the price per sample are thus greatly reduced.

Diagenode's Premium RRBS technology

- Positive and negative spike-in controls are included for the monitoring of bisulfite conversion efficiency
- Size selection has been optimized to keep small fragments of interest and to remove adaptor dimers, resulting in a better coverage.
- The pooling protocol includes a quantification of the samples and a pooling application, available on our website, to help you to group your samples, depending on the DNA amount and adaptor barcode of each sample.
- The bisulfite conversion protocol has been improved to decrease DNA degradation while keeping a highly efficient conversion of unmethylated cytosines.
- The minimum number of amplification cycles needed for each pool is determined to avoid amplification biases. Our MethylTaq Plus enzyme was developed to amplify bisulfite converted DNA with high efficiency, and reduces the number of PCR cycles required.

Kit Method Overview and Time Table

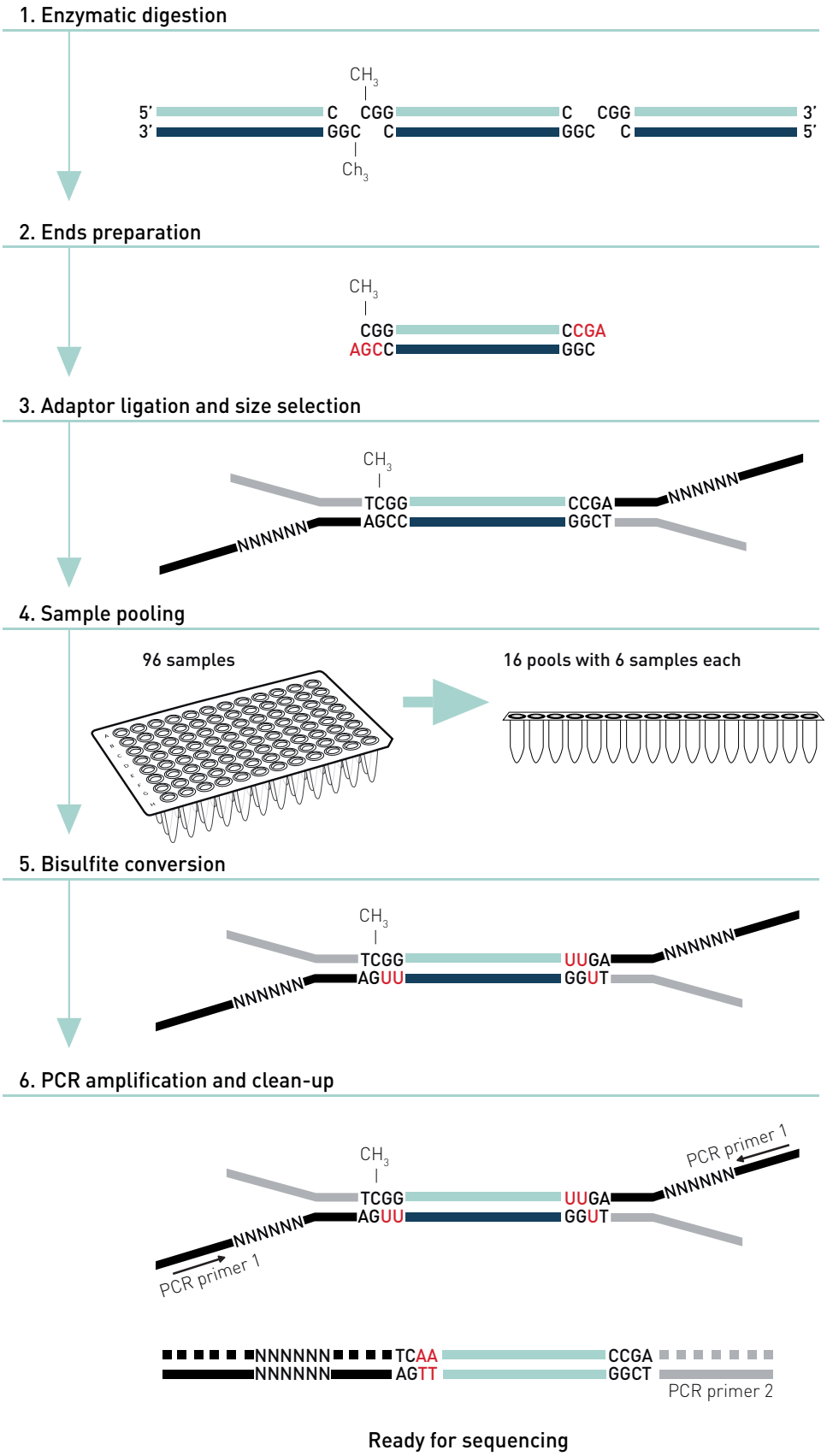


***Figure 2**: Reduced Representation Bisulfite Sequencing (RRBS) workflow*

Table 1. Premium RRBS protocol overview

		Incubation times	1-24 samples	25-96 samples
STEP 1	Enzymatic digestion - Preparation of the 96-well plate with all the samples at the right concentration - Enzymatic digestion	12 hours (overnight)	DAY 0	DAY 1
STEP 2	Ends preparation	1 hour	DAY 1	DAY 2
STEP 3	Adaptor ligation	30 minutes		
STEP 4	Size selection	15 minutes		
STEP 5	Quantification - qPCR in duplicates	1 or 2 qPCR runs		
STEP 6	Pooling - Calculations - Manual pooling - Clean-up to reduce the volumes	15 minutes		DAY 3
STEP 7	Bisulfite conversion	Overnight		
		30 minutes	DAY 2	DAY 4
STEP 8	Determination of the optimal cycle number for the enrichment PCR	1 qPCR run		
STEP 9	Enrichment PCR	Several PCR runs		DAY 4 or 5
STEP 10	Clean-up	25 minutes		

Kit materials

CAUTION *The content of the kit is sufficient to perform 24 or 96 RRBS reactions, starting with genomic DNA and finishing with sequencing-ready libraries (Table 2).*

Store the components at the indicated temperature upon receipt.

Table 2. Components supplied with the Premium RRBS Kit

Component	Quantity (x24)	Quantity (x98)	Storage
Restriction Enzyme (violet)	35 µl	115 µl	-20°C
Ends Preparation Enzyme (blue)	35 µl	115 µl	-20°C
Enzyme Buffer (violet)	100 µl	335 µl	-20°C
dNTP Mix (blue)	35 µl	115 µl	-20°C
Unmethylated spike-in control (blue)	35 µl	115 µl	-20°C
Methylated spike-in control (blue)	35 µl	115 µl	-20°C
Adaptor tube strip A	5 µl each	21 µl each	-20°C
Adaptor tube strip B	5 µl each	21 µl each	-20°C
Adaptor tube strip C	5 µl each	21 µl each	-20°C
Adaptor tube strip D	5 µl each	21 µl each	-20°C
Adaptor tube strip E	5 µl each	21 µl each	-20°C
Adaptor tube strip F	5 µl each	21 µl each	-20°C
Ligase (yellow)	35 µl	115 µl	-20°C
Ligation Buffer (yellow)	1.3 ml	4.5 ml	-20°C
Primer Mix (green)	47 µl	162 µl	-20°C
2X MethylTaq Plus Master Mix (green)	113 µl	413 µl	-20°C
Water (clear)	1390 µl	5.5 ml	4°C/-20°C
Resuspension Buffer (white)	820 µl	3.3 ml	4°C/-20°C
BS Conversion Reagent	1 tube	2 tubes	Room temperature
BS Dilution Buffer (black)	300 µl	600 µl	Room temperature
BS Solubilization Buffer (black)	790 µl	1580 µl	Room temperature
BS Reaction Buffer (black)	160 µl	320 µl	Room temperature
BS Binding Buffer (white)	4.8 ml	12 ml	Room temperature

BS Wash Buffer w/o ethanol (white)	1.0 ml	2.5 ml	Room temperature
BS Desulphonation Buffer (clear)	1.6 ml	4 ml	Room temperature
BS Elution Buffer (white)	176 µl	440 µl	Room temperature
BS Spin Columns	8 columns	20 columns	Room temperature
BS Collection Tubes	8 tubes	20 tubes	Room temperature

Table 3. Distribution of the 24 adaptors in the tube strips and sequences of the 24 corresponding indexes

Adaptor tube strips	Adaptor ID	Index sequences
Strip A	17	GTAGAG
	24	GGTAGC
	26	ATGAGC
	28	CAAAAG
Strip B	29	CAACTA
	30	CACCGG
	31	CACGAT
	32	CACTCA
Strip C	33	CAGGCG
	34	CATGGC
	35	CATTTT
	36	CCAACA

Adaptor tube strips	Adaptor ID	Index sequences
Strip D	37	CGGAAT
	38	CTAGCT
	39	CTATAC
	40	CTCAGA
Strip E	41	GCGCTA
	42	TAATCG
	43	TACAGC
	44	TATAAT
Strip F	45	TCATTC
	46	TCCCGA
	47	TCGAAG
	48	TCGGCA

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- Autoclaved tips
- Nuclease-free 1.5 ml tubes
- Nuclease-free 15 ml tubes
- 96-well plates and 96-well qPCR plates
- Multichannel pipettes (12 channels)
- 12-tube strips
- Racks for 0.2 ml tube strips
- Centrifuge for 0.2 ml tube strips
- Thermocycler and qPCR thermocycler
- qPCR reagents
- Centrifuge for 96-well plates
- Centrifuge for 1.5 and 2 ml tubes
- 96 well plate magnetic rack
- DiaMag1.5 ml – magnectic rack (Diagenode, Cat. No. B04000003)
- AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- 100% ethanol
- Nuclease-free water

Equipment required for quality control

- Fluorescence-based assay for DNA concentration measurement, e.g. the Qubit High Sensitivity assay (Life Technologies #Q32851)
- Library analysis assay such as Agilent High Sensitivity DNA Kit for BioAnalyzer (Agilent, # 5067-4626)

Remarks before starting

Number of samples

This protocol has been optimized for a high number of samples which will be pooled during library preparation to limit the manipulation and reduce the cost. The optimized number of samples per pool is 6 for human and mouse. For this reason it is better to perform your experiment on a number of samples which is a multiple of 6.

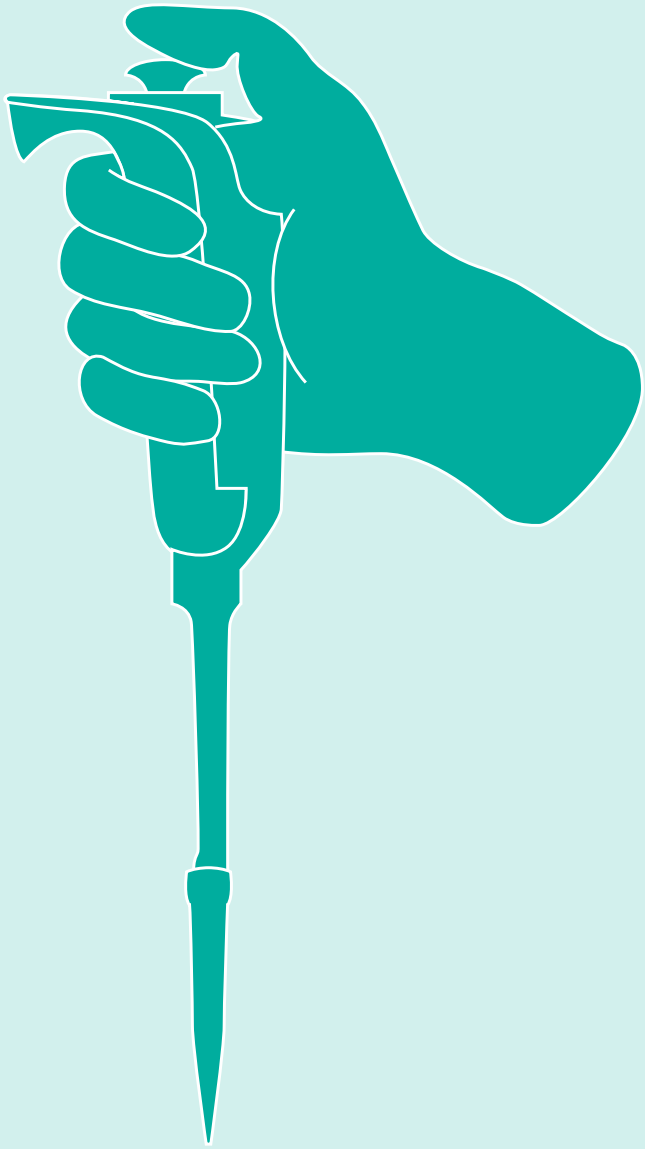
Starting material: high molecular weight genomic DNA

The quality of the gDNA to be used in RRBS is important, we therefore highly recommend the use of our XL GenDNA Extraction module (Cat. No. C03030020) for the DNA extraction. It has been optimized for the preparation of genomic DNA from cultured cells.

***NOTES**:*

- *When the number of cells is limited, a phenol-chloroform extraction can be used.*
- *Regardless the choice of DNA extraction protocol, proteinase K digestion is mandatory.*
- *Do not use Trizol during your DNA extraction as it inhibits the enzymatic digestion.*
- *Do not vortex high molecular weight DNA as this might lead to fragmentation. Mix by pipetting.*
- *Quantify the double-stranded genomic DNA by the use of a fluorescence-based assay such as the Qubit High Sensitivity assay (Life Technologies #Q32851) since spectrophotometric measurements (e.g. NanoDrop) might overestimate the amount of dsDNA.*
- *We recommend to check for genomic degradation by analysis of a small aliquot of each sample on a 0.8 % agarose gel.*
- *It is possible to start with FFPE samples as soon as you do not see DNA fragments smaller than 2000 bp on agarose gel.*

PROTOCOL



STEP 1

Enzymatic digestion

1.1 Transfer your samples to a 96-well plate. For each sample, provide exactly **100 ng of DNA** in **26 µl of water**. Start loading your samples in well A01 and proceed in rows.

CAUTION *It is necessary to proceed in rows when distributing the samples as the adaptors will be distributed the same way. This will help to limit the barcodes incompatibilities between samples of the same project.*

1.2 Prepare the Digestion Mix in a 1.5 ml tube as described in Table 4. The volumes presented here include a small excess.

Table 4.

		Mix for # of samples (µl)							
Components	**Volume /reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Enzyme Buffer (violet cap)	3 µl	60	96	150	192	225	264	300	330
Restriction Enzyme (violet cap)	1 µl	20	32	50	64	75	88	100	110
Total	4 µl	80	128	200	256	300	352	400	440

1.3 Mix by pipetting and distribute the indicated volume (see table 5) of Digestion Mix to each tube of one 12-tube strip. This will allow for multichannel pipette use.

Table 5.

	Mix for # of samples (µl)							
# of samples	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Volume of Digestion Mix (µl)	6	10	16	20	24	28	32	36

1.4 Distribute **4 µl of the Digestion Mix** (12-tube strip) to each sample of the 96-well plate using a multichannel pipette.

***NOTE**: When processing 12 samples or less, 4 µl of Digestion Mix can be added directly to each well without distribution in a 12-tube strip and without multichannel pipette.*

1.5 Mix by pipetting up and down 10 times. Seal, spin, place in a thermocycler and run the following program overnight:

- 12 hours at 37°C
- Hold at 8°C

STEP 2

Ends Preparation

2.1 Prepare the Ends Preparation Mix in a 1.5 ml tube as indicated in Table 6. The volumes presented here include a small excess.

***NOTE**: A methylated control DNA and an unmethylated control DNA are included. They are to be added to each sample to control for under- and overconversion during sodium bisulfite treatment.*

Table 6.

		Mix for # of samples (µl)							
Components	**Volume /reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Ends Preparation Enzyme (blue cap)	1 µl	20	32	50	64	75	88	100	110
dNTP mix (blue cap)	1 µl	20	32	50	64	75	88	100	110
Unmethylated spike-in control (blue cap)	1 µl	20	32	50	64	75	88	100	110
Methylated spike-in control (blue cap)	1 µl	20	32	50	64	75	88	100	110
Total	4 µl	80	128	200	256	300	352	400	440

2.2 Mix by pipetting and distribute the indicated volume (see table 7) of Ends Preparation Mix to each tube of one 12-tube strip.

Table 7.

	Mix for # of samples (µl)							
# of samples	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Volume of Ends Preparation Mix (µl)	6	10	16	20	24	28	32	36

2.3 Distribute **4 µl of Ends Preparation Mix** (12-tube strip) to each sample of the 96-well plate, using a multichannel pipette.

***NOTE**: When processing 12 samples or less, 4 µl of Ends Preparation Mix can be added directly to each well without distribution in a 12-tube strip and without multichannel pipette.*

2.4 Mix by pipetting up and down 10 times, seal and spin. Place in a thermocycler and run the following program:

- 20 minutes at 30°C
- 20 minutes at 37°C
- 20 minutes at 75°C
- Hold at 8°C

***CAUTION** Do not stop at this step and proceed quickly to adaptor ligation.*

STEP 3

Adaptor ligation and size selection

3.1 Thaw the Ligation Buffer about 15 minutes before the ends preparation reaction finishes. Then thaw the six 4-tube strips containing the 24 adaptors and centrifuge them briefly. Put the Adaptor strips in a rack, to form two rows of 12 wells. We recommend to put strips A, B and C (in this order) in row 1 and strips D, E and F (in this order) in row 2.

3.2 **Add 5 µl of the adaptors per well** of the 96-well plate. Use a multichannel pipette to pipette 12 adaptors at one time and distribute the adaptors, alternating between strips A-B-C and strips D-E-F.

***NOTE**: There is no need to decide now which samples will be pooled together.*

3.3 Prepare the Ligation Mix in a 15 ml or 1.5 ml tube as indicated in Table 8. The volumes presented here include a small excess.

Table 8.

		Mix for # of samples (µl)							
Components	**Volume /reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Ligation Buffer (yellow cap)	40 µl	800	1280	2000	2560	3000	3520	4000	4400
Ligase (yellow cap)	1 µl	20	32	50	64	75	88	100	110
Total	41 µl	820	1312	2050	2614	3075	3608	4100	4510

3.4 Mix using a pipette set to 1000 µl and distribute the indicated volume (see table 9) to each tube of a 12-tube strip. When processing 60 or more samples, distribute the master mix in two strips due to maximum well capacity.

Table 9.

	Mix for # of samples (µl)							
# of samples	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Volume of Ligation Mix in the 1st strip (µl)	62	103	144	185	130	134	170	175
Volume of Ligation Mix in the 2nd strip (µl)	0	0	0	0	90	134	130	175

3.5 Distribute **41 µl of the Ligation Mix** (12-tube strip) to each sample of the 96-well plate using a multichannel pipette. The resulting reaction volume is 80 µl.

***NOTE**: When processing 12 samples or less, 41 µl of Ligation Mix can be added directly to each well without distribution in a 12-tube strip and without multichannel pipette.*

3.6 Mix by pipetting up and down 10 times. Seal, spin, place in a thermocycler and run the following program:

- 20 minutes at 25°C
- 10 minutes at 65°C
- Hold at 8°C

Size selection

3.7 Add **60 µl of Room Temperature AMPure XP Beads**, in each well, using a multichannel pipette. Mix by pipetting up and down at least 10 times and incubate 15 minutes to allow DNA binding to the beads.

3.8 Place the 96-well plate on a 96-well magnet for 5 minutes or until the supernatant appears clear.

3.9 Remove the supernatants using a multichannel pipette taking care not to touch the beads.

3.10 Wash the pellets of the first row twice for 5 seconds with **100 µl of freshly prepared 80% ethanol**. When processing more than 12 samples, proceed the same way for the following rows, keeping the pellets from the first row airdrying (3 minutes at least).

***CAUTION** Do not leave the samples longer than 5 seconds in 80% Ethanol as it may already elute DNA from the beads.*

3.11 Add **25 µl of Resuspension Buffer** (clear cap) to each well using a multichannel pipette. Vortex to resuspend the beads and spin shortly. Incubate 5 minutes out of the magnet to elute DNA.

3.12 Place the 96-well plate on the magnet for 5 minutes.

3.13 Transfer the supernatants to a new 96-well plate, using a multichannel pipette and discard beads.

***NOTE**: This plate can be conserved at -20°C for several days.*

STEP 4

Quantification and sample pooling

4.1 Put **3 µl of each sample** in a new 96-well PCR plate using a multichannel pipette. Leave the rest of the samples at -20°C during the quantification by qPCR.

4.2 Add **6 µl of nuclease-free water** in each well using a multichannel pipette and mix by pipetting up and down.

4.3 Prepare the Quantification Mix in a 2 ml tube. An example using a 2X qPCR Master Mix is indicated in Table 10. If your qPCR Master Mix is provided at another concentration adapt the volumes during the Quantification Mix preparation. The indicated volumes are calculated to perform the qPCR in duplicates and include a small excess.

Table 10.

		Mix for # of samples (µl)							
Components	**Volume /reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
2X qPCR Master Mix	5 µl	200	320	500	640	750	880	1000	1100
Primer mix (green cap)	0.5 µl	20	32	50	64	75	88	100	110
Water (clear cap)	1.5 µl	60	96	150	192	225	264	300	330
Total	7 µl	280	448	700	896	1050	1232	1400	1540

4.4 Add the indicated volume of Quantification Mix (see table 11) to each tube of a 12-tube strip. Each library will be quantified in duplicates and the average Ct value will be used to calculate the volumes for pooling.

***NOTE**: You can prepare additional qPCR reactions for a positive control (e.g. a previously tested library) and a negative control (water).*

Table 11.

	Mix for # of samples (µl)							
# of samples	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Volume of Quantification Mix (µl)	21	35	56	70	84	98	112	120

4.5 Transfer **7 µl of the Quantification Mix** to 2 wells of a new 96-well qPCR plate using a multichannel pipette.

4.6 Add **3 µl of the 1:3 dilution of each library** to the qPCR plate using a multichannel pipette.

***NOTE**: If processing less than 12 samples you do not need to use a multichannel pipette. In this case you do not need to dilute the samples before the qPCR because a single channel pipette can pipette 1 µl precisely. If doing that way, adapt the volume of water in the Quantification Mix by adding 2 µl by reaction.*

4.7 Seal the plate, mix by vortexing, centrifuge briefly and place it in a thermocycler. Run the program described in Table 12.

Table 12.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	3 minutes	1
Denaturation	95°C	15 seconds	25
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Hold	4°C	∞	

***NOTE**: When processing more than 48 samples two qPCR plates are needed. You can keep the second plate at 4°C during the first run.*

Sample pooling

The goal of this step is to pool the samples to reduce handling time and reagents consumption. As up to 6 human samples can be sequenced in the same lane, the protocol is optimized for pools of 6. This number can be changed when working with samples from different species, according to the size of their genome. The pooling application is using the Ct values from the qPCR to pool together samples with similar concentration and

to calculate the volumes of each library to use to have the same amount of each sample in the pool.

We propose 2 options for this step. To use our application for automated pooling choose option 1 follow the steps 4.8 to 4.10 and then go directly to step 4.12.

You can also decide to do everything manually, in this case follow option 2 and go directly to step 4.11.

Option 1: Use of our Pooling Aid in Microsoft Excel for Windows

4.8 Download our automated pooling file called "RRBS pooling aid" on our website : (https://www.diagenode.com/documents/rrbs-pooling-aid) and fill in the Input sheet as shown in figure 3. Document the features of each sample in front of the corresponding position in the plate:

- Enter the sample names in column B
- Enter the adaptor ID in column C
- Enter the Ct values from the two qPCR replicates in columns D and E respectively

If there are samples that you absolutely do not want to pool together (coming from different projects, different species...) just use several pooling files.

	A	B	C	D	E
1					
2	well	sample name	adaptor ID	Ct 1	Ct 2
3	A01	sample 1	17	9.43	9.46
4	A02	sample 2	24	9.2	9.23
5	A03	sample 3	26	9.48	9.51
6	A04	sample 4	28	9.35	9.34
7	A05	sample 5	29	9.04	9.08
8	A06	sample 6	30	9.29	9.29
9	A07	sample 7	31	9.38	9.49
10	A08	sample 8	32	9.49	9.57
11	A09	sample 9	33	9.51	9.49
12	A10	sample 10	34	9.5	9.38
13	A11	sample 11	35	9.58	9.61
14	A12	sample 12	36	9.56	9.52

***Figure 3**: Screenshot of the Input sheet of the RRBS pooling aid with the different types of data in it.*

4.9 To pool the samples by group of 6 and determine the volume of each library, use our application:

- Press Ctrl+s: the Sample Sheet opens and shows the pools and the volumes as shown in figure 4
- If one pool contains several times the same barcode the software highlights it in red (see figure 4). You can change the pooling manually to resolve that or press Ctrl+d to do it automatically. You can use the Ctrl+d command several times if one barcode is present 3 or more times in the same pool. When changing some lines manually, just move the A,B,C and D columns to keep intact the formulae in columns E and F.

***NOTE**: If you are doing the barcode's exchanges manually it can happen that you move the wrong lines. In this case you can just press Ctrl+s again to restart. You can also decide to remove some samples because of strange values. To do that, just remove the samples from the Input sheet and press Ctrl+s again. You can add new lines in the same way. The file will calculate new pools and new volumes every time you press Ctrl+s.*

	A	B	C	D	E	F
1						
2	well	sample name	adaptor ID	Ct mean	pooling volume	target well
3	A05	sample 5	29	9.06	13.95260735	P01
4	H05	sample 89	41	9.16	14.95403429	P01
5	A02	sample 2	24	9.215	15.53513465	P01
6	B01	sample 13	30	9.265	16.08297999	P01
7	A06	sample 6	30	9.29	16.36410553	P01
8	A04	sample 4	28	9.345	17	P01
9	A07	sample 7	31	9.435	15.91662821	P02
10	A10	sample 10	34	9.44	15.97188674	P02
11	A01	sample 1	17	9.445	16.02733711	P02
12	A03	sample 3	26	9.495	16.59253993	P02
13	A09	sample 9	33	9.5	16.65014506	P02
14	A08	sample 8	32	9.53	17	P02

***Figure 4**: Screenshot of the Sample Sheet of the RRBS pooling aid containing the sorted samples, the pooling volumes and the barcodes clashes in red.*

4.10 Print the Pooling Sheet to help you during the pipetting step of the pooling. Pool combinations of 6 libraries in a new 1.5 ml tube using the pooling volumes. Add water to reach a volume of 120 µl as calculated in the Pooling Sheet (see figure 5).

Target wells	Source plate wells						Water to add
P01	A05	H05	A02	B01	A07	A04	
Volumes	13.95	14.95	15.54	16.08	15.92	17.00	26.56
P02	A06	A10	A01	A03	A09	A08	
Volumes	16.36	15.97	16.03	16.59	16.65	17.00	21.39
P03	B02	A12	B05	H06	A11	B07	
Volumes	16.31	16.36	16.59	16.88	17.00	17.00	19.85
P04	B08	B09	B04	B06	B03	B12	
Volumes	15.92	15.97	16.54	16.71	16.77	17.00	21.10
P05	C01	B10	C03	C08	C02	B11	
Volumes	15.97	16.03	16.36	16.59	16.88	17.00	21.16
P06	C06	C04	C07	C09	C05	H07	
Volumes	16.03	16.19	16.71	16.71	17.00	17.00	20.36
P07	C12	D02	D03	C10	C11	D01	
Volumes	16.36	16.48	16.71	16.94	16.94	17.00	19.57
P08	D08	D07	D09	D04	D06	D05	
Volumes	15.81	16.14	16.31	16.59	16.77	17.00	21.39
P09	D11	E01	E02	D10	E09	E03	
Volumes	16.25	16.42	16.54	16.77	16.88	17.00	20.14
P10	D12	E07	E08	E06	E05	E04	
Volumes	15.37	15.81	16.59	16.71	16.82	17.00	21.69
P11	E12	F03	E10	F01	F05	F02	
Volumes	14.34	15.01	15.64	15.86	16.59	17.00	25.55
P12	E11	F04	F06	F08	F09	G03	
Volumes	14.34	14.65	14.85	15.11	15.64	17.00	28.40
P13	H10	F10	F11	F12	G02	H02	
Volumes	16.03	16.25	16.42	16.48	16.65	17.00	21.17
P14	G05	H11	G07	G04	G06	G08	
Volumes	14.05	15.48	15.75	15.92	16.71	17.00	25.09
P15	G09	G10	G01	G11	G12	H01	
Volumes	9.50	9.56	10.83	11.45	12.88	17.00	48.77
P16	H09	H12	H03	H08	F07	H04	
Volumes	0.45	0.60	0.77	1.45	7.96	17.00	91.77

***Figure 5**: Screenshot of the Pooling Sheet of the RRBS pooling aid containing the volumes for pooling and for water.*

Option 2: Manual pooling

4.11 If you prefer to decide the pooling manually follow those rules:

- Calculate the average of the Ct values from the duplicates of each sample.
- Sort the samples according to the mean Ct value and decide how to pool them by group of 6. In the same pool, samples must have similar Ct values, similar features (species, FFPE or not...) and different adaptors.
- Identify the samples that you do not want to sequence at the same time and treat them separately (different projects, different species...).
- Find the sample with the maximum value in the pool (CTmax). For this sample the maximum volume of 17 µl will be used.
- For the other samples of the pool calculate the difference in Ct value as dCt = Ctmax – Ctsample. The volume to be used is then calculated as 17*2^(-dCt).
- Pool combinations of 6 libraries in 1.5 ml tubes according to the calculated volumes.

***NOTE**: It is possible to create a pool of 5 or 7 samples if you wish. For a pool of 7 we recommend to use the samples with the smallest Ct values and for a pool of 5 the samples with the highest Ct values.*

- Add water to reach a volume of 120 µl per pool.

4.12 You have now your libraries pooled in 1.5 ml tubes.

***NOTE**: If your number of pools is high you can also put them in a 96-well plate and perform the purification step using multichannel pipettes. Nevertheless you will need to split each pool into two wells of the plate and to divide the volumes of reagent used by 2 because of the small volume capacity of the plate. The protocol is then the same as for 1.5 ml tubes.*

4.13 Add **240 µl of AMPure XP Beads** to each pool, mix well by pipetting up and down and incubate for 15 minutes at room temperature to allow DNA binding to the beads.

4.14 Place the tubes on the DiaMag1.5 magnetic rack (#B04000003) for 5 minutes or until the supernatant appears clear and then remove and discard the supernatant.

4.15 Wash twice with **500 µl of freshly prepared 80% ethanol** for 5 seconds.

4.16 Allow the beads to air-dry for 10 minutes, the tubes standing open in the magnet.

4.17 Add **36 µl of Resuspension Buffer** (clear cap) to each tube and mix by pipetting 10 times. Spin shortly and incubate 5 minutes out of the magnet to elute DNA.

4.18 Place the tubes on the magnet for 5 minutes or until the supernatant is clear.

4.19 Transfer the supernatant in a new 1.5 ml tube.

STEP 5

Bisulfite conversion

5.1 Before the first use add **4 ml of 100 % ethanol** to 1 ml BS Wash Buffer concentrate for C02030032 or **10 ml of 100 % ethanol** to 2.5 ml BS Wash Buffer concentrate for C02030033. The BS Conversion Reagent supplied within this kit is a solid mixture and must be prepared prior to first use.

CAUTION *The BS conversion reagent has to be manipulated under a fume hood.*

5.2 Add **790 µl of BS Solubilization Buffer** (black cap) and **300 µl of BS Dilution Buffer** (black cap) to a tube of BS Conversion Reagent.

5.3 Mix at room temperature with frequent vortexing or shaking for 10 minutes.

5.4 Add **160 µl of BS Reaction Buffer** (black cap) and mix an additional 1 minute.

NOTE*: It is normal to see trace amounts of undissolved reagent in the BS Conversion Reagent. Each tube of BS Conversion Reagent is designed for 8 separate DNA treatments including an excess.*

STORAGE*: The BS Conversion Reagent is light sensitive, so minimize its exposure to light. For best results, the BS Conversion Reagent should be used immediately following preparation.*

5.5 Put **33 µl of each library pool** in tube strips (or in a new 96-well plate).

5.6 Add **117 µl of BS Conversion reagent**, mix, centrifuge briefly and incubate in a thermocycler as indicated in Table 13.

Table 13.

Cycle Step	Temperature	Time	Cycles
Denaturation	95°C	1 minute	20
Conversion	60°C	10 minutes	
Hold	4°C	∞	

5.7 Place BS Spin columns into the provided BS Collection Tubes.

5.8 Add **600 µl of BS Binding Buffer** (white cap) into a BS Spin Column.

5.9 Load bisulfite-converted library pools into the BS Spin Columns containing the BS Binding Buffer. Close the cap and mix by inverting the column several times.

5.10 Centrifuge at full speed (›10,000 x g) for 30 seconds. Discard the flow-through.

5.11 Add **100 µl of BS Wash Buffer** (white cap) to each column. Centrifuge at full speed for 30 seconds.

5.12 Add **200 µl of BS Desulphonation Buffer** (clear cap) to each column and let stand at room temperature (20-30°C) for exactly 30 minutes. After the incubation, centrifuge at full speed for 30 seconds.

5.13 Add **200 µl of BS Wash Buffer** (white cap) to each column. Centrifuge at full speed for 30 seconds. Add another 200 µl of BS Wash Buffer and centrifuge for an additional 30 seconds.

5.14 Place each column into a 1.5 ml tube. Add **22 µl of BS Elution Buffer** (white cap) directly to the center of the column matrix and wait for 2 minutes. Centrifuge for 30 seconds at full speed to elute the DNA.

5.15 Elute one more time by transferring the eluate to the column's membrane. Wait 2 minutes, then centrifuge for 30 seconds at full speed. Bisulfite-converted DNA is highly unstable and we recommend to proceed with the enrichment PCR as soon as possible.

Determination of the optimal cycle number for the enrichment PCR

5.16 Prepare the Quantification Mix in a 2 ml tube. An example using a 2X qPCR Master Mix is indicated in Table 14. The calculated volumes include a small excess. If your qPCR Master Mix is provided at another concentration adapt the volumes during the Quantification Mix preparation.

***NOTE**: A single qPCR per library pool is sufficiently accurate, there is no need to perform it in duplicate.*

Table 14.

		Mix for # of samples (µl)			
Components	**Volume /reaction**	**2**	**4**	**8**	**16**
2X qPCR Master Mix	5 µl	12.5	22.5	42.5	82.5
Primer mix (green cap)	0.5 µl	1.3	2.3	4.3	8.3
Water (clear cap)	3.5 µl	8.8	15.8	29.8	57.8

5.17 Transfer **9 µl of the Quantification Mix** to a new 96-well qPCR plate.

5.18 Add **1 µl of each bisulfite converted library** to the qPCR plate. Leave the rest of the samples at 4°C during the quantification.

5.19 Seal the plate, mix by vortexing, centrifuge briefly and place it in a thermocycler. Run the program described in Table 15.

Table 15.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	3 minutes	1
Denaturation	95°C	15 seconds	30
Annealing	60°C	30 seconds	
Extension	72°C	30 seconds	
Hold	4°C	∞	

5.20 Analyse the Ct values. The optimal cycle number for the enrichment PCR is typically Ct – 1, but this might need some optimization depending on your setup.

***NOTE**: The Ct value is highly dependent on the mastermix and the thermocycler you use, as well as the way you analyze the qPCR results. Thus when using the kit for the first time you may need to verify that the Ct-1 rule can be applied in your conditions.*

STEP 6

Enrichment PCR, clean-up and quality control

Enrichment PCR

6.1 Prepare the Amplification Mix as described in Table 16. The volumes presented here include a small excess.

CAUTION: *The BS conversion reagent has to be manipulated under a fume hood.*

Table 16.

Components	Volume /reaction	Mix for # of samples (µl)			
		2	4	8	16
2X MethylTaq Plus Master Mix (green cap)	25 µl	62.5	112.5	212.5	412.5
Primer mix (green cap)	2.5 µl	6.25	11.25	21.25	41.25
Water (clear cap)	3.5 µl	8.75	15.75	29.75	57.75

6.2 Determine the number of amplification runs you need, according to the different cycle numbers you will have to apply. For each run, corresponding to one number of cycles, use a different 96-well PCR plate.

6.3 Add in each well **31 µl of Amplification Mix** and 1**9 µl of a bisulfite converted RRBS library** and mix.

6.4 Incubate in a thermocycler as indicated in Table 17.

Table 17.

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95°C	5 minutes	1
Denaturation	98°C	20 seconds	Ct from the 2nd qPCR -1
Annealing	60°C	15 seconds	
Extension	72°C	45 seconds	
Final extension	72°C	7 minutes	1
Hold	4°C	∞	

Clean-up

6.5 Add **50 µl of AMPure XP Beads** and mix by pipetting at least 10 times.

6.6 Incubate for 15 minutes at room temperature to allow DNA binding to the beads.

6.7 Place the 96-well plate on a 96-well magnet for 5 minutes or until the supernatant appears clear.

6.8 Remove and discard the supernatant.

6.9 Wash twice for 5 seconds with **100 µl of freshly prepared 80% ethanol**.

6.10 Allow the beads to air-dry for 10 minutes.

6.11 Resuspend the beads in **15 µl of Resuspension Buffer** (clear cap) and incubate for 5 minutes to elute the DNA from the beads.

6.12 Place the 96-well plate on a 96-well magnet for 5 minutes or until the supernatant appears clear.

6.13 Transfer the clear supernatant to a new 1.5 ml tube for each pool.

Quality control

6.14 Determine the concentration of your sample by the use of a fluorescence-based assay such as the Qubit High Sensitivity assay (Life Technologies #Q32851).

6.15 Run a part of each library pool on a chip as the Agilent High Sensitivity for BioAnalyzer according to the manufacturer instructions. An example of the expected results for human libraries is shown on figure 6.

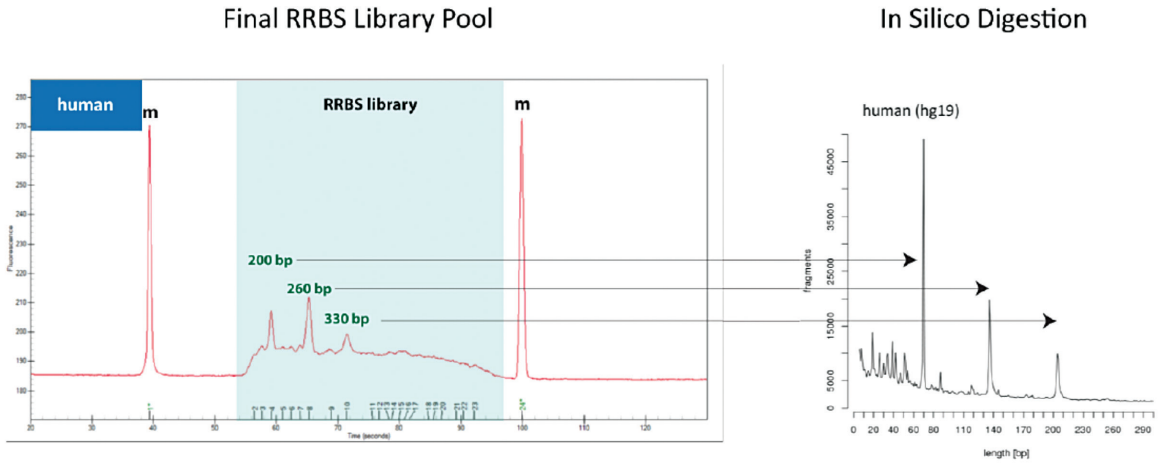


***Figure 6**: Bioanalyzer profile of RRBS library compared to theoretical data obtained in silico.*

***NOTE**: The expected profile of the RRBS library is different for every species, depending on the distribution of CpGs.*

Sequencing recommendations

Single-end sequencing with 50 bp read length is recommended because it is the most cost-effective solution, but it is also possible to use longer reads or paired-end sequencing.

As a consequence of the enzymatic digestion, all RRBS reads are starting by the same bases, which can reduce the efficiency of the clustering during the sequencing run. In order to solve this, sequencing parameters for low diversity libraries should be applied during the sequencing of RRBS libraries. Here are some general guidelines, but we recommend discussing this with the sequencing provider.

Table 18. Recommended parameters for RRBS for various Illumina's sequencers

	MiSeq	HiSeq2000/2500	HiSeq3000/4000	NextSeq500/550
Number of human samples per pool	1	6	8	10
Percentage of Illumina PhiX control	5%	5%	5%	20%
Cluster density	Aim at a 30% beneath the optimal range for the chemistry version and platform			
Software version	RTA 1.17.28 or newer	HCS 2.2.38 or newer	HCS 3.4.0.38 or newer	NCS 1.3 or newer

***NOTES**: When starting with high-quality material and with optimal sequencing parameters, the typical number of reads obtained per sample is around 30 million. Optionally one lane of the flow-cell could be filled with a highÐdiversity library pool to act as an extra control in case of issues with optimal focusing, scanning parameter adjustment and base calling.*

RRBS data analysis recommendations

Read alignment

For the data analysis of an RRBS dataset you have to keep in mind that due to the bisulfite conversion the reads will not mirror the genomic sequence accurately, there could be different versions of the same read based on the the converted base content. Therefore a conventional NGS read aligner like BWA or Bowtie cannot handle bisulfite converted datasets, you have to use specific BS aligners like Bismark or BSMAP (BSMAP has a version specifically designed for RRBS datasets, called RRBSMAP).

An RRBS readset has further distinct features, like the higher duplicate numbers due to lower diversity in comparison of sequencing the whole genome, and because of the dominantly short fragment size - depending on the read length and protocol used - the reads might include an artificially reconstituted MspI site and/or part of the sequencing adapter. Thus RRBS readsets usually need to be trimmed before the alignment; there are many capable tools exist for this purpose, like Trim Galore! or Trimmomatic.

DNA methylation extraction

Once the reads are aligned, the methylation levels of the bases can be extracted. Many aligners, like the aforementioned Bismark and RRBSMAP come with a built-in methylation extractor module that produces this output, along with general methylation statistics. From the output it is easy to proceed with the desired downstream analysis: for example, bedGraph or WIG files can be created which can be viewed in a browser like the UCSC Genome Browser to check the methylation levels visually, or interesting regions can be extracted into BED files based on methylation criteria, and can be annotated for example to see which genes are affected by over or undermethylation in a certain sample.

Spike-in controls

In the Premium RRBS Kit methylated and unmethylated spike-in controls are also available to monitor the efficiency of bisulfite conversion. Because their methylation status is known, they can be used to assess the conversion rate, ie. if you sequence the fully methylated control all reads should align only to the methylated reference sequence if the conversion ratio was 100%. The sequences of these controls are shown in table 19.

Please download our Premium RRBS spike-in controls manual to get the optimal use of our spike-in controls. You can find this document on RRBS product page

Table 19. Sequences of the methylated and unmethylated spike-in controls

Spike-in control	Strand	Sequence (5'-→3')
Unmethylated	Forward	TCGAACGCCCGGTCGCGCGTGTGGAGGATGTCGGTGCTGCAGGGGGCGTTGGAGCCGA AGTAGACCGCGCCGAACGTACGAACGGTCGTTCATATTAGTTATACTGGTTGTCTTGA TGTTGCTTCTCCTAATTT
	Reverse	AAATTAGGAGAAGCAACATCAAGACAACCAGTATAACTAATATGAACGACCGTTCGTA CGTTCGGCGCGGTCTACTTCGGCTCCAACGCCCCCTGCAGCACCGACATCCTCCACAC GCGCGACCGGGCGTTCGA
Methylated	Forward	TmCGAGAGGmCAGTGGGTGmCTGTmCGGmCGAAGTAGGGGTTGAmCGATGmCmCGGAmCmC GTTmCGTAmCGTAmCGmCGmCGAmCmCGmCTAmCTAATTTTAGT TGTGTTTmCTGGTmCmCTATTGATmCATTATmCTTGC
	Reverse	GmCAAGATAATGATCAATAGGAmCmCAGAAACACAAmCTAAAATTAGTAGmCGGTmCG mCGmCGTAmCGTAmCGAAmCGGTmCmCGGmCATmCGTCAAmCCCCTAmCCTTmCGCmCG ACAGmCAmCmCmCAmCTGmCmCTmCTmCGA

Useful Links:

Bismark: *http://www.bioinformatics.babraham.ac.uk/projects/bismark*

BSMAP: *https://code.google.com/p/bsmap*

RRBSMAP: *http://omictools.com/rrbsmap-s996.html*

Trim Galore!: *http://www.bioinformatics.babraham.ac.uk/projects/trim_galore*

Trimmomatic: *http://www.usadellab.org/cms/index.php?page=trimmomatic*

UCSC Genome Browser: *http://genome.ucsc.edu/cgi-bin/hgGateway*

Additional Protocol

Manual Calculation of volumes for pooling

1.1 Copy the following informations concerning your samples in a new Excel file:

- Column A - Position in the 96-well plate (A01 to H12)
- Column B - Sample name
- Column C - Adaptor number (17, 24, 26, 28, 29...)
- Column D - Ct values from your qPCR analysis software (replicate 1)
- Column E - Ct values from your qPCR analysis software (replicate 2)

1.2 Calculate the average Ct value in column F.

1.3 Use conditional formatting on columns D, E and F: Home › Conditionnal Formatting › Color Scales (optional).

1.4 Identify projects that should be sequenced separately and color the rows accordingly.

1.5 For each project, do the following:

- Select all rows belonging to the project and sort them by column F (average Ct value) in descending order.

***NOTE**: This ensures that samples with equal Ct values are pooled.*

- Decide on pools of six samples and color their rows accordingly. In the same pool, samples must have similar Ct values and different adaptors ID.

***NOTE**: If the project sample number is not a multiple of six, five or seven samples are also acceptable. For a pool of 7 we recommend to use the samples with the smallest Ct values and for a pool of 5 the samples with the highest Ct values.*

- For each pool of samples, identify adaptor barcodes clashes by selecting the adaptor IDs in column C and applying Conditional Formatting › Highlight Cells Rules › Duplicate Values.

- Resolve adaptor clashes by exchanging rows via cut and paste. Ct values should be as similar as possible.
- Sort each pool by column F (swapping rows might have messed up the order).
- In column G, calculate the difference in Ct values (dCt) for each sample as: highest average Ct in pool – average Ct of the sample (Ctmax-Ctsample). Use $ signs to fix the Ctmax in the formula inside each pool.
- Calculate the pooling volume in column H as 17*2^(-dCt).
- In column I calculate the volume of water to add to each pool to reach 120 µl. You can use the following formula: water volume = 120-(pool vol 1+pool vol 2+pool vol 3+pool vol 4+pool vol 5+pool vol 6) where pool vol is the pooling volume of each sample.
- In column J (pool number), specify the pool (e.g. P01) in which you will combine the samples.

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