

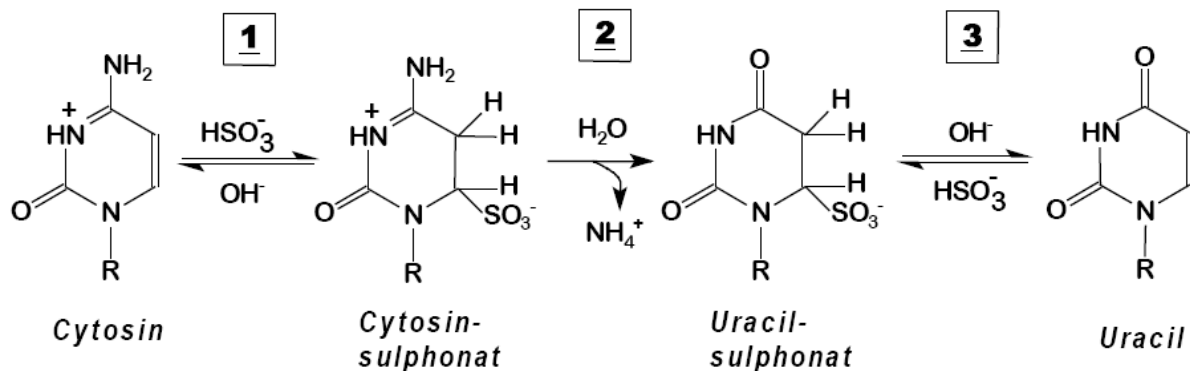
## Schumacher's guide for: Bisulfite conversion of DNA for methylation fine-mapping.

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Version 1.b; Last modified 10<sup>th</sup> of February 2009

### 1. Introduction

The method described in detail below is based on traditional bisulfite modifications, however several new improvements and many comments are included. In this protocol, sodium bisulphite is used to convert cytosine residues to uracil residues in single-stranded DNA, under conditions whereby 5-methylcytosine remains non-reactive (Fig. 1). The converted DNA is then amplified with specific primers (where uracil corresponds to thymine in its base pairing behaviour), followed by downstream detection techniques, such as sequencing (with or without cloning) or microarrays. All the cytosine residues remaining in the interrogated sequence represent methylated cytosines in the genome.

This protocol can also be applied for small sample sizes of a few nanograms or below. The standard protocol uses 500 ng. The eluted DNA is suited for all techniques currently used for the analysis of DNA methylation; including PCR, real-time PCR, MSP-PCR, bisulfite sequencing, COBRA, microarrays and pyrosequencing.



**Fig. 1:** Step 1: Sulphonation; Step 2: hydrolytic deamination and Step 3: alkali-desulphonation. Bisulfite conversion is performed under acidic conditions and preferentially deaminates cytosine in a nucleophilic attack whilst the methyl group on 5-methylcytosine is protecting the amino group from the deamination.

Advantages of this protocol: Under known standard conditions at least 80% (up to ~98%) of the DNA is usually degraded (due to non-specific degradation under acidic conditions), which poses severe problems for subsequent analyses. Hence, this protocol was developed to improved several critical steps, which leads to higher recovery rates, a higher conversion rate (99.5-100%) and a much faster procedure. This method uses the presence of additional denaturing reagents and scavengers.

## 2. Materials

### 2.1 Required chemicals:

Chemical	Company
Sodium bisulfite (mixture of NaHSO <sub>3</sub> and Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub> )	Sigma, # 243973
Hydroquinone	Sigma, #H9003
NaOH pellets	Sigma
Guanidine Hydrochloride	Fluka #50937 (25 ml)
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonsäure (called Trolox)	Sigma-Aldrich, #23,881-3 (1 g)
Tetraethylammoniumchloride (TAC)	Sigma, #T2265 (25 g)
Tetraethylenepentaminepenta-hydrochloride (Also known as TETRAEN)	Sigma-Aldrich, #375683 (10 g)
Degassed water	

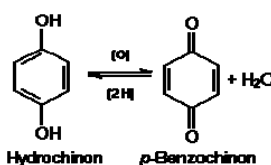
### 2.2 Preparation of the solutions:

- First, degas ddH<sub>2</sub>O by applying a vacuum (e.g. by using a compressor or water-pump) on a flask containing about 50-100 ml water. Stir at the same time for about 30 minutes.

*Note:* Although it is not mentioned in most bisulfite protocols, it is highly recommend to degas the water since the oxygen (O<sub>2</sub>) in the water reacts with the bisulfite, reducing the bisulfite conversion rate significantly. The oxygen slips between the loose hydrogen-bonded network of water molecules without forcing them apart. The oxygen is then caged by water molecules, which weakly pin it in place. For example, at room temperature, about 8.24 mg oxygen is dissolved in every litre of water.

- 3M NaOH: Prepare freshly by dissolving 3 g NaOH pellets (MW: 40g) in 25 ml of the degassed water.
- 320 mM Hydrochinone (MW: 110.1): Dissolve 0.88g hydrochinone in 25 ml of the degassed H<sub>2</sub>O. Wrap in aluminium foil and heat to 50°C in a waterbath or incubator.

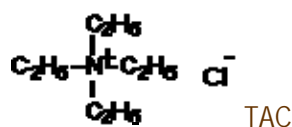
*Note A:* This unsaturated di-keton is an alkalizing antioxidant and forms the energetically similar *p*-benzochinone, thereby working as inhibitor of the unwanted bisulfite-oxidation reaction. Additionally, it prevents DNA strand breakage that can occur through depurination.



*Note B:* Instead of Hydrochinone it is also possible to use quinol (dihydroxy benzene), which is a white crystalline substance, C<sub>6</sub>H<sub>4</sub>(OH)<sub>2</sub> and is obtained by the reduction of quinone. It is a diacid phenol, resembling, and metameric with, pyrocatechin and resorcin.

- 100 mM Tetraethylammonium-chloride (TAC; MW: 165.7): Dissolve 0.42 g in 25 ml of the degassed H<sub>2</sub>O.

*Note:* TAC is a quaternary ammonium compound used as a catalyst of the conversion reaction. Other quaternary amine compounds (i.e. quaternary alkyl ammonium salts) include quaternary alkyl ammonium chlorides such as quaternary methyl ammonium bromide, quaternary alkyl ammonium bromides, quaternary ammonium chlorides, tetraethyl ammonium hydroxide, tetraethyl ammonium chloride, tetrabutyl ammonium chloride, tetrabutyl ammonium bromide, among others.



*Note:* After using the chemicals, if possible, sodium bisulfite and hydroquinone powders should be stored under vacuum or non-reactive nitrogen.

## 3. Method



### 3.2 Preparation of the DNA

First of all, ensure that only high-quality DNA (preferentially phenol-chloroform extracted if applicable) is used for the conversion reaction. Other DNA sources will work too, but may be more difficult to analyze.

- Split your DNA in four tubes.
- For each tube, digest ca. 250 ng to 2 µg genomic DNA with a restriction enzyme outside the region of interest for 2h. A good combination is the following: Tube 1: EcoRI; Tube 2: PvuII; Tube 3: HindIII and Tube 4: No digest.

*Note A:* The purpose of digesting the DNA is to lower the viscosity and hence to facilitate a complete DNA denaturation by creating single stranded DNA molecules. Sodium bisulfite can only react with pyrimidines that are not involved in base-pairing or for inaccessible 3D-structures. By using several different enzymes, only one BS preparation has to be performed, even if many different primer combinations will be used in downstream experiments. If only one enzyme would be used, the danger of having a restriction site within your PCR product would be too big.

*Note B:* This enzyme combination is most cost-efficient and reliable, i.e the fragment sizes are appropriate.

- For the 4<sup>th</sup> tube, either sonicate the DNA briefly or press the DNA to a small syringe needle (e.g. a 19G or 21G syringe needle) to chop it to smaller pieces. Do not add any enzyme to this sample.
- Heat-inactivate the restriction enzymes for at least 20 minutes according to the manufacturers guidelines.
- Pool the DNA from all 4 tubes and mix. The DNA is now ready to be used for BS-conversion.

*Note A:* If enough DNA is present, I strongly recommend purifying/precipitating the DNA before proceeding to the BS reaction. Purification ensures that the DNA has a higher conversion rate (i.e. bound enzymes or remaining RNA molecules may interfere with the BS-conversion).

*Note B:* Some protocols use special matrices to attach the DNA during BS conversion (e.g. glass beads, agarose, silicon oxide, for example glass fleeces or fibers or magnetic glass particles among others). In the case of low-melting point agarose, the DNA is automatically kept in single-stranded conformation, therefore ensuring conversion. However, with the presented protocol it is not necessary to include a 3D-matrix, since the DNA will be kept single stranded in solution.

### 3.3 Bisulfite conversion

- Heat 500 ng of the DNA for 3 minutes to 95°C in a thermal-cycler or waterbath.
- Chill on ice to keep the DNA single stranded.

*Note:* It is not necessary to add NaOH to denature the DNA (but it also doesn't hurt).

- Add 100 µl of the freshly prepared bisulfite solution to the DNA sample.

*Note A:* Since the bisulfite reaction is not overlaid with mineral oil and the volume is relatively large, a thermal-cycler with a heated lid should be used. It is also important to use PCR tubes that really close tightly.

*Note B:* To ensure complete denaturation, no more than 2 µg of starting material should be used.

- Close tightly and heat the sample in a thermocycler with these conditions: [30 sec. 95°C → 20 min 58°C → 10 sec 95°C → 20 min 58°C → 10 sec 95°C → 20 min 58°C → 10 sec 95°C → 20 min 58°C →]. → hold at 20°C.

*Note A (Temperature):* Full cytosine deamination can be achieved under several time/temperature combinations. The sulphonation step itself is exothermic, however the overall deamination rate increases with raising temperature. I recommend a reaction temperature of 55 to 60°C, although higher temperatures are also possible. The risk with increasing the temperature is that also a higher degree of the DNA will degrade. Since this protocol is optimized for increased denaturation (remember: double-stranded DNA is non-reactive to bisulfite treatment), it seems not necessary to expose the bisulfite DNA to very high temperatures.

*Note B (Conversion time):* Since DNA degradation increases with time (other parameters: oxidative decay under acidic conditions, bisulfite concentration and reaction temperature) it is evident to shorten the incubation time as much as possible. In our lab we could produce PCR products from DNA that was bisulfite treated for merely 30 minutes. Yet, to ensure a complete conversion of all non-methylated cytosines, we

usually increase the conversion time up to 1.5 hours, using a thermo-cycler. Longer incubation periods than 3 hours are not recommended since bisulfite will also deaminate methylcytosine if it has enough time.

**Note C:** The converted DNA should be processed as soon as possible, but in emergency, the converted DNA may be left in the thermal-cycler overnight without any significant loss of performance.

**Note D:** It is barely known that under certain conditions, also Thymine reacts with bisulfite, although with lower intensity, producing a dihydrothymine-6-sulfonate structure.

**Note E:** After bisulphite treatment, the strands are no longer complementary and strand specific primers can be designed. This allows amplification, cloning + sequencing of the individual strands (sense and antisense) to determine their methylation patterns. It can be assumed that methylation of both strands is identical (due to symmetric de novo methylation of a newly synthesized strand by the methyltransferase). Hence, usually there is no such need to sequence both strands. We sequenced many genomic regions to study this situation and could not find any significant difference between the strands for all interrogated genes.

### 3.4 Purification, Desulphonation and Neutralization

There are many ways of purifying the BS-DNA, but we will only cover one method that does not include the desulphonation step in a column, which are used by some kits. On one hand, to desulphonate during the purification has the benefit that the procedure is faster, on the other hand there is the risk that the desulphonation may be incomplete (e.g. due to the fact that the DNA may be inaccessible in the matrix or because it is difficult to heat the sample to the required temperature of 42°C) or that the column gets damaged which will lead to the loss of the BS-DNA sample. One has to keep in mind that the DNA has to be single stranded for the desulphonation, however in the denatured form it will likely slip through the silica matrix of a conventional purification column. A good purification is necessary to remove the bisulfite salt and the other chemicals used in the conversion process that may inhibit downstream procedures.

#### Purification (using the NucleoSpin Extract II kit)

- Add 250 µl NT binding buffer to the BS-sample, mix and then add the mixture to a column.  
*Note:* NucleoSpin columns work practically the same as the more widely used Qiagen columns but are much cheaper.
- Centrifuge 1 min at 11.000g.
- Discard flow-through and wash with 600 µl buffer NT3.
- Dry silica membrane for 2 min at 11.000 g.
- Elute in 50 µl H<sub>2</sub>O (not the NT buffer).

#### Desulphonation

- Denature the BS-DNA by adding 5 µl 3M NaOH [ $\rightarrow$  0.3M] and incubating it at 42°C for 20 minutes.

**Note A:** Most protocols use 37°C; however, it was shown that an increase of the incubation temperature to 42°C is necessary to obtain full Denaturation/conversion of GC-rich DNA regions such as CpG islands.

**Note B:** This alkali treatment with NaOH is necessary to remove the bisulfite adduct from the uracil ring (uracil sulphonate). It is important that this desulphonation step is complete.

- Neutralise solution by addition of 0.25x volume of 5 M ammonium acetate (pH 8.0). Ammonium acetate will desulphonate the uracil sulphonate to uracil. The sulphonated uracil derivative is inhibitory to any downstream BS-PCR reaction.
- Precipitate the BS-DNA for 1-2 hours by adding 3x volume of >95% Ethanol.

**Note A:** In case you work with low DNA concentrations (<200 ng), the addition of a carrier molecule (e.g. tRNA or Glycogen) in the precipitation is essential for the complete recovery of the bisulfite treated DNA, followed by two volumes Ethanol.

**Note B:** Also, in case of very low amounts of DNA, it is recommended to use Isopropanol instead of Ethanol for precipitation. Isopropanol has the advantage of precipitating DNA at lower concentrations. Instead of mixing two volumes of ethanol with the DNA-salt solution, addition of one volume of isopropanol will suffice.

*Note C: Many older manuals indicate that precipitation should be allowed to occur in the cold (e.g. -20°C). This has been shown to be unnecessary, especially when using ammonium acetate.*

*Note D: Check the pH of your salt as it may affect the degree of precipitation significantly.*

- Centrifuge for 20 min and then discard the supernatant by inverting the tube (carefully !) on a paper towel.
- Add 500 µl of 75% ethanol, centrifuge for an additional 10 min, remove the supernatant again, and then air-dry the samples (or use a speedvac). Do not dry the pellet too long as it will be difficult to resolve.
- Resuspend in 50 µl elution buffer (e.g. TE or use Column elution buffer).

*Note: A following BS-PCR will work even better if the BS-DNA is eluted in water. However, this will, of course, decrease the lifetime of the converted DNA dramatically.*

### 3.5 Storage conditions of bisulfite-treated DNA.

The DNA can be stored at -20°C. Different labs report very different times for how long the DNA can be used, from several days to up to 2 years. In my experience, bisulfite treated DNA can be stored for at least 1 month without affecting the PCR yield significantly, yet –the fresher, the better. I recommend to aliquot the BS-product into smaller samples which will be thawed only one by one when used. It may also be advisable to add the cyclic ether Tetrahydrofurfuryl alcohol (THFA, a solvent that is sometimes used in make up), to the storage/elution buffer, which helps partly to protect DNA degradation. THFA is a clear, colorless liquid that is used extensively in various industries as a high-purity, water miscible solvent, and as a chemical intermediate. (However, it is unknown to me what percentage of THFA is best. As a solvent it is often used with 2-20% of the total volume).

*Note: Bisulfite treated DNA can not be easily quantified by spectrometry. BS-DNA is a mostly single stranded mixture of nucleic acids that has features of DNA and RNA. A good method to get some idea how much DNA is left is to run a few µl on an agarose gel. For more precise measurements, a HPLC analysis is required.*

### Your Notes:

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