Step 3: Protocol of sodium bisulfite conversion of DNA

Protocol

The bisulfite conversion of DNA is carried out with the EZ DNA Modification kit (Zymo Research). The kit can be stored at room temperature (15-25°C) for up to 12 months.

Equipment and Reagents to Be Supplied by User:

■ EZ DNA Methylation Kit (Zymo Research), (Ozyme_catalog Nos. D5001 (50) & D5002 (200))

■ Tube C (pink cap) of Urodiag® Multiplex PCR Kit (OncoDiag) = Methylated human control DNA

■ Absolute ethanol (96-100%)

■ 1.5 ml microcentrifuge tubes and MicroAmp 8-Tube strip, 0.2 ml (ThermoFisher Scientific_N8010580) and 8-Cap Strip (ThermoFisher Scientific_N8010535)

■ Micropipets and Pipet tips with aerosol barrier

■ Microcentrifuge (with rotor for 1.5 ml tubes)

■ Vortexer

■ Water bath or heating block (37°C and 50°C) or thermocycler (50°C)

Buffer Preparation:

Preparation of the conversion solution (CT-Conversion tube) – The **CT Conversion** tube supplied is a solid mixture. The conversion solution must be prepared as follows:

1. Add 750 µl sterile water and 210 µl of **M-Dilution Buffer** to a tube of **CT Conversion** tube.

2. Mix at room temperature (15-25°C) with frequent vortexing for 5-10 minutes.

   Note: *It is normal to see trace amonts of undissolved reagent in the CT-Conversion tube. The CT-Conversion solution is designed for 10 separate reactions and must be used immediately following preparation (recommended) or stored for one week at +4°C and one month at -20°C.*

Preparation of M-Wash Buffer– Add 24 ml of ethanol to the 6 ml **M-Wash Buffer** concentrate (D5001) or 96 ml of ethanol to the 24 ml **M-Wash Buffer** concentrate (D5002) before use.

1. Add 5 µl of M-Dilution Buffer to the DNA sample and adjust the final volume to 50 µl with sterile water (see table below) in a 1.5 ml microcentrifuge tube.

	Tube C	Patient	
	Conc. 1.25 ng/µl	Conc. 1.25 ng/µl	Conc. 0.625-1.25 ng/µl
DNA sample	24 µl (30 ng)	24 µl (30 ng)	X µl (15- 30 ng)
M-Dilution Buffer	5 µl	5 µl	5 µl
Sterile water	21 µl	21 µl	X µl (adjust the volume to 50 µl)
Mix the sample by pipetting up and down.			

Note: Optimal patient DNA input is 30 ng *(24 µl of DNA sample at 1.25 ng/µl). DNA amount of 15-30 ng (0.625-1.25 ng/µl) can be also used.*

2. Incubate the sample at 37°C for 15 minutes (water bath or heating block).

3. After the above incubation, centrifuge briefly the tube for 2 seconds to remove drops from the lid or sides.

4. Add 100 µl of the conversion solution to DNA sample and mix by pipetting up and down 10 times.

5. Incubate the sample in the dark at 50°C for 15 hours and 30 minutes in water bath or heating block.

6. Centrifuge the tube for 2 seconds to remove drops from the lid or sides.

7. Incubate the sample at 4°C (e.g., on ice or fridge) for at least 10 minutes.

   Note: Alternative incubation condition. *For steps 5 & 6 of the protocol, transfer the sample in a 0.2 ml tube (e.g., MicroAmp 8-Tube strip and 8-Cap Strip, applied Biosystems).*

   Incubate the sample in a thermocycler at 50°C for 15 hours and 30 minutes, then « hold » at 4°C.

8. Add 400 µl of **M-Binding Buffer** to a **Zymo-Spin IC Column** and place the column into a provided **Collection tube**.

9. Load the sample (~150 µl) into the Zymo-Spin IC Column containing the M-Binding Buffer. Close the column and mix by inverting the column at least 5 times.

10. Centrifuge the column at full speed (14,000 rpm, 16,800 rcf) for 30 seconds. Discard the flow-through.

11. Add 100 µl of **M-Wash Buffer** to the column. Close the column and centrifuge at full speed for 30 seconds.

12. Add 200 µl of **M-Desulphonation Buffer** to the column and let stand at room temperature for 20 minutes. After the incubation, centrifuge the column at full speed for 30 seconds.

13. Add 200 µl of **M-Wash Buffer** to the column. Close the column and centrifuge at full speed for 30 seconds. Add another 200 µl of **M-Wash Buffer** to the column and centrifuge at full speed for an additional 30 seconds.

14. Place the column into a 1.5 ml microcentrifuge tube and centrifuge at full speed for 30 seconds to eliminate possible traces of M-Wash Buffer, which will be removed by pipetting.

15. Add 10 µl of **M-Elution Buffer** directly to the column matrix, and let stand at room temperature for 5 minutes. Centrifuge the microcentrifuge tube at full speed for 30 seconds to elute the bisulfite modified DNA.

The bisulfite-converted DNA solution is ready for immediately analysis or can be stored at -20°C for later use.