Bisulfite pyrosequencing protocol for Human sperm DNA

Version 2

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Abstract

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Protocol

Human Sperm DNA Isolation procedure

Step 1.
Extraction buffer of sperm DNA

AMOUNT
21.2 ml Additional info: 6mol/L guanidine thiocyanate
AMOUNT
600 µl Additional info: 5mol/L NaCl
AMOUNT
1 ml Additional info: 30% N-lauroylsarcosine sodium salt
AMOUNT
3 ml Additional info: 1mol/l dithiothreitol (DTT)
AMOUNT
600 µl Additional info: 10mg/ml proteinase K
AMOUNT
3.6 ml Additional info: Doubly deionized water

Human Sperm DNA Isolation procedure

Step 2.
Add 150 µl of semen to 1.5 ml micro-centrifuge tube.

Human Sperm DNA Isolation procedure

Step 3.
Wash with 1 ml of PBS (0.1 mol/L).

Human Sperm DNA Isolation procedure

Step 4.
Centrifuge at 1500 ×g for 10 min at 4°C.

Human Sperm DNA Isolation procedure

Step 5.
Repeat 2 times washing as step 3-4.

Human Sperm DNA Isolation procedure

Step 6.
Add 0.5 ml extraction buffer into sperm pelle

Human Sperm DNA Isolation procedure

Step 7.
Place in a 65°C water bath for 12 h.

Human Sperm DNA Isolation procedure

Step 8.
Cool at room temperature.

Human Sperm DNA Isolation procedure

Step 9.
Add 10 µl of RNase A (10 mg/ml), mix by pulse-vortexing for 15s, and incubate for 10 min at room temperature.

Human Sperm DNA Isolation procedure

Step 10.
Briefly centrifuge the tube.

Human Sperm DNA Isolation procedure

Step 11.
Add 510 µl of isopropanol and centrifuge at 10000 ×g for 10 min at 4°C.

Human Sperm DNA Isolation procedure

Step 12.
Add 800 µl of ethanol (75%), and reverse mixing for dozens of times.

Human Sperm DNA Isolation procedure

Step 13.
Incubate for 12 h at - 20°C.

Human Sperm DNA Isolation procedure

Step 14.
Centrifuge at 10000 ×g for 10 min at 4°C. Then dry sample at room temperature.

Human Sperm DNA Isolation procedure

Step 15.
Sperm DNA is dissolved in 50 µl of Elution Buffer.

Human Sperm DNA Isolation procedure

Step 16.
Incubate in a 65°C water bath for 2 h.

Procedure for bisulfite treatment

Step 17.
Add 130 µl of the CT Conversion Reagent solution to 1000 ng of your DNA sample in a PCR tube.

Procedure for bisulfite treatment

Step 18.
Place the sample tube in a thermal cycler and perform the following steps*: ① 98°C for 10 minutes; ② 64°C for 2.5 hours; ③ 4°C.

Procedure for bisulfite treatment

Step 19.
Add 600 µl of M-Binding Buffer into a Zymo-Spin IC™ Column and place the column into a provided Collection Tube.

Procedure for bisulfite treatment

Step 20.
Load sample (from Step 2) into the Zymo-Spin IC™ Column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.

Procedure for bisulfite treatment

Step 21.

Centrifuge at full speed (>10,000 xg) for 30 seconds. Discard the flow-through.

Procedure for bisulfite treatment

Step 22.

Add 100 µl of M-Wash Buffer to the column. Spin at full speed for 30 seconds.

Procedure for bisulfite treatment

Step 23.

Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20 °C – 30 °C) for 15-20 minutes. After the incubation, spin at full speed for 30 seconds.

Procedure for bisulfite treatment

Step 24.

Add 200 µl of M-Wash Buffer to the column. Spin at full speed for 30 seconds. Add another 200 µl of M-Wash Buffer and spin at top speed for 30 seconds.

Procedure for bisulfite treatment

Step 25.

Add 8 µl of M-Elution Buffer directly to the column matrix. Place the column into a 1.5 ml tube. Spin briefly at full speed to elute the DNA. Add 7 µl of M-Elution Buffer and additional repeated 1 time eluting was subsequently performed.

Procedure for bisulfite treatment

Step 26.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use.

PCR amplification of bisulfite-treated sperm DNAs

Step 27.

All reactions are performed with provided PCR mixtures (total volume at 25µl) provided in Table 1. Each reaction also contain 2.5 ul of CoralLoad Concentrate (10x) for checking amplicons on an agarose gel.

cmd COMMAND (Table 1 Components of PCR mixtures)

Components Volume (µl) Final concentration PyroMark PCR Master Mix, 2× 12.5 1× CoraLoad Concentrate, 10× 2.5 1× Q-solution, 5× 5 1× primer forward (10 uM) 0.5 0.2uM primer reverse (10 uM) 0.5 0.2uM Template DNA 50ng Final volume 25

PCR amplification of bisulfite-treated sperm DNAs

Step 28.

PCR and pyrosequencing primers are designed and listed in Table 2. Reverse primer is conjugated to biotin.

cmd COMMAND (Table 2 Primer sequences for PCR amplification)

DMR Forward primer *Reverse primer Sequencing primer H19 GTATATGGGTATTTTTTGGAGGT ATATCCTATTCCCAAATAA TGGTTGTAGTTGTGGAAT MEG3 GGGATTTTTGTTTTTTTTTGTAGTAGG CCAACCAAAACCCACCTATAAC TTTGGGGTTGGGGTT PEG3 TAATGAAAGTGTTTGAGATTTGTTG CCTATAAACAACCCCACACCTATAC GGGGGTAGTTGAGGTT

PCR amplification of bisulfite-treated sperm DNAs

Step 29.

The PCR conditions are used as following: 94 °C for 15 min, followed by 45 cycles of 94 °C, 30 s, 56 °C, 30 s, 72°C, 30 s, and by a 72 °C final extension step for 10min.

Pyrosequencing

Step 30.

Add 40 µl of Binding Buffer, 3 µl of streptavidin-sepharose beads and 17 µl DDW into 20 µl of PCR

products.

Pyrosequencing

Step 31.

Seal film and shake at 1400 rpm for 10 min at room temperature.

Pyrosequencing

Step 32.

PCR products on streptavidin-sepharose beads are washed with ethanol (10%) for 5s.

Pyrosequencing

Step 33.

Place sample (from step 3) into denaturation solution for 5s.

Pyrosequencing

Step 34.

Place sample (from step 4) into Wash Buffer for 10s for getting purified biotinylated single stranded PCR products. These single stranded PCR products are isolated using the Pyrosequencing Work Station.

Pyrosequencing

Step 35.

Transfer purified biotinylated single stranded PCR products into PSQ 96 Plate Low with 40 µl annealing buffer and 1.6 µl sequencing primer (10µmol/L).

Pyrosequencing

Step 36.

Heat PSQ 96 Plate Low at 80 ℃ for 2 minutes.

Pyrosequencing

Step 37.

Undergo pyrosequencing on a Pyromark Q96 MD pyrosequencing instrument and sequence using PyroMark Gold Q96 kit.

Pyrosequencing

Step 38.

The degree of methylation at each CpG site is determined using PyroMark CpG Software (Biotage AB, Uppsala, Sweden). Pyrosequencing assays are performed in duplicate in sequential runs (technical replicates), and the values show represent the mean methylation for each individual CpG site.