FeBABE-mediated hydroxyl radical cleavage assay

DNA hybridization

1. DNA containing at least two phosphorothioates at alternate bases can be obtained from Invitrogen.
   The following codes are used to modify bases

   A-Phosphorothioate =F
   C-Phosphorothioate =O
   G-Phosphorothioate =E
   T-Phosphorothioate =Z

2. All oligos mentioned here have the same bottom strand annealed to different top strands. The top strands contain biotin on the 5' end.
3. Anneal oligos in 100 µl of 1X TE+ 0.1M NaCl by heating at 100°C for 5 mins and then slow cooling overnight. Add 10µg of biotinylated top strand and 20µg of the bottom strand so that it is always forced into the hybridized state
4. Ethanol precipitate the annealed oligo by adding 6 µl of 3M NaOAc and 300 µl of 100% EtOH to 100 ul of annealed oligo. Store the pellet at -20°C for at least 4 hours and then wash with ~500 µl of 80% EtOH and dry at room temperature.
5. Resuspend the pellet in 20mM MOPS pH 7.9 to a final concentration of 300 ng/ µl.

Coupling to FeBABE

6. FeBABE is obtained from Dojindo (catalog no: F279-10). Resuspend the yellowish powder in 50 µl of water (to a final concentration of 34mM) and store at -80 °C till needed.
7. The coupling reaction is done in 20mM MOPS pH 7.9. To 600 ng of the oligo, 3.5 µl of 34mM of FeBABE is added.

   2 µl hybridized biotinylated oligo

   3.5 µl of FeBABE (34mM)

   0.8 µl of 0.5M MOPS pH7.9

13.7 µl of H_2O

8. Incubate at 50°C for 16 hours (Put the reaction in the thermocycler and use the program INC under user ROS)

Coupling FeBABE conjugated DNA to beads

9. Wash 40 µl of Dynabeads M-280 Streptavidin (catalog no: 112.05D) for each reaction with 1X B&W buffer (manufacturer recommended) twice and once with 20mM MOPS pH 7.9

10. Pull down the beads on a magnet and remove the washes. Resuspend the beads in 300 µl of 20mM MOPS pH7.9

11. Add the FeBABE-coupled DNA to the beads and let it incubate by rotating at 4°C for 4 hours

12. Then pull down the beads, remove the supernatant and wash the beads with 20mM MOPS pH7.9 twice to get rid of the unbound DNA and the excess FeBABE reagent.

13. Then, wash the beads once with the final reaction buffer (1X Reaction buffer -DTT).

FEBABE cleavage reaction

14. Pull down and remove the wash. Resuspend the beads in 300 µl of Reaction mix+water (containing DTT).

15. Add 6 µl of 1 µM TBP (20nM final concentration). Incubate the tubes on a rotator at room temperature for 20 minutes.

16. Pull down the beads using the magnet and remove the flow-through.

17. Resuspend the beads in 285 µl of Reaction mix+water. Add 15 µl of 145nM Mot1 and incubate at room temperature on the rotator for 30 minutes.

18. Then pull down the beads and remove the supernatant.

19. Wash the beads with 1 ml of 1X Reaction buffer (-DTT) once. (If reactions need to be split, this is a good point to split it.)

20. Pull down the beads on the magnet and remove the wash completely.

21. Resuspend the beads in 7.5 µL of 1X Reaction buffer (-DTT). Add 2.5 µL of 50% glycerol to each. Spin down quickly to bring down the beads (The glycerol is added to localize the free radicals; DTT is a radical scavenger, so you don't want it at this stage.)

22. To do the reaction, add 1.25 µL of 50mM sodium ascorbate. Mix and then quickly add 1.25 µL of 50mM H_2O_2/10mM EDTA. React for 5 minutes at 37°C (This can vary according to the cleavage required. I have tried 5 mins at 30°C or 37°C)

23. Quench with 6 µL of 4X SB with 1 µL of 1M DTT.

24. Separate the cleaved fragments using SDS-PAGE (I used 14% gels for Mot1). Western blot using an antibody that recognizes one terminus of the protein (For Mot1, I used the antibody that recognized the C terminus (Auble et al., 1997))

1X binding and washing (B&W) buffer

5mM Tris HCl pH7.5

0.5mM EDTA

1M NaCl

5X Reaction buffer (same as 5X binding buffer)

20% glycerol	400µL of 50% glycerol
20mM Tris-Cl, pH 8	20µL of 1M
300mM KCl	300µL of 1M
25mM MgCl2	25 µL of 1M
500mg/ml BSA	10 µL of 50mg/ml
	245 µL water

1X Reaction buffer (for washes)

Dilute 5X reaction buffer in water to 1X

Reaction mix (For 300 µL)

60 µL 5X binding buffer

3 µL 100mM DTT

30 µL 1% Brij

Adjust volume with water

50mM H_2O_2/10mM EDTA

974 µL water

20 µL 500mM EDTA

5.7 µL of 30% H_2O_2

50mM ascorbate

99mg of sodium ascorbate in 10mL of water. (You can store the ascorbate at -80°C. But I just remake it every time as it is easier than to thaw it.)

ADP-AlF_4

1 µL of 2mM ADP

0.5 µL of 50mM NaF

0.1 µL of 10 mM $AlCl_3$

Mix well and add 1.6 µL to the reaction

4X SB+DTT

To 6 µL of 4XSB, add 1 µL of 1M DTT (Add that to quench the reaction)