Protocol for demethylation of an m6A-containing RNA by FTO RNA Demethylase (NEB #M0616)

Overview

1. Preparation of Buffer Reagents and Fe(II) Cofactor.

    a. To prepare 1 M Tris HCl, pH 7.5 buffer solution, dissolve 157.60 g of Tris HCl (Tris HCl, Millipore Sigma Catalog # T5941 [M.W. = 157.60 $g.mol^{-1}$]) in 750 mL of Nuclease-free H_2O (NEB #B1500S). Adjust to pH 7.5 using 10 N NaOH (Fisher Scientific Catalog # SS255-1). Adjust final volume to 1 L with Nuclease-free H_2O. Filter sterilize and store at 4ºC.

    b. Dissolve 23 mg α-ketoglutaric acid disodium salt dihydrate (αKG) (Millipore Sigma Catalog # 75892 [M.W. = 226.09 $g.mol^{-1}$]) in 1 mL of Nuclease-free H_2O to generate a 100 mM stock solution. Store at -20ºC. Shelf life 5 months.

    c. Dissolve 20 mg sodium L-ascorbate (L-Asc) (Millipore Sigma Catalog # A7631 [M.W. = 198.11 $g.mol^{-1}$]) in 1 mL of Nuclease-free H_2O to generate a 100 mM stock solution. Store at -20ºC. Shelf life 5 months.

    d. Dissolve 200 mg of ammonium iron(II) sulfate hexahydrate (Fe(II)) (Millipore Sigma Catalog # 203505 [M.W. = 392.14 $g.mol^{-1}$]) in 1 mL of 5 mN sulfuric acid (H_2SO_4) to generate a 500 mM stock solution. Store at -20ºC.
    To prepare 5 mN H_2SO_4, use a graduated glass cylinder to pour 6.7 mL of concentrated H_2SO_4 (Millipore Sigma Catalog # 339741 [MW = 98.08 $g.mol^{-1}$; density = 1.84 g/mL]) into 1043.3 mL of Nuclease-free H_2O.

2. Preparation of 5X FTO RNA Demethylase Buffer.*

Component	Volume (µL)	Final Concentration (mM)
1 M Tris HCl, pH 7.5	100	250
100 mM αKG	20	5
100 mM L-Asc	20	5
Nuclease-free H_2O (NEB #B1500S)	260	-
Total Volume	400	-

* The shelf-life of 5X FTO Buffer is 5 months at –20 °C.

3. Preparation of Fe(II).

When ready to perform the FTO RNA Demethylase activity assay, dilute 1.5 µL of 500 mM Fe(II) stock solution in 998.5 µL of Nuclease-free H_2O to obtain a 750 µM working solution. Prolonged storage of Fe(II) solution at room temperature will result in its oxidation leading to lower FTO RNA Demethylase activity. The 750 µM Fe(II) working solution is discarded after usage.

4. **Protocol:**

   Set up a 25 µL reaction as follows:

Component	25 µL reaction	Final Concentration or Amount
FTO Buffer (5X)	5 µL	1X
EDTA-free m6A-RNA*	100 ng	4 ng/µL
FTO RNA Demethylase	1 µL	1 mg/µL
Fe (II) (750 µM)	2.5 µL	75 µM
Nuclease-free H_2O (NEB #B1500S)	to 25 µL	-

   *It is imperative that the m6A-RNA substrate be EDTA-free. Presence of EDTA will reduce FTO activity.

   The reaction is incubated for 1 hour at 37°C.

5. 1 µl of 0.8 U/mL Proteinase K (NEB #P8107S) is added to the assay solution and the reaction is incubated for 30-60 min at 37°C.

6. The product RNA is purified using Monarch® RNA Cleanup Kit (NEB #T2040), according to instructions.