

the subsequent processing into mature hFXN after translocation into the mitochondria. The FXN fusion protein is described in US Provisional Patent Application No. 62/891,029 and US Patent Application No. 16/942,276, the entire contents of each of which are hereby incorporated herein by reference.

To facilitate development of FXN replacement therapies, methods are needed to accurately and reliably quantify activity of FXN and FXN fusion proteins. For example, it is desirable to quantify activity of various batches of FXN fusion proteins, as a quality control measure of the manufacturing process for producing the FXN fusion proteins. It is also desirable to quantify activity of various batches of FXN fusion proteins to identify appropriate storage conditions for the manufactured batches of FXN fusion proteins. The present invention addresses this unmet need.

SUMMARY

In one aspect, the present disclosure refers to a method for measuring enzymatic activity of a Frataxin (FXN) protein, wherein said enzymatic activity comprises enzymatically catalyzed generation of reactive oxygen species (ROS), said method comprising (a) combining said FXN protein with a reducing agent and a rROS detector compound at a pH of 7.4 to 9, thereby producing an assay mixture; (b) measuring the amount of ROS generated in the assay mixture over time; and (c) determining an enzymatic parameter associated with the ROS generating activity of the FXN protein.

In another aspect, the present disclosure refers to a method for measuring enzymatic activity of a Frataxin (FXN) protein, wherein said enzymatic activity comprises enzymatically catalyzed generation of reactive oxygen species (ROS), said method comprising (a) combining said FXN protein with a reducing agent and a ROS detector compound in the absence of metal ions, thereby producing an assay mixture; (b) measuring the amount of ROS generated in the assay mixture over time; and (c) determining an enzymatic parameter associated with the ROS generating activity of the FXN protein.

In another aspect, the present disclosure refers to a method for measuring enzymatic activity of a Frataxin (FXN) protein, wherein said enzymatic activity comprises enzymatically catalyzed generation of reactive oxygen species (ROS), said method comprising (a) combining said FXN protein with a reducing agent and a ROS detector compound in the absence of iron ions, thereby producing an assay mixture; (b) measuring the amount of ROS generated in the assay mixture over time; and (c) determining an enzymatic parameter associated with the ROS generating activity of the FXN protein.

In another aspect, the present disclosure refers to a method for performing quality control on a sample comprising a Frataxin (FXN) protein, said method comprising measuring enzymatic activity of an FXN protein in accordance with the method as disclosed herein.

In another aspect, the present disclosure refers to a composition for measuring enzymatic activity of an FXN protein, said composition comprising an FXN protein, a reducing agent and a reactive oxygen species (ROS) detector compound, wherein pH of the composition is 7.4 to 9.

5 In another aspect, the present disclosure refers to a composition for measuring enzymatic activity of an FXN protein, said composition comprising an FXN protein, a reducing agent and a reactive oxygen species (ROS) detector compound, wherein the composition is substantially free of metal ions.

10 In another aspect, the present disclosure refers to a composition for measuring enzymatic activity of an FXN protein, said composition comprising an FXN protein, a reducing agent and a reactive oxygen species (ROS) detector compound, wherein the composition is substantially free of iron ions.

15 In another aspect, the present disclosure refers to use of a kit for determining enzymatic activity of an FXN protein, said kit comprising a reducing agent, an ROS detector compound, instructions to combine the reducing agent, the ROS detector compound and the FXN protein to produce an assay mixture; and instructions to determine an enzymatic parameter of the ROS generating activity of the FXN protein in the assay mixture.

20 In another aspect, the present disclosure refers to a method for identifying a compound capable of modulating activity of a Frataxin (FXN) protein, said method comprising the steps of: (i) combining said FXN protein with a test compound, a reducing agent and a reactive oxygen species (ROS) detector compound, thereby producing an assay mixture; (ii) measuring the amount of ROS generated in the assay mixture over time in the presence of the test compound; (iii) determining an enzymatic parameter associated with the ROS generating activity of the FXN protein in the presence of the test compound; (iv)
25 comparing the enzymatic parameter associated with the ROS generating activity of the FXN protein in the presence of the test compound with an enzymatic parameter associated with the ROS generating activity of the FXN protein in the absence of the test compound; and (v) identifying the test compound as a compound capable of modulating enzymatic activity of the FXN protein when the enzymatic parameter associated with the ROS generating activity of
30 the FXN protein in the presence of the test compound is different from the enzymatic parameter associated with the ROS generating activity of the FXN protein in the absence of the test compound.

35 In another aspect, the present disclosure refers to a method for identifying a compound capable of modulating enzymatic activity of a Frataxin (FXN) protein, said method comprising the steps of: (i) combining said FXN protein with a test compound, a reducing agent and a reactive oxygen species (ROS) detector compound, thereby producing an assay mixture; (ii) measuring the amount of ROS generated in the assay mixture over time in the presence of the test compound and determining V_i of the ROS generating activity of the FXN protein (iii) comparing the V_i of the ROS generating activity of the FXN protein in
40 the presence of the test compound with V_i of the ROS generating activity of the FXN protein in the absence of the test compound; and (iv) identifying the test compound as a compound capable of modulating enzymatic activity of the FXN protein when V_i of the ROS generating activity of the FXN protein in the presence of the test compound is different from V_i of the ROS generating activity of the FXN protein in the absence of the test compound.

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In yet another aspect, the present disclosure refers to a method of preparing a pharmaceutical composition comprising an FXN protein, the method comprising measuring enzymatic activity of the FXN protein according to the method as disclosed herein, and formulating the FXN protein, thereby preparing the pharmaceutical composition.

5 Accordingly, in some examples the present disclosure provides a method for measuring activity of a Frataxin (FXN) protein, the method comprising: combining the FXN protein with a reducing agent and a reactive oxygen species (ROS) detector compound, thereby producing an assay mixture, wherein the FXN protein facilitates oxidation of the
10 reducing agent and generation of ROS, such that the amount of ROS generated is higher than the amount of ROS generated in an assay mixture comprising the reducing agent and the ROS detector compound without the FXN protein.

 In some examples, the present disclosure provides a method for measuring activity of a Frataxin (FXN) protein, the method comprising combining the FXN protein with a reducing
15 agent and a reactive oxygen species (ROS) detector compound at a pH of greater than about 7.4, thereby producing an assay mixture, wherein the FXN protein facilitates oxidation of the reducing agent and generation of ROS, such that the amount of ROS generated is higher than the amount of ROS generated in an assay mixture comprising the reducing agent and the ROS detector compound without the FXN protein.

 In some examples, the method comprises combining the FXN protein with a reducing
20 agent and a reactive oxygen species (ROS) detector compound at a pH of about 7.9 or greater.

CLAIMS

What is claimed is:

1. A method for measuring enzymatic activity of a Frataxin (FXN) protein, wherein said enzymatic activity comprises enzymatically catalyzed generation of reactive oxygen species
5 (ROS), said method comprising
 - (a) combining said FXN protein with a reducing agent and a ROS detector compound at a pH of 7.4 to 9, thereby producing an assay mixture;
 - (b) measuring the amount of ROS generated in the assay mixture over time; and
 - (c) determining an enzymatic parameter associated with the ROS generating activity
10 of the FXN protein.
2. A method for measuring enzymatic activity of a Frataxin (FXN) protein, wherein said enzymatic activity comprises enzymatically catalyzed generation of reactive oxygen species (ROS), said method comprising
 - (a) combining said FXN protein with a reducing agent and a ROS detector
15 compound, in the absence of metal ions, thereby producing an assay mixture;
 - (b) measuring the amount of ROS generated in the assay mixture over time; and
 - (c) determining an enzymatic parameter associated with the ROS generating activity of the FXN protein.
3. A method for measuring enzymatic activity of a Frataxin (FXN) protein, wherein said
20 enzymatic activity comprises enzymatically catalyzed generation of reactive oxygen species (ROS), said method comprising
 - (a) combining said FXN protein with a reducing agent and a ROS detector compound, in the absence of iron ions, thereby producing an assay mixture;
 - (b) measuring the amount of ROS generated in the assay mixture over time; and

(c) determining an enzymatic parameter associated with the ROS generating activity of the FXN protein.

4. The method of claim 2 or 3, further comprising adjusting pH of the assay mixture to pH of 7.4 to 9.

5 5. The method of claim 1 or 4, further comprising adding a metal ion into the assay mixture.

6. The method of claim 5, wherein the metal ion is not Fe^{2+} .

7. The method of claim 5, wherein the metal ion is selected from the group consisting of Mn^{2+} , Fe^{3+} , Zn^{2+} , Cu^{2+} and Mg^{2+} .

8. The method of claim 7, wherein the metal ion is Mn^{2+} .

10 9. The method of claim 7, wherein the metal is Fe^{3+} .

10. The method of any one of claims 1-9, further comprising incubating said assay mixture.

11. The method of any one of claims 1-10, comprising measuring the amount of ROS generated at regular time intervals.

12. The method of claim 11, comprising measuring the amount of ROS generated every 0.1
15 second to every 10 minutes after the start of incubation of the assay mixture.

13. The method of claim 11 or 12, further comprising determining maximum initial velocity (V_i) of ROS generation in the assay mixture.

14. The method of claim 13, further comprising determining time to maximum initial velocity (T_i).

20 15. The method of claim 14, further comprising determining the unit activity of the FXN protein at pH of 7.4 to 9.

16. The method of any one of claim 1-15, further comprising determining the specific activity of the FXN protein.

17. The method of any one of claims 1-16, wherein the reducing agent is an organic compound.
18. The method of claim 17, wherein the reducing agent is a reduced quinone compound.
19. The method of claim 18, wherein the reduced quinone compound is selected from the group consisting of a reduced benzoquinone compound, a reduced naphthoquinone compound and a reduced anthraquinone compound.
20. The method of claim 19, wherein the reduced quinone compound is a reduced benzoquinone compound.
21. The method of claim 20, wherein the reduced benzoquinone compound is hydroquinone.
22. The method of any one of claims 1-21, wherein the ROS detector compound is a fluorescent probe or a chemiluminescent probe.
23. The method of any one of claims 1-21, wherein the ROS detector compound is a superoxide anion detection reagent or a hydrogen peroxide detection reagent.
24. The method of any one of claims 1-23, wherein the ROS detector compound is selected from the group consisting of coelenterazine; dihydroethidium; 2',7'-dichlorodihydrofluorescein (H₂DCF), lucigenin, luminol, Cypridina Luciferin Analog (CLA), Cypridina Luciferin methoxy-analogue (MCLA), methylthiazolyldiphenyl-tetrazolium bromide (MTT), p-nitrotetrazolium blue (NBT), and RedoxSensor Red CC-1, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT).
25. The method of claim 24, wherein the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).
26. The method of any one of claims 1-25, wherein the reducing agent and the ROS detector compound are the same compound.
27. The method of any one of claims 1-25, wherein the reducing agent and the ROS detector compound are different compounds.

28. The method of claim 27, wherein the reducing agent is dihydroquinone and the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).
29. The method of any one of claims 1-28, wherein the FXN protein is frataxin (FXN).
30. The method of any one of claims 1-29, wherein the FXN protein is an FXN fusion protein comprising full-length hFXN and a cell penetrating peptide (CPP).
31. The method of claim 30, wherein the CPP comprises a peptide selected from the group of CPPs listed in the Database of Cell-Penetrating Peptides CPPsite 2.0.
32. The method of claim 31, wherein the CPP comprises a peptide selected from the group consisting of HIV-TAT, galanin, mastoparan, transportan, penetratin, polyarginine, VP22.
33. The fusion protein of claim 32, wherein the CPP comprises HIV-TAT.
34. The method of any one of claims 30-33, wherein the FXN fusion protein comprises SEQ ID NO: 12.
35. A method for performing quality control on a sample comprising a Frataxin (FXN) protein, said method comprising measuring enzymatic activity of an FXN protein in accordance with the method of any one of claims 1-34.
36. A composition for measuring enzymatic activity of an FXN protein, said composition comprising an FXN protein, a reducing agent and a reactive oxygen species (ROS) detector compound, wherein pH of the composition is 7.4 to 9.
37. The composition of claim 36, further comprising a metal ion.
38. The composition of claim 37, wherein the metal ion is not Fe²⁺.
39. The composition of claim 37, wherein the metal ion is selected from the group consisting of Mn²⁺, Fe³⁺, Zn²⁺, Cu²⁺ and Mg²⁺.
40. The composition of claim 39, wherein the metal ion is Fe³⁺.

41. The composition of claim 39, wherein the metal ion is Mn^{2+} .
42. A composition for measuring enzymatic activity of an FXN protein, said composition comprising an FXN protein, a reducing agent and a reactive oxygen species (ROS) detector compound, wherein the composition is substantially free of metal ions.
- 5 43. A composition for measuring enzymatic activity of an FXN protein, said composition comprising an FXN protein, a reducing agent and a reactive oxygen species (ROS) detector compound, wherein the composition is substantially free of iron ions.
44. The composition of claim 42 or 43, wherein pH of the composition is 7.4 to 9.
45. The composition of any one of claims 36-44, wherein the reducing agent is an organic
10 compound.
46. The composition of claim 45, wherein the reducing agent is a reduced quinone compound.
47. The composition of claim 46, wherein the reduced quinone compound is selected from the group consisting of a reduced benzoquinone compound, a reduced naphthoquinone
15 compound and a reduced anthraquinone compound.
48. The composition of claim 47, wherein the reduced quinone compound is a reduced benzoquinone compound.
49. The composition of claim 48, wherein the reduced benzoquinone compound is hydroquinone.
- 20 50. The composition of any one of claims 36-49, wherein the ROS detector compound is a fluorescent probe or a chemiluminescent probe.
51. The composition of any one of claims 36-49, wherein the ROS detector compound is a superoxide anion detection reagent or a hydrogen peroxide detection reagent.

52. The composition of claim 50 or 51, wherein the ROS detector compound is selected from the group consisting of coelenterazine; dihydroethidium; 2',7'-dichlorodihydrofluorescein (H₂DCF), lucigenin, luminol, Cypridina Luciferin Analog (CLA), Cypridina Luciferin methoxy-analogue (MCLA), methylthiazolyldiphenyl-tetrazolium bromide (MTT), p-nitrotetrazolium blue (NBT), RedoxSensor Red CC-1, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT).
53. The composition of claim 52, wherein the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).
54. The composition of any one of claims 36-53, wherein the reducing agent and the ROS detector compound are the same compound.
55. The composition of any one of claims 36-53, wherein the reducing agent and the ROS detector compound are different compounds.
56. The composition of claim 55, wherein the reducing agent is dihydroquinone and the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).
57. The composition of any one of claims 36-56, wherein the FXN protein is frataxin (FXN).
58. The composition of any one of claims 36-56, wherein the FXN protein is an FXN fusion protein comprising full-length hFXN and a cell penetrating peptide (CPP).
59. The composition of claim 58, wherein the CPP comprises a peptide selected from the group of CPPs listed in the Database of Cell-Penetrating Peptides CPPsite 2.0.
60. The composition of claim 59, wherein the CPP comprises a peptide selected from the group consisting of HIV-TAT, galanin, mastoparan, transportan, penetratin, polyarginine, VP22.
61. The composition of claim 60, wherein the CPP comprises HIV-TAT.
62. The composition of claim 61, wherein the FXN fusion protein comprises SEQ ID NO: 12.

63. Use of a kit for determining enzymatic activity of an FXN protein, said kit comprising a reducing agent, an ROS detector compound, instructions to combine the reducing agent, the ROS detector compound and the FXN protein to produce an assay mixture; and instructions to determine an enzymatic parameter of the ROS generating activity of the FXN protein in the assay mixture.
64. The use of claim 63, wherein the kit further comprises a metal ion compound.
65. The use of claim 64, wherein the metal ion compound comprises an ion selected from the group consisting of Mn^{2+} , Fe^{3+} , Zn^{2+} , Cu^{2+} and Mg^{2+} .
66. The use of claim 65, wherein the metal ion compound comprises Fe^{3+} .
67. The use of claim 65, wherein the metal ion compound comprises Mn^{2+} .
68. The use of any one of claims 63-67, wherein the reducing agent is an organic compound.
69. The use of claim 68, wherein the reducing agent is a reduced quinone compound.
70. The use of claim 69, wherein the reduced quinone compound is selected from the group consisting of a reduced benzoquinone compound, a reduced naphthoquinone compound and a reduced anthraquinone compound.
71. The use of claim 70, wherein the reduced quinone compound is a reduced benzoquinone compound.
72. The use of claim 71, wherein the reduced benzoquinone compound is hydroquinone.
73. The use of any one of claims 63-72, wherein the ROS detector compound is a fluorescent probe or a chemiluminescent probe.
74. The use of any one of claims 63-72, wherein the ROS detector compound is a superoxide anion detection reagent or a hydrogen peroxide detection reagent.
75. The use of claim 73 or 74, wherein the ROS detector compound is selected from the group consisting of coelenterazine; dihydroethidium; 2',7'-dichlorodihydrofluorescein (H_2DCF),

lucigenin, luminol, Cypridina Luciferin Analog (CLA), Cypridina Luciferin methoxy-analogue (MCLA), methylthiazolyldiphenyl-tetrazolium bromide (MTT), p-nitrotetrazolium blue (NBT), and RedoxSensor Red CC-1, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT).

5 76. The use of claim 75, wherein the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).

77. The use of any one of claims 63-76, wherein the reducing agent and the ROS detector compound are the same compound.

78. The use of any one of claims 63-76, wherein the reducing agent and the ROS detector
10 compound are different compounds.

79. The use of claim 78, wherein the reducing agent is dihydroquinone and the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).

80. A method for identifying a compound capable of modulating enzymatic activity of a Frataxin (FXN) protein, said method comprising the steps of:

15 (i) combining said FXN protein with a test compound, a reducing agent and a reactive oxygen species (ROS) detector compound, thereby producing an assay mixture;

(ii) measuring the amount of ROS generated in the assay mixture over time in the presence of the test compound;

(iii) determining an enzymatic parameter associated with the ROS generating activity
20 of the FXN protein in the presence of the test compound;

(iv) comparing the enzymatic parameter associated with the ROS generating activity of the FXN protein in the presence of the test compound with an enzymatic parameter associated with the ROS generating activity of the FXN protein in the absence of the test compound; and

(v) identifying the test compound as a compound capable of modulating enzymatic
25 activity of the FXN protein when the enzymatic parameter associated with the ROS generating

activity of the FXN protein in the presence of the test compound is different from the enzymatic parameter associated with the ROS generating activity of the FXN protein in the absence of the test compound.

81. The method of claim 80, further comprising incubating said assay mixture.

5 82. The method of claim 80 or 81, wherein step (ii) comprises measuring the amount of ROS generated at regular time intervals.

83. The method of claim 82, wherein step (ii) comprises measuring the amount of ROS generated every 0.1 second to every 10 minutes.

84. The method of any one of claims 80-83, further comprising determining maximum
10 initial velocity (V_i) of ROS generation in the assay mixture.

85. The method of claim 84, further comprising determining time to maximum initial velocity (T_i).

86. The method of claim 85, further comprising determining the unit activity of the FXN protein.

15 87. The method of any one of claims 80-86, further comprising determining the specific activity of the FXN protein.

88. The method of claim 84, further comprising the steps of:

20 comparing V_i of the ROS generating activity of the FXN protein in the presence of the test compound with V_i of the ROS generating activity of the FXN protein in the absence of the test compound; and

identifying the test compound as a compound capable of modulating enzymatic activity of the FXN protein when the V_i of the ROS generating activity of the FXN protein in the presence of the test compound is different from the V_i of the ROS generating activity of the FXN protein in the absence of the test compound.

89. A method for identifying a compound capable of modulating enzymatic activity of a Frataxin (FXN) protein, said method comprising the steps of:

(i) combining said FXN protein with a test compound, a reducing agent and a reactive oxygen species (ROS) detector compound, thereby producing an assay mixture;

5 (ii) measuring the amount of ROS generated in the assay mixture over time in the presence of the test compound and determining V_i of the ROS generating activity of the FXN protein;

(iii) comparing the V_i of the ROS generating activity of the FXN protein in the presence of the test compound with V_i of the ROS generating activity of the FXN protein in the
10 absence of the test compound; and

(iv) identifying the test compound as a compound capable of modulating enzymatic activity of the FXN protein when V_i of the ROS generating activity of the FXN protein in the presence of the test compound is different from V_i of the ROS generating activity of the FXN protein in the absence of the test compound.

15 90. The method of any one of claims 80-89, further comprising adjusting pH of the assay mixture to pH of 7.4 to 9.

91. The method of any one of claims 80-90, further comprising adding a metal ion to the assay mixture.

20 92. The method of claim 91, wherein the metal ion is selected from the group consisting of Mn^{2+} , Fe^{3+} , Zn^{2+} , Cu^{2+} and Mg^{2+} .

93. The method of claim 92 wherein the metal ion is Mn^{2+} .

94. The method of claim 92, wherein the metal ion is Fe^{3+} .

95. The method of any one of claims 80-94, wherein the reducing agent is an organic compound.

96. The method of claim 95, wherein the reducing agent is a reduced quinone compound.
97. The method of claim 96, wherein the reduced quinone compound is selected from the group consisting of a reduced benzoquinone compound, a reduced naphthoquinone compound and reduced anthraquinone compound.
98. The method of claim 97, wherein the reduced quinone compound is a reduced benzoquinone compound.
99. The method of claim 98, wherein the reduced benzoquinone compound is hydroquinone.
100. The method of any one of claims 80-99, wherein the ROS detector compound is a fluorescent probe or a chemiluminescent probe.
101. The method of any one of claims 80-99, wherein the ROS detector compound is a superoxide anion detection reagent or a hydrogen peroxide detection reagent.
102. The method of any one of claims 80-101, wherein the ROS detector compound is selected from the group consisting of coelenterazine; dihydroethidium; 2',7'-dichlorodihydrofluorescein (H₂DCF), lucigenin, luminol, Cypridina Luciferin Analog (CLA), Cypridina Luciferin methoxy-analogue (MCLA), methylthiazolyldiphenyl-tetrazolium bromide (MTT), p-nitrotetrazolium blue (NBT), and RedoxSensor Red CC-1, 2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT).
103. The method of claim 102, wherein the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).
104. The method of any one of claims 80-103, wherein the reducing agent and the ROS detector compound are the same compound.
105. The method of any one of claims 80-103, wherein the reducing agent and the ROS detector compound are different compounds.

106. The method of claim 105, wherein the reducing agent is dihydroquinone and the ROS detector compound is 2',7'-dichlorodihydrofluorescein (H₂DCF).
107. The method of any one of claims 80-106, wherein the FXN protein is frataxin (FXN).
- 5 108. The method of any one of claim 80-106, wherein the FXN protein is an FXN fusion protein comprising full-length hFXN and a cell penetrating peptide (CPP).
109. The method of claim 108, wherein the CPP comprises a peptide selected from the group of CPPs listed in the Database of Cell-Penetrating Peptides CPPsite 2.0.
- 10 110. The method of claim 108, wherein the CPP comprises a peptide selected from the group consisting of HIV-TAT, galanin, mastoparan, transportan, penetratin, polyarginine, VP22.
111. The method of claim 110, wherein the CPP comprises HIV-TAT.
112. The method of claim 111, wherein the FXN fusion protein comprises SEQ ID NO: 12.
- 15 113. A method of preparing a pharmaceutical composition comprising an FXN protein, the method comprising measuring enzymatic activity of the FXN protein according to the method of any one of claims 1-34, and formulating the FXN protein, thereby preparing the pharmaceutical composition.