ForenSeq Kintelligence

1. Purpose

This document describes setup of the MiSeq FGx using the ForenSeq Kintelligence kit.

2. Summary

The Verogen ForenSeq Kintelligence amplification kit is used to generate SNP (Single Nucleotide Polymorphism) data that can be uploaded into the GEDMatch Pro Database in order to be used for Forensic Genetic Genealogy investigations. This describes the setup and run of this assay.

3. Procedure

PCR1

Preparation

1. Bring reagents to room temperature:
    a. NA24385
    b. kPCR1
    c. KPM
    d. FEM

Note: Remove FEM from freezer only for addition to the master mix. Do not vortex FEM.

2. Prepare Positive Control Dilutions
    a. In the Control DNA Dilution 1 tube, combine the following volumes to prepare 50 µl 400 pg/µl NA24385:
        i. 10 ng/µl NA24385 (2 µl)
        ii. Nuclease-free water (48 µl)
    b. Gently pipette to mix, and then cap and centrifuge briefly.
    c. In the Control DNA Dilution 2 tube, combine the following volumes to prepare 100 µl 40 pg/µl NA24385:
        i. 400 pg/µl NA24385 (10 µl)
        ii. Nuclease-free water (90 µl)
    d. Gently pipette to mix, and then cap and centrifuge briefly.

Procedure

3. Normalize samples to 0.04ng/µl

4. Create a master mix for each Sample, including overage.

Reagent	Volume
kPCR1	18.5µl
KPM	5.0µl
FEM	1.5µl

5. Pipette to mix and then centrifuge briefly.
    a. If processing more than eight samples, the master mix may be evenly distributed into each well of an eight-tube strip and dispensed using a multichannel pipette.

6. Add 25µl master mix to each well of the plate for each sample.

7. Add 25µl diluted NA24385 as a positive control, 25µl water as a negative control and 25µl of each sample dilution to the appropriate well on the plate.
    a. Pipette to mix while dispensing.

8. Seal the plate and centrifuge at 1000 × g for 30 seconds.

9. Place the plate on the thermal cycler and run the K-PCR1 program.

Note: This is a safe stopping point. Plate can be stored at 2°C to 8°C for up to 2 days.

Purify Targets

Preparation

10. Bring reagents to room temperature:
    a. ProK
    b. RSB
    c. SPB2

11. Prepare 80% EtOH
    a. 1.5ml per sample with Purify Libraries included
    b. 1.0ml per sample for Purify Targets only

12. Prepare SPB2, first use only
    a. Add 7.5 µl ProK to the SPB2 tube. Mark the checkbox on the SPB2 label

Procedure

Perform First Cleanup

13. Add 75 µl ProK/SPB2 to each well of the Purification Bead Plate

14. Transfer 45 µl reaction from each well of the ForenSeq Sample Plate to the corresponding well of the Purification Bead Plate 1.

15. Seal the ForenSeq Sample Plate to protect the unused wells until PCR2.

16. Seal the Purification Bead Plate 1 and shake at 1800 rpm for **2** minutes.

17. Incubate at room temperature for **10** minutes.

18. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).

19. Remove and discard all supernatant.

20. Keep on the magnetic stand and wash as follows.
    a. Add 200 µl fresh 80% EtOH to each well.
    b. Incubate for 30 seconds.
    c. Remove and discard all supernatant.

21. Wash a second time.

22. Seal and centrifuge at 1000 × g for 30 seconds.

23. Place on the magnetic stand.

24. With a 20 µl pipette, remove residual EtOH from each well.

25. Remove from the magnetic stand.

26. Add 30 µl RSB to each sample well, mixing the samples before removing the pipette tip.

27. Seal and shake at 1800 rpm for **2** minutes.

28. Incubate at room temperature for **2** minutes.

29. Place on the magnetic stand.

30. Transfer 28 µl supernatant from each well of the Purification Bead Plate 1 to a fresh well in the same plate. Some bead carryover into the second cleanup is normal.

Perform Second Cleanup

31. Add 45 µl ProK/SPB2 to each sample well.
32. Seal and shake at 1800 rpm for **2** minutes.
33. Incubate at room temperature for **5** minutes.
34. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
35. Remove and discard all supernatant.
36. Keep on the magnetic stand and wash as follows.
    a. Add 200 µl fresh 80% EtOH to each well.
    b. Incubate for 30 seconds.
    c. Remove and discard all supernatant.
37. Wash a second time.
38. Seal and centrifuge at 1000 × g for 30 seconds.
39. Place on the magnetic stand.
40. With a 20 µl pipette, remove residual EtOH from each well.
41. Add 27 µl RSB to each sample well, mixing the samples before removing the pipette tip.
42. Seal and shake at 1800 rpm for **2** minutes.
43. Incubate at room temperature for **2** minutes.
44. Place on the magnetic stand.
45. Transfer 25 µl supernatant from each well of the Purification Bead Plate to the corresponding well of the Purified Targets Plate.

Note: This is a safe stopping point. Sealed plate can be stored at -25°C to -15°C overnight.

Enrich Targets

Preparation

46. Bring reagents to room temperature:
    a. KPCR2
    b. UDI adapters

Procedure

47. Add 5 µl UDI adapters to each sample well of the Purified Targets Plate.
48. Briefly centrifuge kPCR2 and then pipette to mix.
49. Add 20 µl kPCR2 to each well.
50. Pipette to mix.
51. Seal and centrifuge at 1000 × g for 30 seconds.
52. Place on the preprogrammed thermal cycler and run the K-PCR2 program.

Note: This is a safe stopping point. Sealed plate can be stored at 2°C to 8°C for up to 7 days. Alternatively, leave on the thermal cycler overnight.

Purify Libraries

Preparation

53. Bring reagents to room temperature:
    a. RSB
54. Prepare EtOH if not prepared the same day
    a. 0.5ml per sample
55. Plates
    a. Purification Bead Plate – Midi
    b. Purified Library Plate – PCR

Procedure

56. Add 45 µl ProK/SPB2 to each well of the Purification Bead Plate.
57. Transfer 45 µl reaction from each well of the Purified Targets Plate to the corresponding well of the Purification Bead Plate.
58. Seal and shake at 1800 rpm for **2** minutes.
59. Incubate at room temperature for **5** minutes.
60. Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
61. Remove and discard all supernatant.
62. Keep on the magnetic stand and wash as follows.
    a. Add 200 µl fresh 80% EtOH to each well.
    b. Incubate for 30 seconds.
    c. Remove and discard all supernatant.
63. Wash a second time.
64. With a 20 µl pipette, remove residual EtOH from each well.
65. Remove from the magnetic stand.
66. Add 52.5 µl RSB to each sample well, mixing the samples before removing the pipette tip.
67. Seal and shake at 1800 rpm for **2** minutes.
68. Incubate at room temperature for **2** minutes.
69. Place on the magnetic stand.
70. Transfer 50 µl supernatant from each well of the Purification Bead Plate 2 to the corresponding well of the Purified Library Plate.
71. Seal and centrifuge at 1000 × g for 30 seconds.

Note: This is a safe stopping point. Sealed plate can be stored at -25°C to -15°C for up to 1 year.

Normalize Libraries

Preparation

72. Bring reagents to room temperature:
    a. RSB
73. Plates/Tubes:
    a. Normalized Library Plate – PCR plate

b. Normalized Library – Tube

Procedure

74. Place on the magnetic stand.

75. Quantify the library using a fluorometric method.

    a. QuantiFluor® One dsDNA System

    Note: QuantiFluor® One Dye should be protected from light as much as possible through the process.

    i. Label tubes for each sample and if necessary, 2 for calibration
    ii. Add 200µl QuantiFluor® One Dye to each tube
    iii. Add 2µl of each sample or 1µl DNA Standard
    iv. Vortex and incubate protected from light for 5 minutes
    v. Select dsDNA protocol on the Quantus™ Fluorometer
    vi. Calibrate if necessary
    vii. Measure the fluorescence of the sample and record the final sample concentration

76. For libraries with a concentration > 0.75 ng/µl, calculate the volume of RSB needed to dilute the library to 0.75 ng/µl as follows.
    a. Use the formula $C_1V_1=C_2V_2$ to calculate the value for V_2, where:
        i. C_1 is the library quantification result
        ii. V_1 is 8 µl undiluted library
        iii. C_2 is 0.75 ng/µl
        iv. V_2 is the final volume of diluted library
    b. Calculate the amount of RSB (V_2 - 8 µl) required to dilute each library to 0.75 ng/µl.

77. Add the calculated volume of RSB to the corresponding well of the Normalized Library Plate or tube. Use the tube when the library is ≥ 15 ng/µl.

78. Transfer 8 µl of each purified library from the Purified Library Plate to the corresponding well of the Normalized Library Plate or tube. The result is a Normalized Library Plate or containing 0.75 ng/ul libraries.

Note: This is a safe stopping point. Sealed plate or tube can be stored at -25°C to -15°C for up to 6 months.

Pool Libraries

Procedure

79. Transfer 5 µl each library to the Pooled Libraries tube.

80. Seal the Normalized Library Plate or cap the Normalized Library tube and store in the post-PCR area at -25°C to -15°C for ≤ 30 days.

81. Cap vortex to mix, and then centrifuge briefly.

Note: This is a safe stopping point. Sealed tube can be stored at -25°C to -15°C for up to 6 months.

Denature and Dilute Libraries

Preparation

82. Bring reagents to room temperature:
    a. HP3
    b. HSC
    c. HT1

83. Tubes
    a. 12pM Denatured Library
    b. 20pM Denatured Library
    c. Denatured HSC

d. Diluted NaOH

84. Prepare Diluted NaOH tube (0.2 N)
    a. 90µl Nuclease-free water
    b. 10µl HP3
    c. Invert several times to mix and use within 12 hours.

Procedure

85. Prepare the Denatured HSC tube:
    a. Combine HSC and 0.2 N NaOH
        i. 2 µl HSC
        ii. 2 µl 0.2 N NaOH
    b. Cap vortex to mix, and then centrifuge briefly.
    c. Incubate at room temperature for **5** minutes.
    d. Add 36 µl HT1 to the Denatured HSC tube.

    Note: Denatured HSC can be stored at room temperature for ≤ 1 day.

86. Prepare the 20 pM Denatured Library tube:
    a. Combine Library Pool and 0.2 N NaOH
        i. 5 µl 0.75 ng/µl library pool
        ii. 5 µl 0.2 N NaOH
    b. Cap and vortex briefly, and then centrifuge briefly.
    c. Incubate at room temperature for **5** minutes.
    d. Add 990 µl HT1 to the 20 pM Denatured Library tube to prepare 1 ml 20 pM denatured library.

    Note: The 20 pM denatured library can be stored at -15°C to -25°C for ≤ 3 weeks.

87. Prepare the 12 pM Denatured Library tube:
    a. Combine 20 pM library and HT1
        i. 360 µl 20 pM library pool
        ii. HT1 (238 µl)
        iii. Denatured HSC (2 µl)
    b. Cap vortex to mix, and then centrifuge briefly.
    c. Immediately transfer the entire volume to the reagent cartridge.

4. References

ForenSeq Kintelligence Kit Reference Guide Document # VD2020053 Rev. B, March 2021

5. Definitions

N/A