ForenSeq DNA Signature Prep

1. *Purpose*

This document describes setup of the MiSeq FGx using the ForenSeq DNA Signature (and DNA Signature Plus) next generation sequencing kit.

2. *Summary*

Next Generation Sequencing is the primary amplification assay of the IMF laboratory. The Verogen ForenSeq NGS amplification kit is used for NGS comparison of evidentiary items with known profiles and potential upload to CODIS. This describes the setup and run of this assay.

3. *Procedure*

PCR1

Preparation

1. Bring reagents to room temperature
   a. DPMA or DPMB
   b. PCR1
   c. 1X TBE Buffer
   d. 2800M

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

2. Prepare Positive Control dilution
   a. Mix 98µl water with 2µl of 2800m positive control

Procedure

3. Normalize samples to 0.2ng/ul

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

Reagent	Volume
PCR1	4.7µl
FEM	0.3µl
DPMA or DPMB	5.0µl

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 10µl master mix to each well of the plate for each sample.

7. Add 5µl diluted 2800M as a positive control, 5µl water as a negative control and 5µl of each sample 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 PCR1 program.

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

PCR2

Preparation

10. Bring reagents to room temperature, vortex, and centrifuge briefly.
    a. Index Adapters
    b. PCR2

Procedure

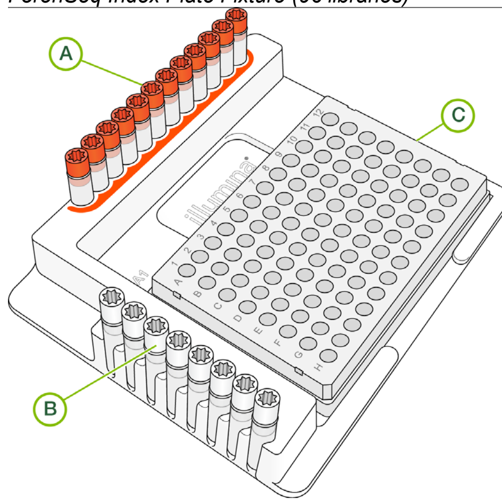
11. Centrifuge the PCR1 plate at 1000 × g for 30 seconds.

12. Arrange Index 1 (i7) adapters in columns 1–12 of the ForenSeq Index Plate Fixture.

13. Arrange Index 2 (i5) adapters in rows A–H of the ForenSeq Index Plate Fixture.

14. Place the plate on the ForenSeq Index Plate Fixture.

    ForenSeq Index Plate Fixture (96 libraries)



    a. Columns 1–12: Index 1 (i7) adapters (orange caps)
    b. Rows A–H: Index 2 (i5) adapters (white caps)
    c. PCR1 plate

15. Add 4µl Index 1 (i7) adapters to each column. Replace the caps on i7 adapter tubes with new orange caps.
    a. Use a multichannel pipette whenever possible.

16. Add 4µl Index 2 (i5) adapters to each row. Replace the caps on i5 adapter tubes with new white caps.
    a. Use a multichannel pipette whenever possible.

17. Vortex PCR2 and then centrifuge briefly.

18. Add 27µl PCR2 to each well.

19. Seal the plate with adhesive film.

20. Centrifuge the PCR2 plate at 1000 × g for 30 seconds.

21. Place the plate on the thermal cycler and run the PCR2 program.

    Note: This is a safe stopping point. Plate can be stored at 2°C to 8°C for up to 7 days.

Purify Libraries

Preparation

22. Bring reagents to room temperature
    a. RSB (Resuspension Buffer)
    b. SPB (Sample Purification Beads)
        i. Mix thoroughly by vortex
    c. Prepare 80% EtOH by diluting 100% EtOH with water.
        i. 12ml EtOH and 3ml water

23. Label plates
    a. PBP (Purification Bead Plate) – Midi Plate
    b. PLP (Purified Library Plate) – PCR Plate

Procedure

24. Centrifuge the PCR2 plate at 1000 × g for 30 seconds.
25. Pour SPB into a reagent reservoir and add 45µl to each well of the PBP plate according to the sample sheet.
26. Transfer 45µl from PCR2 plate to the corresponding well of the PBP plate.
27. Seal the PBP plate with Microseal 'B' and shake at 1800 rpm for **2** minutes.
28. Incubate at room temperature for **5** minutes.
29. Place the PBP plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
30. Remove and discard all supernatant from each well.
31. Wash two times:
    a. Add 200µl freshly prepared 80% EtOH to each well.
    b. Incubate on the magnetic stand for 30 seconds.
    c. Remove and discard all supernatant from each well.

    Note: Do not remove the plate from the magnet stand during the washing.
32. Seal the PBP plate and quick centrifuge at 1000 × g.
33. Place the plate on the magnetic stand and remove residual EtOH from each well.
34. Remove the plate from the magnetic stand.
35. Add 52.5µl RSB to each well.
    a. Mix to resuspend the beads using caution to completely expel beads before discarding the tip.
36. Seal the plate with Microseal 'B' and shake at 1800 rpm for **2** minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 2 minutes.
37. Incubate at room temperature for **2** minutes.
38. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
39. Transfer 50µl to the corresponding well of the PLP plate.
40. Seal the PLP plate and centrifuge at 1000 × g for 30 seconds.

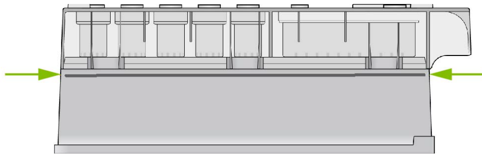
    Note: This is a safe stopping point. Plate can be stored at -25°C to -15°C for up to 1 year.

Normalize Libraries

Preparation

41. If loading on the same day, remove reagent cartridge and thaw in water bath. Place the reagent cartridge in a water bath containing enough room temperature water to submerge the base of the reagent cartridge up to the water line printed on the reagent cartridge. Do not allow the water to exceed the maximum water line.

    Maximum Water Line



42. Bring reagents to room temperature
    a. HP3
    b. LNA1
    c. LNB1
    d. LNW1
    e. LNS2

43. Label tubes and plates
    a. LNA1/LNB1 Master Mix — 1.5 ml microcentrifuge tube or 15 ml conical tube
    b. NWP (Normalization Working Plate) — midi plate
    c. NLP (Normalization Library Plate) — PCR plate

Procedure

44. Create a master mix in the LNA1/LNB1 Master Mix tube.
    a. LNA1 (46.8µl per sample)
    b. LNB1 (8.5µl per sample)

    Note: Mix LNB1 using 1000ul pipette, pipexing 15-20 times to ensure no bead pellets are in the mix before combining with LNA1.

45. Vortex and then invert the tube several times to mix.

46. Pour into a reagent reservoir.

47. Transfer 45µl to each well of the NWP plate that will contain a library according to the sample sheet.

48. To clear any beads that might have been aspirated during purification, place the PLP plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

49. Transfer 20µl from each well of the PLP plate to the corresponding well of the NWP plate.

50. Seal the plate with Microseal 'B' and shake at 1800 rpm for **30** minutes.

51. While the plate is shaking, perform the following steps:
    a. Prepare 0.1 N HP3 in a new 1.5 ml microcentrifuge tube, as follows:
        i. Nuclease-free water (33.3µl per sample)
        ii. HP3 (1.8µl per sample)
        iii. Invert the tube several times to mix.
        iv. Set aside.
    b. Add 30µl LNS2 to each well of the NLP plate that will contain a library according to the sample sheet.

52. Immediately after the NWP has finished shaking, place the NWP plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

53. Remove and discard all supernatant from each well.

54. Remove the plate from the magnetic stand.

55. Wash two times with 45µl LNW1 as follows:
    a. Add 45µl LNW1 to each well.
    b. Seal the plate with Microseal 'B' and shake at 1800 rpm for **5** minutes.
    c. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
    d. Remove and discard all supernatant from each well.
    e. Remove the plate from the magnetic stand.

56. Seal the plate and quick centrifuge at 1000 × g.

57. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

58. Remove residual supernatant from each well.

59. Remove the plate from the magnetic stand.

60. Add 32µl freshly prepared 0.1 N HP3 to each well. Discard tube.

61. Seal the plate with Microseal 'B' and shake at 1800 rpm for **5** minutes. If the beads are not resuspended, pipette to mix or repeat shake at 1800 rpm for 5 minutes.

62. Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).

63. Transfer 30µl to the corresponding well of the NLP plate. Pipette to mix.

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

    Note: This is a safe stopping point. Plate can be stored at -25°C to -15°C for up to 30 days.

Pool Libraries

Preparation

65. Determine which libraries to pool for sequencing.

66. Label the tube PNL to indicate Pooled Normalized Libraries.

Procedure

67. Transfer 5µl of each library to the PNL tube.

68. Vortex and then centrifuge briefly.

69. **Note:** This is a safe stopping point. Tube can be stored at -25°C to -15°C for up to 30 days.

Denature and Dilute Libraries

Preparation

70. Ensure that the Reagent Cartridge is fully thawed before beginning the Denature steps.
    a. Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge.
    b. Dry the base of the cartridge. Make sure that no water has splashed on the top of the reagent cartridge.
    c. Invert the reagent cartridge ~10 times to mix the thawed reagents, and then visually inspect that all positions are thawed.

    NOTE: It is critical that the reagents in the cartridge are thoroughly thawed and mixed to ensure proper sequencing. Once thawed, the reagent cartridge can be stored at 2°C to 8°C (up to 6 hours) until ready to set up the run. For best results, proceed directly to loading the sample and setting up the run.

71. Bring reagents to room temperature
    a. HP3
    b. HSC
    c. HT1
    d. Reagent Cartridge – Thaw in water bath for ~1.5 hrs or in the refrigerator overnight.

72. Preheat the heat block to 100°C.

73. Prepare either of the following:
    a. Remove a tube benchtop cooler from -25°C to -15°C storage or ice bucket.
    b. Prepare an ice-water bath by combining 3 parts ice and 1 part water.

74. Label tubes:
    a. HSC mixture
    b. DNL to indicate Denatured Normalized Libraries.

Procedure

75. Create an HSC denaturation reaction in the HSC mixture tube.
    a. HSC (2µl)
    b. HP3 (2µl)
    c. Nuclease-free water (36µl)

76. Vortex and then centrifuge briefly.

77. Incubate at room temperature for **5** minutes.

78. Add 591µl HT1 to the DNL tube.

79. Transfer 7 from the PNL tube to the DNL tube. Pipette to mix.

    a. Up to 12ul of PNL may be used to enhance cluster generation when needed
    b. For each ul of PNL above 7ul, the HT1 volume should be reduced to ensure concentration remains the same
        i. Example. If 10ul of PNL is used, 588ul HT1 should be added to the mix

80. Transfer 4µl HSC mixture to the DNL tube. Pipette to mix. Do not store HSC mixture long term, which results in a significant reduction of cluster density.

81. Vortex and then centrifuge briefly.

82. Place on the heat block for at least **4** minutes.

83. Invert the DNL tube several times to mix.

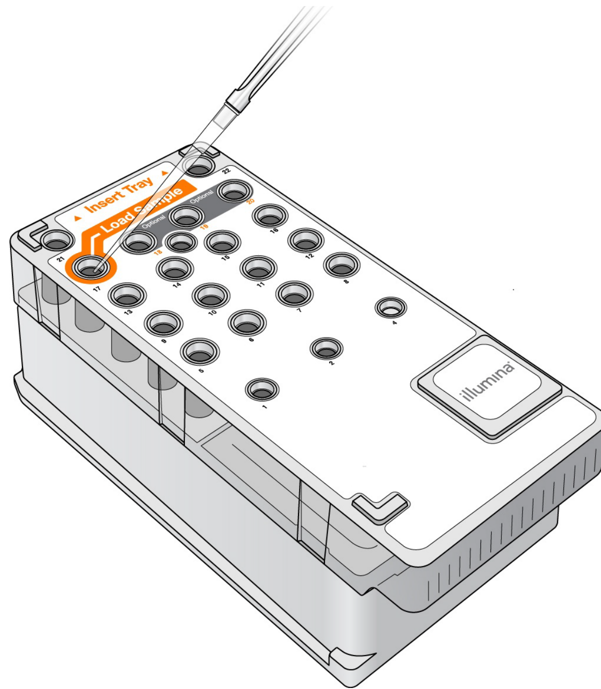
84. Immediately place in the ice-water bath or on the -25°C to -15°C benchtop cooler for **5** minutes.

85. Use a clean 1 ml pipette tip to pierce the foil seal covering the reservoir labeled **Load Samples** (well 17).

    NOTE: Do not pierce any other reagent positions. Other reagent positions are pierced automatically during the run.

86. Pipette the entire contents of the **DNL** sample libraries into the **Load Samples** reservoir. Avoid touching the foil seal as you dispense the sample.

87. Gently tap the cartridge on the bench to release any bubbles and check for air bubbles in the reservoir after loading sample.



88. The cartridge is ready for loading on the FGx instrument.

NOTE: This is **NOT** a safe stopping point. Proceed directly to the run setup steps.

4. References

ForenSeq™ DNA Signature Prep Reference Guide

5. Definitions

N/A