ForenSeq mtDNA Control Region Kit Checklist

For Research, Forensic, or Paternity Use Only

Amplify and Tag Targets

- ☐1 Using nuclease-free water, dilute 100 pg gDNA to a volume of ≥ 12 µl at 8.33 pg/µl.
- ☐2 In the Master Mix CRS1 tube, combine the following volumes per sample.
  - mtPCR1 (3.7 µl)
  - FEM (0.3 µl)
  - CRS1 (5 µl)
- ☐3 In the Master Mix CRS2 tube, combine the following volumes per sample.
  - mtPCR1 (3.7 µl)
  - FEM (0.3 µl)
  - CRS2 (5 µl)
- ☐4 Pipette each master mix and centrifuge briefly.
- ☐5 [Optional] For > 16 samples, transfer each master mix to an 8-tube strip.
- ☐6 Add master mixes to the mtDNA CR Sample Plate:
  - ☐a Split the plate into two even sections (samples will be divided).
  - ☐b Add 9 µl CRS1 Master Mix to the first section.
  - ☐c Add 9 µl CRS2 Master Mix to the second section.
- ☐7 Dilute HL60:
  - ☐a In the Control DNA Dilution 1 tube, combine the following volumes to prepare 100 pg/µl HL60:
    - 10 ng/µl HL60 (2 µl)
    - Nuclease-free water (198 µl)
  - ☐b Gently pipette and centrifuge briefly.
  - ☐c In the Control DNA Dilution 2 tube, combine:
    - 100 pg/µl HL60 (5 µl)
    - Nuclease-free water (55 µl)
  - ☐d Gently pipette and centrifuge briefly.
- ☐8 Add the reagent blank:
  - ☐a Add 6 µl reagent blank to the CRS1 set.
  - ☐b Add 6 µl reagent blank to the CRS2 set.
  - ☐c Pipette to mix.
- ☐9 Divide each sample:
  - ☐a Add 6 µl 8.33 pg/µl gDNA to the CRS1 set.
  - ☐b Add 6 µl 8.33 pg/µl gDNA to the CRS2 set.
  - ☐c Pipette to mix.
- ☐10 Add the positive amplification control:
  - ☐a Add 6 µl 8.33 pg/µl HL60 to the CRS1 set.
  - ☐b Add 6 µl 8.33 pg/µl HL60 to the CRS2 set.
  - ☐c Pipette to mix.
- ☐11 Add the negative amplification control:
  - ☐a Add 6 µl nuclease-free water to the CRS1 set.
  - ☐b Add 6 µl nuclease-free water to the CRS2 set.
  - ☐c Pipette to mix.
- ☐12 Centrifuge at 1000 × g for 30 seconds.
- ☐13 Place on the thermal cycler and run the mtPCR1 program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Enrich Targets

- ☐1 Centrifuge the mtDNA CR Sample Plate at 1000 × g for 30 seconds.
- ☐2 Arrange the index adapters in the ForenSeq Index Plate Fixture.
- ☐3 Place the mtDNA CR Sample Plate on the ForenSeq Index Plate Fixture.
- ☐4 Add 4 µl R7XX down each column.
- ☐5 Add 4 µl A50X across each row.
- ☐6 Invert mtPCR2 several times, and then centrifuge briefly.
- ☐7 [Optional] Evenly divide mtPCR2 among an 8-tube strip.
- ☐8 Add 27 µl mtPCR2.
- ☐9 Pipette to mix.
- ☐10 Centrifuge at 1000 × g for 30 seconds.
- ☐11 Place on the thermal cycler and run the mtPCR2 program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 7 days. Alternatively, leave on the thermal cycler overnight.

Purify Libraries

- ☐1 Add 90 µl SPB2/ProK to the Purification Bead Plate.
- ☐2 Centrifuge the mtDNA CR Sample Plate at 1000 × g for 30 seconds.
- ☐3 Transfer 45 µl from the mtDNA CR Sample Plate to the corresponding column of the Purification Bead Plate.
- ☐4 Shake the Purification Bead Plate at 1800 rpm for 2 minutes.
- ☐5 Incubate at room temperature for 5 minutes.
- ☐6 Place on the magnetic stand until transparent.
- ☐7 Remove and discard all supernatant.
- ☐8 Wash as follows.
  - ☐a Add 200 µl fresh 80% EtOH.
  - ☐b Incubate for 30 seconds.
  - ☐c Remove and discard all supernatant.
- ☐9 Wash a **second** time.
- ☐10 Remove residual EtOH.
- ☐11 Remove from the magnetic stand.
- ☐12 Add 52.5 µl RSB.
- ☐13 Shake at 1800 rpm for 2 minutes.
- ☐14 If necessary, pipette or reshake.

☐15 Incubate at room temperature for 2 minutes.
☐16 Place on the magnetic stand until clear.
☐17 Transfer 50 µl supernatant from the Purification Bead Plate to the Purified Library Plate.
☐18 Centrifuge at 1000 × g for 30 seconds.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 1 year.

Normalize Libraries

Bead-Based Method

☐1 Vortex LNB1 to resuspend.
☐2 In the LNA1/LNB1 Master Mix tube, combine the following volumes per sample without overage.
- LNA1 (46.8 µl)
- LNB1 (8.5 µl)

☐3 Vortex and invert several times to mix.
☐4 Transfer LNA1/LNB1 Master Mix to a reservoir.
☐5 Add 45 µl LNA1/LNB1 Master Mix to the Normalization Working Plate.
☐6 Place the Purified Library Plate on the magnetic stand until clear.
☐7 Transfer 20 µl from the Purified Library Plate to the Normalization Working Plate.
☐8 Seal the Purified Library Plate and store at -25°C to -15°C for up to 1 year.
☐9 Shake the Normalization Working Plate at 1800 rpm for 30 minutes.
☐10 While shaking, perform steps 11–13.
☐11 In the 0.1 N HP3 tube, combine the following volumes per sample without overage.
- Nuclease-free water (33.3 µl)
- HP3 (1.8 µl)

☐12 Invert several times to mix, and then set aside.
☐13 Add 30 µl LNS2 to the Normalization Library Plate and set aside.
☐14 Place the Normalization Working Plate on the magnetic stand until clear.
☐15 Remove and discard all supernatant.
☐16 Wash as follows.
- ☐a Remove from the magnetic stand.
- ☐b Add 45 µl LNW1 to each well.
- ☐c Shake at 1800 rpm for 5 minutes.
- ☐d Place on the magnetic stand until clear.
- ☐e Remove and discard all supernatant.

☐17 Wash a **second** time.
☐18 Remove from the magnetic stand.
☐19 Centrifuge at 1000 × g for 30 seconds.
☐20 Place on the magnetic stand until clear.
☐21 Remove residual supernatant.
☐22 Remove from the magnetic stand.
☐23 Add 32 µl 0.1 N HP3.
☐24 Shake at 1800 rpm for 5 minutes.
☐25 If necessary, pipette or reshake.
☐26 Place on the magnetic stand until clear.
☐27 Transfer 30 µl supernatant from the Normalized Working Plate to the Normalization Library Plate.
☐28 Centrifuge at 1000 × g for 30 seconds.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Manual Quantification Method

☐1 Quantify using a fluorometric method.
☐2 For concentrations > 0.75 ng/µl, calculate the volume of RSB to dilute to 0.75 ng/µl.
- ☐a Use the formula $C_1V_1=C_2V_2$ to calculate the value for V_2.
- ☐b Calculate the amount of RSB (V_2 - 4 µl) to dilute to 0.75 ng/µl.

☐3 Add RSB to the Quant Normalized Library Plate or a 1.7 ml tube.
☐4 Transfer 4 µl each library from the Purified Library Plate to the Normalized Library Plate or 1.7 ml tube.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Pool Libraries

☐1 Transfer 5 µl each library to an 8-tube strip.
☐2 Store the Normalized Library Plate at -25°C to -15°C for up to 30 days.
☐3 Transfer libraries from the 8-tube strip to the Pooled Normalized Libraries tube.
☐4 Vortex to mix, and then centrifuge briefly.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 30 days.

Denature and Dilute

Bead-Based Normalized Libraries

☐1 In the Denatured HSC tube, combine:
- HSC (2 µl)
- HP3 (2 µl)
- Nuclease-free water (36 µl)

☐2 Vortex to mix, and then centrifuge briefly.
☐3 Incubate at room temperature for 5 minutes.
☐4 Add 600 µl HT1 to the Denatured Normalized Libraries tube.
☐5 Incubate the Pooled Normalized Libraries tube on the heat block for 2 minutes.

☐6 Transfer 5 µl library from the Pooled Normalized Libraries tube to the Denatured Normalized Libraries tube.
☐7 Pipette to mix.
☐8 Store the Pooled Normalized Libraries tube at -25°C to -15°C for ≤ 30 days.
☐9 Add 4 µl HSC to the Denatured Normalized Libraries tube.
☐10 Pipette to mix.
☐11 Vortex to mix, and then centrifuge briefly.
☐12 Immediately transfer to the reagent cartridge.

Manually Quantified Libraries

☐1 In the Denatured HSC tube, combine:
- HSC (2 µl)
- HP3 (2 µl)
- Nuclease-free water (36 µl)

☐2 Vortex to mix, and then centrifuge briefly.
☐3 Incubate at room temperature for 5 minutes.
☐4 In the 20 pM Denatured Normalized Libraries tube, combine:
- 0.75 ng/µl normalized library pool (5 µl)
- 0.2 N HP3 (5 µl)

☐5 Vortex briefly.
☐6 Centrifuge at 280 × g for 1 minute.
☐7 Incubate at room temperature for 5 minutes.
☐8 Add 990 µl HT1 to the 20 pM Denatured Normalized Libraries tube.
☐9 In the 6 pM Denatured Normalized Libraries tube, combine:
- 20 pM library (180 µl)
- HT1 (416 µl)
- Denatured HSC (4 µl)

☐10 Vortex to mix, and then centrifuge briefly.
☐11 Immediately transfer to the reagent cartridge.

Acronyms

Acronym	Definition
A50X	i5 Index Adapter
CRS1	Control Region Set 1
CRS2	Control Region Set 2
FEM	ForenSeq Enzyme Mix
HL60	Control DNA HL60
HP3	2 N NaOH
HSC	Human Sequencing Control
HT1	Hybridization Buffer
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LNS2	Library Normalization Storage Buffer 2
LNW1	Library Normalization Wash 1
mtPCR1	mtPCR1 Reaction Mix
mtPCR2	mtPCR2 Reaction Mix
ProK	Proteinase K
R7XX	i7 Index Adapter
RSB	Resuspension Buffer
SPB2	Sample Purification Beads 2