Moving Implementation Mountains: Experiencing the Forensic Laboratory NGS and Bioinformatics Workflow through Simulation

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Chair:

Donia Slack, M.S. (RTI International)

Presenters:

Bruce Budowle, Ph.D. (University of North Texas Health Science Center)

Jennifer Churchill, Ph.D. (University of North Texas Health Science Center)

Seth Faith, Ph.D. (North Carolina State University)

Walther Parson Ph.D. (Institute of Legal Medicine, Innsbruck Medical University)

Frank Wendt, Ph.D. Candidate (University of North Texas Health Science Center)

NGS Simulation Technical Lead:

Sarah Norsworthy, M.S. (RTI International)

Description:

Attendees will be provided with a unique opportunity to learn best practices to implement Next-Generation Sequencing (NGS) into a typical forensic laboratory workflow. Supported by the National Institute of Justice (NIJ) and its Forensic Technology Center of Excellence (FTCoE), RTI International has developed an immersive and interactive virtual simulation tool that guides DNA practitioners through three commercially available NGS forensic laboratory processes amenable to two massively parallel sequencing instruments. In collaboration with the Dr. Bruce Budowle laboratory at the University of North Texas Health Science Center, Dr. Seth Faith of North Carolina State University, and Dr. Walther Parson of Innsbruck Medical University, attendees will be given the opportunity to virtually employ laboratory protocols as well as analyze forensically relevant genetic loci through commercially available and third-party software applications. Additionally, attendees will hear how NGS was implemented in a laboratory and used in an important immigration case.

Learning Outcomes:

Attendees will interactively learn laboratory best practices processes that demonstrate the workflow of NGS techniques. This exposure will be accomplished through a virtual software simulation tool that guides the end-user through several steps (liquid transfer, bead separation, etc.), which provides virtual "hands-on" experience that connects the physical activity with the theoretical chemistry across the entire workflow process. Thus, one of the major knowledge gaps and barriers to the adoption of NGS to the field of forensics will be addressed.

Attendees also will learn how to analyze real data obtained from NGS technologies through vendor and third-party software applications. The instructors will lead informative and engaging discussion on the bioinformatics processes and attendees will gain "hands-on" experience with the software applications. Applications such as the Illumina Universal Analysis Software package, STRait RAZOR, Ion Torrent Plug-Ins, etc. will be demonstrated and participants will manipulate data to make the connection between standard size fragment analysis and sequence analysis of STRs and SNPs. They will learn how NGS may provide additional mitochondrial DNA information on a sample of interest beyond what Sanger Sequencing may provide.

Attendees will learn how to navigate through the physical workflow of the Promega PowerSeq (STR) kit, the Illumina ForenSeq (STR, Y-STR, SNP) kit, and the Life Technologies AmpliSeq mtDNA kit. This exercise will include immersive and interactive virtual demonstrations through the simulation tool of how to load onto the Illumina MiSeq (Powerseq and Forenseq) and the Ion S5/Ion Chef (AmpliSeq) platforms. By demonstrating multiple technologies on two platforms, a more comprehensive experience will be provided so that the attendees will have a better foundation to select the best platform to meet their agency's needs and thereby further facilitate adoption.

Despite various workshops and presentations, a substantial barrier to the adoption of NGS technology is bridging the knowledge gap between the laboratory bench workflow and the theoretical basis of the NGS chemistry. Through the developed interactive simulation tool, attendees will be able to make the connection between the chemistry of NGS (MID design, bead manipulation, emulsion PCR, etc.) with respect to the molecular biological mechanisms of the technologies (bridge amplification, adaptor ligation, etc.). Additionally, attendees will learn how these technologies are both similar and distinct from currently employed forensic protocols.

Attendees will learn the steps and lessons learned from a laboratory that has implemented NGS into its forensic workflow. Key points that will be discussed include training requirements, validation strategies, and data storage considerations.

Intended Audience: The target audience for this workshop is DNA technical leaders, DNA analysts, and DNA laboratory directors. An understanding of forensic DNA methodologies and theories is required.

Schedule

Topic	Presenter	Time
General introduction	Donia Slack	8:30-8:40
Introduction of NGS for forensic applications	Bruce Budowle	8:40-8:55
How to use the virtual environment and simulation of Illumina's ForenSeq technology	Donia Slack	8:55-10:15
BREAK	**REFRESHMENTS**	**10:15-10:30**
Simulation of the ThermoFisher Ion S5/Ion Chef workflow for mtDNA	Jennifer Churchill	10:30-11:30
Simulation of the Promega PowerSeq technology and begin Bioinformatics lesson	Seth Faith	11:30-12:00
LUNCH	**NORTHSTAR BALLROOM**	**12:00-1:00**
Continuation of post-analysis Bioinformatics exercises	Seth Faith	1:00-3:00
BREAK	**REFRESHMENTS**	**3:00-3:15**
Post-analysis through STRait Razor	Frank Wendt	3:15-3:45
Implementing Mitochondrial DNA Massively Parallel Sequencing into Forensic Casework	Walther Parsons	3:45-4:30
Round Table / Q&A	All Presenters	4:30 – 5:00

Simulation Tool Standard Operating Procedures

ForenSeq

Ion Torrent

PowerSeq

ForenSeq DNA Signature Prep

Library Prep Workflow

Legend: Pre-PCR ● Post-PCR ●
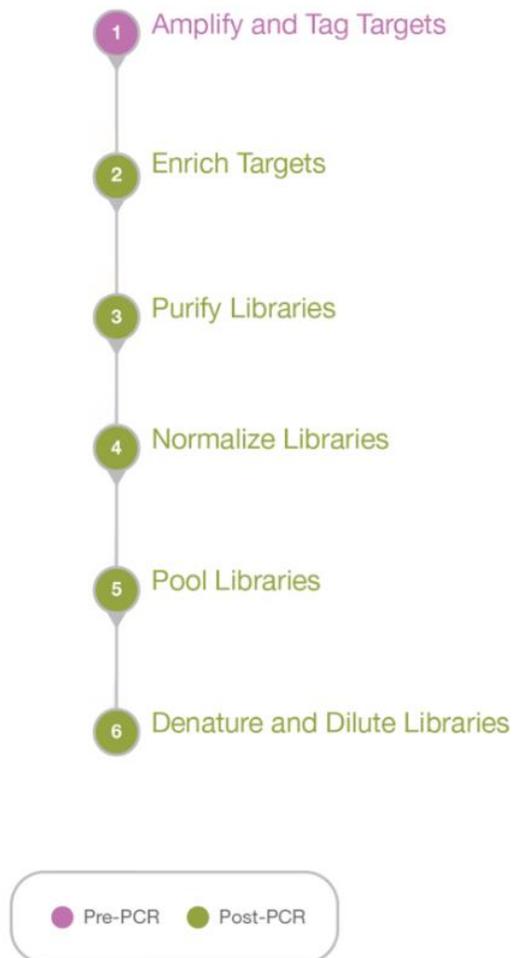
Protocol

Part 1 – Amplify and Tag Targets

This process amplifies and tags gDNA using a ForenSeq primer mix with regions specific to DNA sequences upstream and downstream of STRs and SNPs. Targets are enriched after amplification, and then libraries are normalized prior to pooling them together in one tube. For simplicity, only one sample will be processed throughout this simulation.

Consumables

96-Well Plates
- ► Plate 1, ForenSeq Sample Plate (FSP)

Microcentrifuge Tubes
- ► Tube 1, Master Mix
- ► Tube 2, PCR1 Reaction Mix (PCR1)
- ► Tube 3, Enzyme Mix (FEM)
- ► Tube 4, DNA Primer Mix (DPMA)
- ► Tube 5, Purified DNA

Procedure

1. Move tubes 1-5 to the working (empty) tube rack
2. Prepare master mix in tube 1
   a. PCR1 (tube 2)
      - ► Open master mix & PCR1 tubes
      - ► Open pipette tip box
      - ► Pick up pipette
      - ► Pick up pipette tip
      - ► Pipette PCR1 into master mix tube
      - ► Discard pipette tip & place pipette on pipette rack
      - ► Discard PCR1 tube
   b. FEM (tube 3)
      - ► Open FEM tube
      - ► Pick up pipette & tip
      - ► Pipette FEM into master mix tube
      - ► Discard pipette tip & place pipette on pipette rack
      - ► Discard FEM tube
   c. DPMA (tube 4)
      - ► Open DPMA tube
      - ► Pick up pipette & tip
      - ► Pipette DPMA into master mix tube
      - ► Discard pipette tip & place pipette on pipette rack
      - ► Close master mix tube
      - ► Discard DPMA tube
   d. Vortex master mix & then centrifuge briefly
      - ► Pick up master mix tube & vortex for 5 seconds
      - ► Place tube in minifuge & close lid
      - ► After 5 seconds, minifuge lid will automatically open

      ▶   Remove tube from minifuge & place in working tube rack

3   Prepare sample for PCR

   a   Add master mix to FSP plate (plate 1)

- ▶ Open master mix tube
- ▶ Pick up pipette & tip
- ▶ Pipette master mix into FSP plate well A1
- ▶ Discard pipette tip & place pipette on pipette rack
- ▶ Discard master mix tube

   b   Add purified DNA (tube 5) to FSP plate

- ▶ Open purified DNA tube
- ▶ Pick up pipette & tip
- ▶ Pipette purified DNA into FSP plate well A1
- ▶ Discard pipette tip & place pipette on pipette rack
- ▶ Discard purified DNA tube

   c   Centrifuge FSP plate at 1000 x g

- ▶ Pick up FSP plate & place in empty cradle in centrifuge
- ▶ Close centrifuge lid & press "Start"
- ▶ After 5 seconds, centrifuge lid will automatically open
- ▶ Remove FSP plate from centrifuge

4   Run PCR1 program

   a   Place FSP plate in thermal cycler & close lid

   b   Press "Start Run"; vignette will play automatically

ForenSeq DNA Signature Prep

Part 2 – Enrich Targets

This process adds index adapters and sequences required for cluster amplification. Index adapters tag DNA with a unique combination of index sequences which allows data from each tagged library to be separated later during analysis.

Consumables

96-Well Plates
  ▸ Plate 1, ForenSeq Sample Plate (FSP)

Microcentrifuge Tubes
  ▸ Tube 6, Index 1 (i7) Adapter
  ▸ Tube 7, Index 2 (i5) Adapter
  ▸ Tube 8, PCR2 Reaction Mix (PCR2)

Procedure

1 Move tubes 6-8 to the working tube rack
2 Open thermal cycler lid, remove FSP plate & place plate on lab bench
3 Add index adapters to sample
   a Index 1 (i7) adapter (tube 6)
      ▸ Open i7 tube
      ▸ Pick up pipette & tip
      ▸ Pipette i7 into FSP plate well A1
      ▸ Discard pipette tip & place pipette on pipette rack
      ▸ discard i7 tube

   b Index 2 (i5) adapter (tube 7)
      ▸ Open i5 tube
      ▸ Pick up pipette & tip
      ▸ Pipette i5 into FSP plate well A1
      ▸ Discard pipette tip & place pipette on pipette rack, then discard i5 tube

4 Prepare sample for PCR
   a Add PCR2 (tube 8) to sample
      ▸ Open PCR2 tube
      ▸ Pick up pipette & tip
      ▸ Pipette PCR2 into FSP plate well A1
      ▸ Discard pipette tip & place pipette on pipette rack
      ▸ Discard PCR2 tube

   b Centrifuge FSP plate at 1000 x g
      ▸ Pick up FSP plate & place in empty cradle in centrifuge
      ▸ Close centrifuge lid & press "Start"
      ▸ After 5 seconds, centrifuge lid will automatically open
      ▸ Remove FSP plate from centrifuge

5 Run PCR2 program
   a Place FSP plate in thermal cycler & close lid
   b Press "Start Run"; vignette will play automatically

ForenSeq DNA Signature Prep

Part 3 – Purify Libraries

SPBs (Sample Purification Beads) purify the amplified libraries from other reaction components. Magnetic SPBs bind DNA and are attracted to magnet, allowing excess primers and reagents to be removed from the sample via two ethanol washes. The purified library is then eluted off the SPBs with the resuspension buffer.

Consumables

96-Well Plates
- ► Plate 1, ForenSeq Sample Plate (FSP)
- ► Plate 2, Purification Bead Plate (PBP)
- ► Plate 3, Purified Library Plate (PLP)

Microcentrifuge Tubes
- ► Tube 9, Sample Purification Beads (SPB)
- ► Tube 10, 80% EtOH
- ► Tube 11, Resuspension Buffer (RSB)

Procedure

1 Move tubes 9-11 to the working tube rack

2 Add SPB (tube 9) to PBP plate (plate 2)

    a Open SPB tube
    b Pick up pipette & tip
    c Pipette SPB into PBP plate well A1
    d Discard pipette tip & place pipette on pipette rack
    e Discard SPB tube

3 Open thermal cycler lid, remove FSP plate & place plate on lab bench

4 Transfer library to PBP plate

    a Pick up pipette & tip
    b Transfer library from FSP plate well A1 to PBP plate well A1
    c Discard pipette tip & place pipette on pipette rack
    d Discard FSP plate

5 Shake PBP plate at 1800 rpm

    a Pick up PBP plate & place in microplate shaker
    b Press "Start"
    c After 5 seconds, shaking will automatically stop
    d Remove PBP plate from microplate shaker & place on lab bench

6 Incubate at room temperature for 5 seconds

7 Purify library

    a Pick up PBP plate & place on magnetic stand; vignette will play automatically
    b Remove & discard all supernatant
       ► Pick up pipette & tip
       ► Pipette out supernatant from PBP plate well A1
       ► Discard pipette tip & place pipette on pipette rack
    c Wash-1 with 80% EtOH (tube 10)

- ▸ Open EtOH tube
- ▸ Pick up pipette & tip
- ▸ Pipette EtOH into PBP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Close EtOH tube

d   Remove & discard all supernatant

- ▸ Pick up pipette & tip
- ▸ Pipette out supernatant from PBP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack

e   Wash-2 with 80% EtOH (tube 10)

- ▸ Open EtOH tube
- ▸ Pick up pipette & tip
- ▸ Pipette EtOH into PBP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Discard EtOH tube

f   Remove & discard all supernatant

- ▸ Pick up pipette & tip
- ▸ Pipette out supernatant from PBP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack

g   Centrifuge PBP plate at 1000 x g

- ▸ Pick up PBP plate & place in empty cradle in centrifuge
- ▸ Close centrifuge lid & press "Start"
- ▸ After 5 seconds, centrifuge lid will automatically open
- ▸ Remove PBP plate from centrifuge & place on magnetic stand; vignette will play automatically

h   Remove residual EtOH

- ▸ Pick up pipette & tip
- ▸ Pipette out residual EtOH from PBP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack

i   Add RSB (tube 11) to library

- ▸ Open RSB tube
- ▸ Pick up pipette & tip
- ▸ Pipette RSB into PBP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Discard RSB tube

j   Shake PBP plate at 1800 rpm

- ▸ Pick up PBP plate & place in microplate shaker
- ▸ Press "Start"
- ▸ After 5 seconds, shaking will automatically stop
- ▸ Remove PBP plate from microplate shaker & place on lab bench

k   Incubate at room temperature for 5 seconds

l   Pick up PBP plate & place on magnetic stand; vignette will play automatically

8   Transfer library to PLP plate (plate 3)

  a Pick up pipette & tip
  b Transfer library from PBP plate well A1 to PLP plate well A1
  c Discard pipette tip & place pipette on pipette rack
  d Discard PBP plate

9 Centrifuge PLP plate at 1000 x g

  a Pick up PLP plate & place in empty cradle in centrifuge
  b Close centrifuge lid & press "Start"
  c After 5 seconds, centrifuge lid will automatically open
  d Remove PLP plate from centrifuge & place on lab bench

Part 4 – Normalize Libraries

After purification, libraries are normalized using Sample Purification Beads (SPBs). This process prepares DNA libraries for cluster generation, and assures samples with varying input amounts or sample types achieve consistent cluster density to optimize the resolution of individual samples when pooled together. As the SPBs only bind a defined amount of DNA, excess DNA will remain in the supernatant and be removed; thus, the need for post-PCR quantification is eliminated with this process.

Consumables

96-Well Plates
  ► Plate 3, Purified Library Plate (PLP)
  ► Plate 4, Normalization Working Plate (NWP)
  ► Plate 5, Normalized Library Plate (NLP)

Microcentrifuge Tubes
  ► Tube 12, LNA1/ LNB1 Master Mix
  ► Tube 13, Library Normalization Additives 1 (LNA1)
  ► Tube 14, Library Normalization Beads 1 (LNB1)
  ► Tube 15, 0.1 N HP3
  ► Tube 16, Nuclease-Free Water
  ► Tube 17, HP3 (2N-NaOH) Stock
  ► Tube 18, Library Normalization Storage Buffer 2 (LNS2)
  ► Tube 19, Library Normalization Wash 1 (LNW1)

Procedure

1   Move tubes 12-19 to the working tube rack

2   Prepare LNA1/LNB1 master mix in tube 12

  a   LNA1 (tube 13)

      ► Open LNA1/LNB1 master mix & LNA1 tubes
      ► Pick up pipette & tip
      ► Pipette LNA1 into master mix tube
      ► Discard pipette tip & place pipette on pipette rack
      ► Discard LNA1 tube

  b   LNB1 (tube 14)

      ► Open LNB1 tube
      ► Pick up pipette & tip
      ► Pipette LNB1 into master mix tube
      ► Discard pipette tip & place pipette on pipette rack
      ► Close master mix tube
      ► Discard LNB1 tube

  c   Vortex master mix & then centrifuge briefly

      ► Pick up master mix tube & vortex for 5 seconds
      ► Place tube in minifuge & close lid
      ► After 5 seconds, minifuge lid will automatically open
      ► Remove tube from minifuge & place in working tube rack

3   Transfer LNA1/LNB1 master mix to NWP plate (plate 4)

   a   Open master mix tube
   b   Pick up pipette & tip
   c   Transfer master mix to NWP plate well A1
   d   Discard pipette tip & place pipette on pipette rack
   e   Discard master mix tube

4   Transfer library to PLP plate

   a   Pick up NWP plate & place on magnetic stand (this assures no SPBs remain in the sample); vignette will play automatically
   b   Pick up pipette & tip
   c   Transfer library from PLP plate well A1 to NWP plate well A1
   d   Discard pipette tip & place pipette on pipette rack
   e   Discard PLP plate

5   Shake NWP plate at 1800 rpm

   a   Pick up NWP plate & place in microplate shaker
   b   Press "Start"
   c   After 5 seconds, shaking will automatically stop
   d   Remove NWP plate from microplate shaker & place on lab bench

6   Prepare 0.1 N HP3 in tube 15

   a   Nuclease-Free Water (tube 16)

      ▸ Open 0.1 N HP3 & nuclease-free water tubes
      ▸ Pick up pipette & tip
      ▸ Pipette nuclease-free water into 0.1 N HP3 tube
      ▸ Discard pipette tip & place pipette on pipette rack
      ▸ Close nuclease-free water tube

   b   HP3 (2N-NaOH) Stock (tube 17)

      ▸ Open HP3 stock tube
      ▸ Pick up pipette & tip
      ▸ Pipette HP3 stock into 0.1 N HP3 tube
      ▸ Discard pipette tip & place pipette on pipette rack
      ▸ Close 0.1 N HP3 & HP3 stock tubes

   c   Vortex & then centrifuge briefly

      ▸ Pick up 0.1 N HP3 tube & vortex for 5 seconds
      ▸ Place tube in minifuge & close lid
      ▸ After 5 seconds, minifuge lid will automatically open
      ▸ Remove tube from minifuge & place in working tube rack

7   Add LNS2 (tube 18) to NLP plate (plate 5) to prepare the plate for library storage

   a   Open LNS2 tube
   b   Pick up pipette & tip
   c   Pipette LNS2 into NLP plate well A1
   d   Discard pipette tip & place pipette on pipette rack
   e   Discard LNS2 tube

8   Wash library

a   Pick up NWP plate (plate 4) & place on magnetic stand; vignette will play automatically

b   Remove & discard all supernatant

- ▸ Pick up pipette & tip
- ▸ Pipette out supernatant from NWP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack

c   Wash-1 with LNW1 (tube 19)

- ▸ Pick up NWP plate from magnetic stand & place on lab bench
- ▸ Open LNW1 tube
- ▸ Pick up pipette & tip
- ▸ Pipette LNW1 into NWP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Close LNW1 tube

d   Shake NWP plate at 1800 rpm

- ▸ Pick up NWP plate and place in microplate shaker
- ▸ Press "start"
- ▸ After 5 seconds, shaking will automatically stop
- ▸ Remove NWP plate from microplate shaker & place on magnetic stand; vignette will play automatically

e   Remove & discard all supernatant

- ▸ Pick up pipette & tip
- ▸ Pipette out supernatant from NWP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack

f   Wash-2 with LNW1 (tube 19)

- ▸ Pick up NWP plate from magnetic stand & place on lab bench
- ▸ Open LNW1 tube
- ▸ Pick up pipette & tip
- ▸ Pipette LNW1 into NWP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Discard LNW1 tube

g   Shake NWP plate at 1800 rpm

- ▸ Pick up NWP plate and place in microplate shaker
- ▸ Press "start"
- ▸ After 5 seconds, shaking will automatically stop
- ▸ Remove NWP plate from microplate shaker & place on magnetic stand; vignette will play automatically

h   Remove & discard all supernatant

- ▸ Pick up pipette & tip
- ▸ Pipette out supernatant from NWP plate well A1
- ▸ Discard pipette tip & place pipette on pipette rack

i   Centrifuge NWP plate at 1000 x g

- ▸ Pick up NWP plate from magnetic stand & place in empty cradle in centrifuge
- ▸ Close centrifuge lid & press "Start"
- ▸ After 5 seconds, centrifuge lid will automatically open

▸ Remove NWP plate from centrifuge & place on magnetic stand; vignette will play automatically

j Remove residual LNW1

▸ Pick up pipette & tip
▸ Pipette out residual LNW1 from NWP plate well A1
▸ Discard pipette tip & place pipette on pipette rack
▸ Pick up NWP plate from magnetic stand & place on lab bench

k Add 0.1 N HP3 (tube 15) to library

▸ Open 0.1 N HP3 tube
▸ Pick up pipette & tip
▸ Pipette 0.1 N HP3 into NWP plate well A1
▸ Discard pipette tip & place pipette on pipette rack
▸ Discard 0.1 N HP3 tube

l Shake NWP plate at 1800 rpm

▸ Pick up NWP plate & place in microplate shaker
▸ Press "Start"
▸ After 5 seconds, shaking will automatically stop
▸ Remove NWP plate from microplate shaker & place on magnetic stand; vignette will play automatically

m Transfer normalized library to NLP plate (plate 5)

▸ Pick up pipette & tip
▸ Transfer library from NWP plate well A1 to NLP plate well A1
▸ Discard pipette tip & place pipette on pipette rack
▸ Discard NWP plate

n Centrifuge NLP plate at 1000 x g

▸ Pick up NLP plate & place in empty cradle in centrifuge
▸ Close centrifuge lid & press "Start"
▸ After 5 seconds, centrifuge lid will automatically open
▸ Remove NLP plate from centrifuge & place on lab bench; vignette will play automatically

Part 5 – Pool Libraries

This process combines equal volumes of normalized libraries to create a pool of libraries that are sequenced together on the same flow cell.

Consumables

96-Well Plates
- ► Plate 5, Normalized Library Plate (NLP)

Microcentrifuge Tubes
- ► Tube 20, Pooled Normalized Libraries (PNL)

Procedure

1   Move tube 20 to the working tube rack

2   Transfer normalized libraries from NLP plate (plate 5) to PNL tube (tube 20)

   a   Open PNL tube
   b   Pick up pipette & tip
   c   Transfer sample from NLP plate well A1 to PNL tube
   d   Discard pipette tip & place pipette on pipette rack
   e   Discard NLP plate

3   Vortex PNL tube & then centrifuge briefly

   a   Pick up PNL tube & vortex for 5 seconds
   b   Place tube in minifuge & close lid
   c   After 5 seconds, minifuge lid will automatically open
   d   Remove tube from minifuge & place in working tube rack; vignette will play automatically

Part 6 – Denature and Dilute Libraries

In this process, libraries are diluted in the hybridization buffer and heat denatured in preparation for sequencing. A human sequencing control is also added to the sample.

Consumables

Microcentrifuge Tubes
- ► Tube 16, Nuclease-Free Water
- ► Tube 17, HP3 (2N-NaOH) Stock
- ► Tube 20, Pooled Normalized Libraries (PNL)
- ► Tube 21, HSC Mixture
- ► Tube 22, Human Sequencing Control (HSC)
- ► Tube 23, Hybridization Buffer (HT1)
- ► Tube 24, Diluted Normalized Libraries (DNL)

MiSeq Reagent Cartridge

Procedure

1   Move tubes 21-24 to the working tube rack

2   Prepare an HSC denaturation reaction in the HSC mixture tube (tube 21)

   a   HSC (tube 22)
- ► Open HSC mixture & HSC tubes
- ► Pick up pipette & tip
- ► Pipette HSC into HSC mixture tube
- ► Discard pipette tip & place pipette on pipette rack
- ► Discard HSC tube

   b   HP3 (2N-NaOH) stock (tube 17)
- ► Open HP3 stock tube
- ► Pick up pipette & tip
- ► Pipette HP3 stock into HSC mixture tube
- ► Discard pipette tip & place pipette on pipette rack
- ► Discard HP3 stock tube

   c   Nuclease-free water (tube 16)
- ► Open nuclease-free water tube
- ► Pick up pipette & tip
- ► Pipette nuclease-free water into HSC mixture tube
- ► Discard pipette tip & place pipette on pipette rack
- ► Close HSC mixture tube
- ► Discard nuclease-free water tube

   d   Vortex HSC mixture tube & then centrifuge briefly
- ► Pick up HSC mixture tube & vortex for 5 seconds
- ► Place tube in minifuge & close lid
- ► After 5 seconds, minifuge lid will automatically open
- ► Remove tube from minifuge & place in working tube rack

   e   Incubate at room temperature for 5 seconds

3   Prepare diluted normalized libraries in DNL tube (tube 24)

    a   HT1 (tube 23)

- ▸ Open HT1 & DNL tubes
- ▸ Pick up pipette & tip
- ▸ Pipette HT1 into DNL tube
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Discard HT1 tube

    b   Transfer pooled normalized libraries from PNL tube (tube 20) to DNL tube

- ▸ Open PNL tube
- ▸ Pick up pipette & tip
- ▸ Transfer libraries from PNL tube to DNL tube
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Discard PNL tube

    c   Transfer HSC mixture to DNL tube

- ▸ Open HSC mixture tube
- ▸ Pick up pipette & tip
- ▸ Transfer HSC mixture from HSC mixture tube to DNL tube
- ▸ Discard pipette tip & place pipette on pipette rack
- ▸ Close DNL tube
- ▸ Discard HSC mixture tube

    d   Vortex DNL tube & then centrifuge briefly

- ▸ Pick up DNL tube & vortex for 5 seconds
- ▸ Place tube in minifuge & close lid
- ▸ After 5 seconds, minifuge lid will automatically open
- ▸ Remove tube from minifuge & place in working tube rack

4   Denature diluted libraries

    a   Place DNL tube in the 96°C microheating system (thermomixer)

- ▸ Pick up DNL tube & place in thermomixer
- ▸ After 5 seconds, remove tube from thermomixer

    b   Place DNL tube in the ice-water bath

- ▸ Place DNL tube in ice-water bath
- ▸ After 5 seconds, remove tube from ice-water bath & place in working tube rack

5   Load library onto the MiSeq reagent cartridge

    a   Open DNL tube
    b   Pick up pipette & tip
    c   Pipette diluted normalized libraries onto the MiSeq reagent cartridge
    d   Discard pipette tip & place pipette on pipette rack
    e   Discard DNL tube; vignette will play automatically
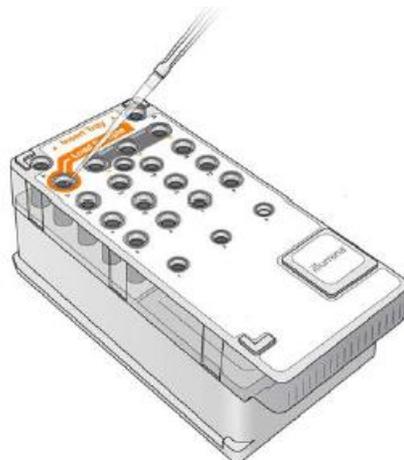
ForenSeq DNA Signature Prep

Part 7 – Start Sequencing Run

In this process, the reagent cartridge and flow cell are loaded into the MiSeq, and the run is started.

### Consumables

MiSeq Reagent Cartridge
MiSeq Flow Cell

Procedure

1   Open flow cell compartment door & press flow cell latch release button
2   Load flow cell, then close flow cell latch & flow cell compartment door
3   Open reagent compartment door & reagent chiller door
4   Load reagent cartridge, then close reagent chiller door & reagent compartment door
5   Press "Start Run"; vignette will play automatically

MiSeq Components
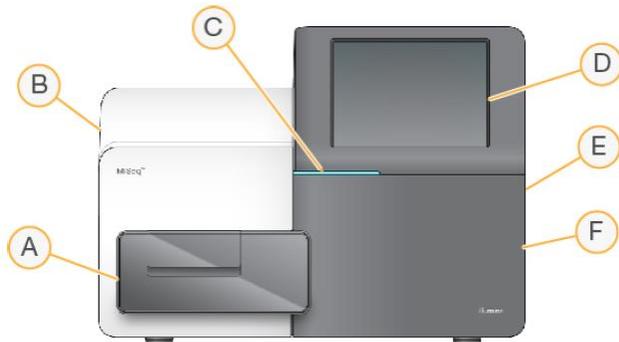
A   Flow cell compartment – Contains the flow cell stage that houses the flow cell throughout the run.
B   Enclosed optics module – Contains optical components that enable imaging of the flow cell.
C   Status bar – Indicates instrument status. Green indicates the instruments is ready to begin the next run, blue indicates the instrument is processing & orange indicates the instruments needs attention.
D   Touch screen monitor
E   External USB ports
F   Reagent compartment – Holds reagents, wash solutions & the waste bottle

Flow Cell Compartment
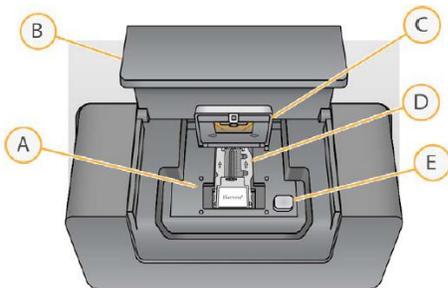
A   Flow Cell Stage
B   Flow Cell Compartment Door
C   Flow Cell Latch
D   Flow Cell
E   Flow Cell Latch Release Button

Reagent Compartment
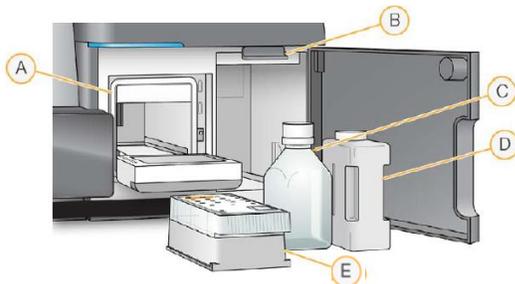
A   Reagent Chiller
B   Sipper Handle
C   Wash Buffer Bottle
D   Waste Bottle
E   Reagent Cartridge

iontorrent

Ion AmpliSeq Library Preparation on the Ion Chef System

The Ion AmpliSeq Kit for Chef DL8 (**D**NA to **L**ibrary, **8** samples per run) is designed for use with the Ion Chef System for automation of Ion AmpliSeq library construction. The Ion Chef instrument prepares a target amplification reaction for each sample with each primer pool, using a different barcode for each sample. Library concentration is normalized to 100 pM using Ion Library Equalizer technology, and libraries, combined in a single tube, are ready to use in an Ion Chef template preparation reaction.

Consumables

- Ion AmpliSeq Chef Reagents DL8
- IonCode Barcode 96 Well Plate
- Precision ID mtDNA Whole Genome Panel (Tube 1)
- gDNA (Tube 2)

Procedure

1. Uncap all tubes in the Ion AmpliSeq Chef Reagents DL8 Cartridge
   - Remove cap from tube in position A and discard
   - Remove cap from tube in position B and discard
   - Remove cap from tube in position C and discard
   - Remove cap from tube in position D and discard
2. Add Precision ID mtDNA Whole Genome Panel (tube 1) to the Reagents Cartridge
   - Remove cap from tube 1 and discard
   - Open pipette tip box, then pick up pipette and tip
   - Pipette primer panel into tube in positions A and B (use the same pipette tip)
   - Discard pipette tip and place pipette on the pipette rack
   - Discard tube 1
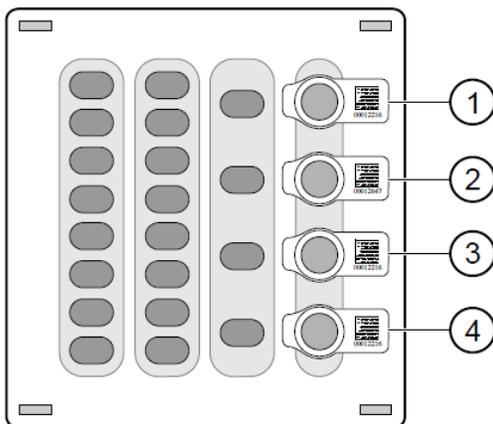
① Position A (150 μL Primer Pool 1)
② Position B (150 μL Primer Pool 1 or 2)
③ Position C (Empty tube)
④ Position D (Output tube)

3. Add gDNA (tube 2) to the IonCode Barcode 96 Well Plate
   - Open gDNA tube
   - Pick up pipette and tip
   - Pipette gDNA into IonCode Barcode plate well A1
   - Discard pipette tip and place pipette on the pipette rack
   - Discard gDNA tube

4. Load the Reagents Cartridge and Ion Barcode plate in the Ion Chef instrument
    - Open the Ion Chef door
    - Pick up the Ion AmpliSeq Chef Reagents DL8 Cartridge and place in the Reagents station
    - Pick up the IonCode Barcode plate and place on the thermal cycler block
    - Close the Ion Chef door
    - Press "Run"; vignette will play automatically
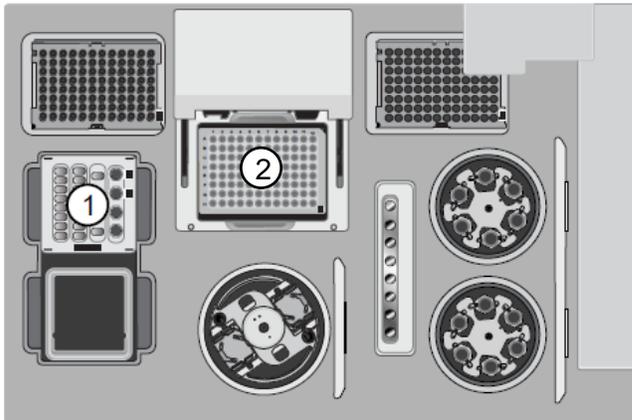
① Reagents station (4°C)
② Thermal cycler sample block

Thermal Cycler Block

Reagents Station
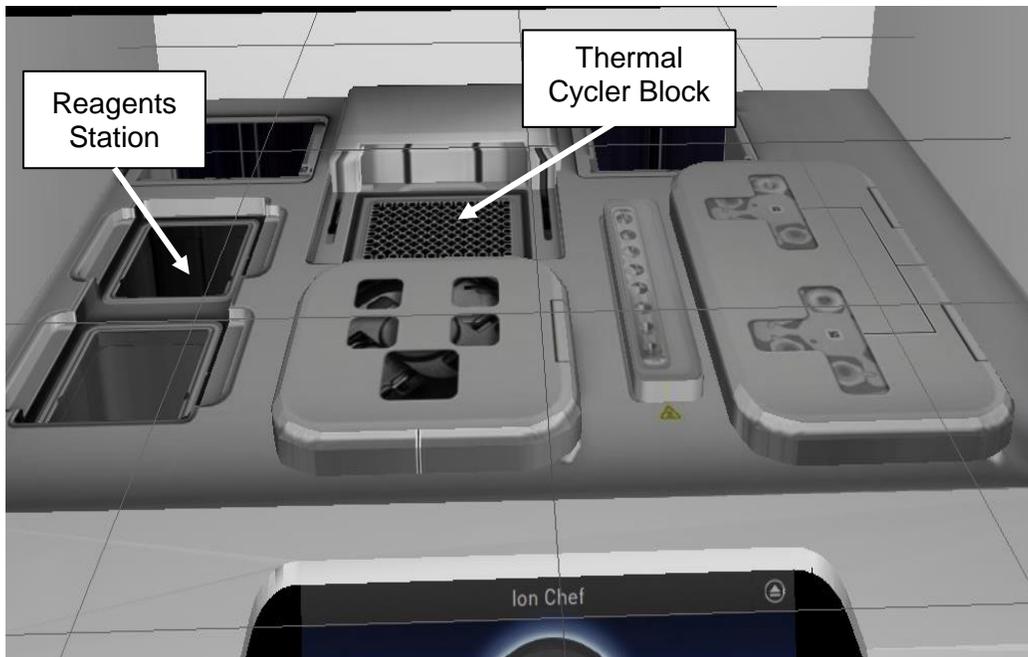
Ion Chef

Template Preparation and Chip Loading on the Ion Chef System

The 100 pM combined library prepared in Part 1 (Ion AmpliSeq Library Preparation on the Ion Chef System) is diluted to 50 pM. With the Ion 530 Kit, the Ion Chef instrument prepares enriched, template-positive Ion Sphere Particles (ISPs) using the diluted library. During this process, a sequencing chip is prepared for the sequencing of libraries on the Ion S5 Sequencer.

Consumables

- Ion Sequencing Chip
- Chip Adapter
- Ion 530 Reagent Cartridge
- Diluted Library (Tube 3)
- Nuclease-Free Water (Tube 4)

Procedure

1. Remove tube containing the 100 pM combined library from the Ion AmpliSeq Chef Reagents DL8 Cartridge
   - Open the Ion Chef door
   - Remove the tube in position D of the Reagents Cartridge and place in tube rack
2. Prepare a 50 pM library dilution in tube 3 with nuclease-free water (tube 4)
   - Open diluted library and nuclease-free water tubes
   - Pick up pipette and tip
   - Pipette the 100 pM library into the diluted library tube
   - Discard pipette tip and then pick up a new pipette tip
   - Pipette nuclease-free water into the diluted library tube
   - Discard pipette tip and place pipette on the pipette rack
   - Close diluted library and nuclease-free water tubes
   - Discard the 100 pM library tube
   - Pick up the diluted library tube and vortex for 5 seconds
   - Place tube in minifuge and close lid
   - After 5 seconds, minifuge lid will automatically open
   - Remove tube from minifuge and place in tube rack
3. Uncap all tubes in the Ion 530 Reagents Cartridge
   - Remove cap from tube in position A and discard
   - Remove cap from tube in position B and discard
   - Remove cap from tube in position C and discard
   - Remove cap from tube in position D and discard
4. Add the diluted library (tube 3) and nuclease-free water (tube 4) to the Reagents Cartridge
   - Open diluted library and nuclease-free water tubes
   - Pick up pipette and tip
   - Pipette diluted library into tube in position A of the Reagents Cartridge
   - Discard pipette tip, then pick up a new pipette tip
   - Pipette nuclease-free water into tube in position B of the Reagents Cartridge
   - Discard pipette tip and place pipette on pipette rack
   - Discard diluted library tube and nuclease-free water tube
5. Load the Reagents Cartridge and Sequencing Chip onto Ion Chef instrument
   - Open the Ion Chef door

- Pick up the Reagents Cartridge and place in the Reagents station
- Open the Chip-Loading Centrifuge door
- Pick up the Sequencing Chip + Adapter and place in the empty Chip-Loading Centrifuge Bucket
- Close the Chip-Loading Centrifuge door, and then close the Ion Chef door
- Press "Run"; vignette will play automatically
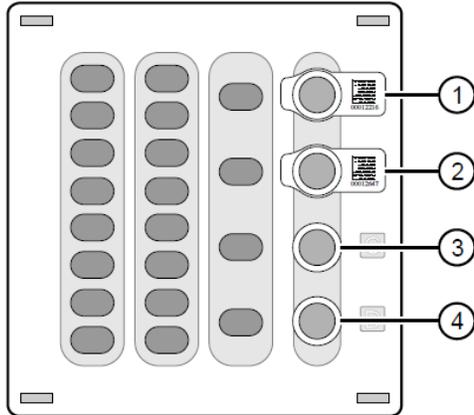
① Position A (Library)
② Position B (Library)
③ Position C (NaOH)
④ Position D (Empty tube)

Chip Adapter
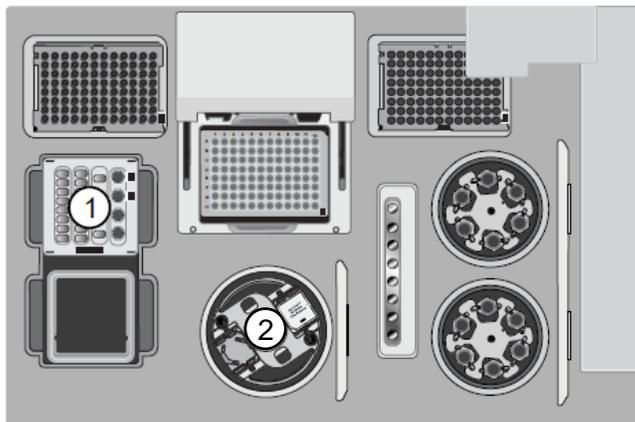
Ion chip

① Reagents station (4°C)
② Chip-loading centrifuge

Reagents Station

Chip-loading Centrifuge

Ion Chef
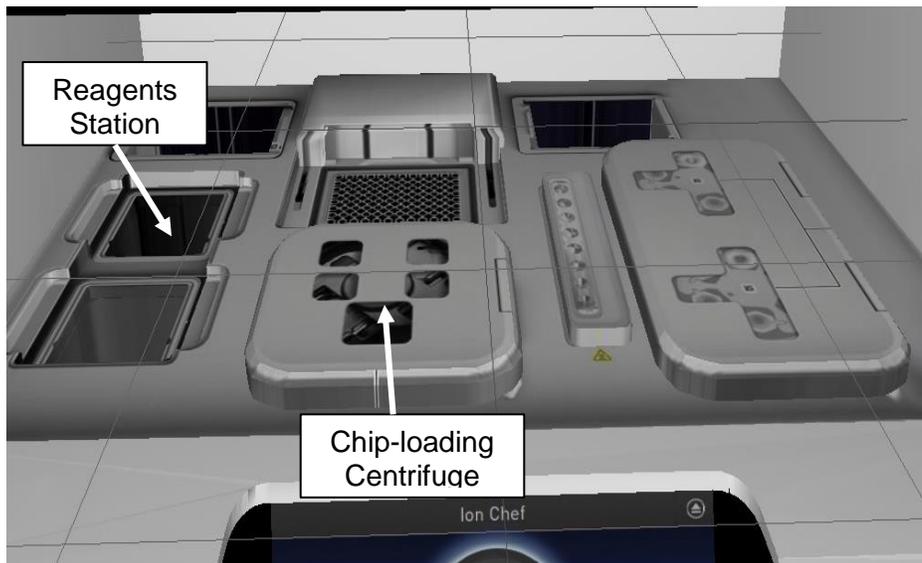
Sequencing on the Ion S5

During the sequencing reaction on the Ion S5, the sequencing chip is flooded with one of the four nucleotides. Whenever a nucleotide is incorporated into a single strand of DNA, a hydrogen ion is released changing the pH of the surrounding solution. The ion semiconductor sequencing chip is able to detect this change in pH and convert it directly to base calls.

Procedure

1. Prepare the Ion S5 for chip loading
   - Open the Ion S5 door
   - Open the Chip Clamp
2. Unload the Sequencing Chip from the Ion Chef
   - Open the Ion Chef door, and then the Chip-Loading Centrifuge door
   - Remove the Sequencing Chip + Chip Adapter and place on the lab bench
   - Remove the Sequencing Chip from the Chip Adapter
   - Place the Sequencing Chip in the Chip Clamp of the Ion S5
   - Close the Chip Clamp
   - Close the Ion S5 door
   - Press "Run"; vignette will play automatically
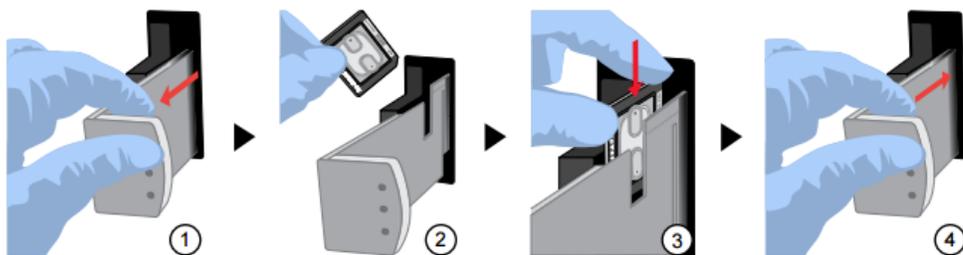
INSTRUCTIONS FOR USE

Part 1 – PCR Setup and Thermal Cycling

This process amplifies the gDNA using the PowerSeq 5X primer mix with regions specific to DNA sequences upstream and downstream of STRs. Targets are enriched after amplification, and then libraries are normalized prior to pooling them together in one tube. For simplicity, only one sample will be processed throughout this simulation.

Consumables

96-Well Plates
- Plate 1, PCR Plate

Microcentrifuge Tubes
- Tube 1, Master Mix
- Tube 2, Nuclease-Free Water
- Tube 3, PowerSeq 5X Master Mix
- Tube 4, PowerSeq 5X Primer Pair Mix
- Tube 5, Template DNA

Procedure

1. Move tubes 1-5 to the working (empty) tube rack

2. Prepare master mix in tube 1

   a. Nuclease-Free Water (tube 2)

      - Open master mix and nuclease-free water tubes
      - Open pipette tip box
      - Pick up pipette and tip
      - Pipette nuclease-free water into master mix tube
      - Discard pipette tip and place pipette on pipette rack
      - Close nuclease-free water tube

   b. PowerSeq 5X Master Mix (tube 3)

      - Open PowerSeq 5X Master Mix tube
      - Pick up pipette and tip
      - Pipette PowerSeq 5X Master Mix into master mix tube
      - Discard pipette tip and place pipette on pipette rack
      - Discard PowerSeq 5X Master Mix tube

   c. PowerSeq 5X Primer Pair Mix (tube 4)

      - Open PowerSeq 5X Primer Pair Mix tube
      - Pick up pipette and tip
      - Pipette PowerSeq 5X Primer Pair Mix into master mix tube
      - Discard pipette tip and place pipette on pipette rack
      - Close master mix tube
      - Discard PowerSeq 5X Primer Pair Mix tube

    d. Vortex master mix and then centrifuge briefly

- Pick up master mix tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

3. Prepare sample for PCR

    a. Add master mix to PCR plate (plate 1)

- Open master mix tube
- Pick up pipette and tip
- Pipette master mix into PCR plate well A1
- Discard pipette tip and place pipette on pipette rack
- Discard master mix tube

    b. Add template DNA (tube 5) to PCR plate

- Open template DNA tube
- Pick up pipette and tip
- Pipette template DNA into PCR plate well A1
- Discard pipette tip and place pipette on pipette rack
- Discard template DNA tube

    c. Centrifuge PCR plate at 1000 x g

- Pick up PCR plate and place in empty cradle in centrifuge
- Close centrifuge lid and press "Start"
- After 5 seconds, centrifuge lid will automatically open
- Remove PCR plate from centrifuge

4. Run PCR program

    a. Place PCR plate in thermal cycler and close lid
    b. Press "Start Run"; vignette will play automatically

Part 2 – Amplification Product Purification

This process purifies the amplification product created in Part 1 using the Qiagen MinElute PCR Purification kit. The concentration of the purified amplification product is then determined using a NanoDrop spectrophotometer.

Consumables

96-Well Plates
- Plate 1, PCR Plate

Microcentrifuge Tubes
- Tube 6, Amplification Product from Part 1

Procedure

1. Move tube 6 to the working tube rack

2. Open thermal cycler lid, remove PCR plate and place on lab bench

3. Transfer sample from PCR plate to amplification product tube (tube 6)

   a. Open amplification product tube
   b. Pick up pipette and tip
   c. Transfer sample from PCR plate well A1 to amplification product tube
   d. Discard pipette tip and place pipette on pipette rack
   e. Close amplification product tube
   f. Discard PCR plate; vignette will play automatically

Part 3a – Library Preparation, End Repair Reaction

This process converts any overhangs resulting from PCR into blunt ends using the Illumina TruSeq PCR-free LT Solution Preparation Kit (Set A or B) End Repair Mix 2. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the 5' to 3' polymerase activity fills in the 5' overhangs. Following end repair, the appropriate library size is selected using the Qiagen MinElute PCR Purification kit.

Consumables

96-Well Plates
- Plate 2, Library Prep Plate - End Repair

Microcentrifuge Tubes
- Tube 6, Amplification Product from Part 1
- Tube 7, End Repair Mix 2 (ERP2)
- Tube 8, Resuspension Buffer (RSB)

Procedure

1. Move tubes 7-8 to the working tube rack

2. Assemble end repair reaction in library prep plate - end repair (plate 2)

   a. Amplification Product from Part 1 (tube 6)

      - Open amplification product tube
      - Pick up pipette and tip
      - Pipette amplification product into end repair plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard amplification product tube

   b. ERP2 (tube 7)

      - Open ERP2 tube
      - Pick up pipette and tip
      - Pipette ERP2 into end repair plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard ERP2 tube

   c. RSB (tube 8)

      - Open RSB tube
      - Pick up pipette and tip
      - Pipette RSB into end repair plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard RSB tube

   d. Centrifuge end repair plate at 1000 x g

      - Pick up end repair plate and place in empty cradle in centrifuge
      - Close centrifuge lid and press "Start"

- After 5 seconds, centrifuge lid will automatically open
- Remove end repair plate from centrifuge

3. Run end repair program

  a. Place end repair plate in thermal cycler and close lid
  b. Press "Start Run"; vignette will play automatically

Part 3b – Library Preparation, Amplification Product Purification

This process selects the appropriate library size from the end repair reaction using the Qiagen MinElute PCR Purification kit.

Consumables

96-Well Plates

- Plate 2, Library Prep Plate - End Repair

Microcentrifuge Tubes

- Tube 9, End Repair Reaction Amplification Product

Procedure

1. Move tube 9 to the working tube rack

2. Open thermal cycler lid, remove end repair plate and place on lab bench

3. Transfer sample from end repair plate to end repair reaction amplification product tube (tube 9)

   a. Open amplification product tube
   b. Pick up pipette and tip
   c. Transfer sample from end repair plate well A1 to amplification product tube
   d. Discard pipette tip and place pipette on pipette rack
   e. Close amplification product tube
   f. Discard end repair plate; vignette will play automatically

Part 3c – Library Preparation, A-Tailing Reaction

During this process, a single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to each other during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

96-Well Plates
- Plate 3, Library Prep Plate - A-Tailing & Adapter Ligation

Microcentrifuge Tubes
- Tube 9, End Repair Reaction Amplification Product
- Tube 10, A-Tailing Mix (ATL)

Procedure

1. Move tube 10 to the working tube rack

2. Assemble A-tailing reaction in library prep plate - A-tailing & adapter ligation (plate 3)

   a. End Repair Reaction Amplification Product (tube 9)

      - Open amplification product tube
      - Pick up pipette and tip
      - Pipette amplification product into A-tailing & adapter ligation plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard amplification product tube

   b. ATL (tube 10)

      - Open ATL tube
      - Pick up pipette and tip
      - Pipette ATL into A-tailing & adapter ligation plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard ATL tube

   c. Centrifuge A-tailing & adapter ligation plate at 1000 x g

      - Pick up A-tailing & adapter ligation plate and place in empty cradle in centrifuge
      - Close centrifuge lid and press "Start"
      - After 5 seconds, centrifuge lid will automatically open
      - Remove A-tailing & adapter ligation plate from centrifuge

3. Run A-tailing program

   a. Place A-tailing & adapter ligation plate in thermal cycler and close lid
   b. Press "Start Run"; vignette will play automatically

Part 3d – Library Preparation, Adapter Ligation Reaction

This process ligates indexing adapters to the ends of the DNA fragments, preparing them for hybridization onto a flow cell. Index adapters tag DNA with a unique combination of index sequences which allows data from each tagged library to be separated later during analysis.

Consumables

96-Well Plates
- Plate 3, Library Prep Plate – A-Tailing & Adapter Ligation

Microcentrifuge Tubes
- Tube 11, Resuspension Buffer (RSB)
- Tube 12, Ligation Mix 2 (LIG2)
- Tube 13, Adapter
- Tube 14, Stop Ligation Buffer (STL)

Procedure

1. Move tubes 11-14 to the working tube rack

2. Open thermal cycler lid, remove A-tailing & adapter ligation plate and place on lab bench

3. Assemble adapter ligation reaction in A-tailing & adapter ligation plate

   a. RSB (tube 11)

   - Open RSB tube
   - Pick up pipette and tip
   - Pipette RSB into A-tailing & adapter ligation plate well A1
   - Discard pipette tip and place pipette on pipette rack
   - Discard RSB tube

   b. LIG2 (tube 12)

   - Open LIG2 tube
   - Pick up pipette and tip
   - Pipette LIG2 into A-tailing & adapter ligation plate well A1
   - Discard pipette tip and place pipette on pipette rack
   - Discard LIG2 tube

   c. Adapter (tube 13)

   - Open Adapter tube
   - Pick up pipette and tip
   - Pipette Adapter into A-tailing & adapter ligation plate well A1
   - Discard pipette tip and place pipette on pipette rack
   - Discard Adapter tube

   d. Centrifuge A-tailing & adapter ligation plate at 1000 x g

   - Pick up A-tailing & adapter ligation plate and place in empty cradle in centrifuge

- Close centrifuge lid and press "Start"
- After 5 seconds, centrifuge lid will automatically open
- Remove A-tailing & adapter ligation plate from centrifuge

4. Run adapter ligation program

   a. Place A-tailing & adapter ligation plate in thermal cycler and close lid
   b. Press "Start Run"; vignette will play automatically

5. Stop the adapter ligation reaction

   a. Open thermal cycler lid, remove A-tailing & adapter ligation plate and place on lab bench

   b. Add STL (tube 14) to A-tailing & adapter ligation plate

      - Open STL tube
      - Pick up pipette and tip
      - Pipette STL into A-tailing & adapter ligation plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard STL tube

   c. Centrifuge A-tailing & adapter ligation plate at 1000 x g

      - Pick up A-tailing & adapter ligation plate and place in empty cradle in centrifuge
      - Close centrifuge lid and press "Start"
      - After 5 seconds, centrifuge lid will automatically open
      - Remove A-tailing & adapter ligation plate from centrifuge and place on lab bench; vignette will play automatically

Part 3e – Library Preparation, Purify Libraries

In this process, SPBs (Solution Purification Beads) purify the ligation products from other reaction components. Magnetic SPBs bind DNA and are attracted to magnet, allowing excess adapters and reagents to be removed from the solution via two ethanol washes. The purified ligation product is then eluted off the SPBs with the resuspension buffer. A second purification may be completed.

Consumables

96-Well Plates

- Plate 3, Library Prep Plate – A-Tailing & Adapter Ligation
- Plate 4, Purified Library Plate

Microcentrifuge Tubes

- Tube 2, Nuclease-Free Water
- Tube 15, 80% EtOH
- Tube 16, Sample Purification Beads (SPB)
- Tube 17, Resuspension Buffer (RSB)

Procedure

1. Move tubes 15-17 to the working tube rack

2. Purify ligation products with SPB

   a. Add SPB (tube 16) to ligation products (plate 3)

      - Open SPB tube
      - Pick up pipette and tip
      - Pipette SPB into ligation products plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Discard SPB tube

   b. Incubate at room temperature for 5 seconds

   c. Pick up ligation products plate and place on magnetic stand; vignette will play automatically

   d. Incubate at room temperature for 5 seconds

   e. Remove and discard all supernatant

      - Pick up pipette and tip
      - Pipette out supernatant from ligation products plate well A1
      - Discard pipette tip and place pipette on pipette rack

   f. Wash-1 with 80% EtOH (tube 15)

      - Open EtOH tube
      - Pick up pipette and tip
      - Pipette EtOH into ligation products plate well A1

- Discard pipette tip and place pipette on pipette rack
- Close EtOH tube

g. Incubate at room temperature for 5 seconds

h. Remove and discard all supernatant

- Pick up pipette and tip
- Pipette out supernatant from ligation products plate well A1
- Discard pipette tip and place pipette on pipette rack

i. Wash-2 with 80% EtOH (tube 15)

- Open EtOH tube
- Pick up pipette and tip
- Pipette EtOH into ligation products plate well A1
- Discard pipette tip and place pipette on pipette rack
- Discard EtOH tube

j. Incubate at room temperature for 5 seconds

k. Remove and discard all supernatant

- Pick up pipette and tip
- Pipette out supernatant from ligation products plate well A1
- Discard pipette tip and place pipette on pipette rack

l. Air dry ligation products for 5 seconds

m. Add RSB (tube 17) to ligation products

- Pick up ligation products plate from magnetic stand and place on lab bench
- Open RSB tube
- Pick up pipette and tip
- Pipette RSB into ligation products plate well A1
- Discard pipette tip and place pipette on pipette rack
- Discard RSB tube

n. Incubate at room temperature for 5 seconds

o. Pick up ligation products plate and place on magnetic stand; vignette will play automatically

p. Transfer ligation products to purified library plate (plate 4)

- Pick up pipette and tip
- Transfer ligation products to purified library plate well A1
- Discard pipette tip and place pipette on pipette rack
- Discard ligation product plate

Part 3f – Library Preparation, Normalize, Denature & Dilute Libraries

After purification, the library is quantified using the PowerSeq Quant MS System. The library is them normalized to ensure solutions achieve consistent cluster density to optimize the resolution of individual solutions when pooled together. Pooled libraries are sequenced together on the same flow cell. After normalization, libraries are diluted in the hybridization buffer and denatured in preparation for sequencing. A sequencing control is also added to the solution.

Consumables

MiSeq Reagent Cartridge

96-Well Plates
- Plate 4, Purified Library Plate

Microcentrifuge Tubes
- Tube 2, Nuclease-Free Water
- Tube 18, Tris-HCl + 0.1% Tween 20
- Tube 19, 0.2N NaOH
- Tube 20, 2N NaOH
- Tube 21, Pooled Libraries
- Tube 22, Hybridization Buffer (HT1)
- Tube 23, PhiX Control
- Tube 24, Stock PhiX Control (10 nM)
- Tube 25, Sequencing Dilution

Procedure

1. Vignette will play automatically (Library Quantification with PowerSeq Quant MS System)

2. Move tubes 19-25 to the working tube rack

3. Normalize Library

   a. Add Tris-HCl + 0.1% Tween 20 (tube 18) to purified library (plate 4)

      - Open Tris-HCl + 0.1% Tween 20 tube
      - Pick up pipette and tip
      - Pipette Tris-HCl + 0.1% Tween 20 into purified library plate well A1
      - Discard pipette tip and place pipette on pipette rack
      - Close Tris-HCl + 0.1% Tween 20 tube

   b. Centrifuge purified library plate at 1000 x g

      - Pick up purified library plate and place in empty cradle in centrifuge
      - Close centrifuge lid and press "Start"
      - After 5 seconds, centrifuge lid will automatically open
      - Remove purified library plate from centrifuge and place on lab bench

4. Denature and Dilute Library

   a. Prepare 0.2N NaOH in tube 19

i. 2N NaOH (tube 20)

- Open 2N NaOH tube
- Pick up pipette and tip
- Pipette 2N NaOH into 0.2N NaOH tube
- Discard pipette tip and place pipette on pipette rack
- Discard 2N NaOH tube

ii. Nuclease-Free Water (tube 2)

- Open nuclease-free water tube
- Pick up pipette and tip
- Pipette nuclease-free water into 0.2N NaOH tube
- Discard pipette tip and place pipette on pipette rack
- Discard nuclease-free water tube

iii. Vortex 0.2N NaOH tube and then centrifuge briefly

- Pick up 0.2N NaOH tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

b. Pool libraries into tube 21

- Open pooled libraries tube
- Pick up pipette and tip
- Pipette sample from purified library plate well A1 into pooled libraries tube
- Discard pipette tip and place pipette on pipette rack
- Discard purified library plate

c. Denature pooled libraries with 0.2N NaOH (tube 19)

- Open 0.2N NaOH tube
- Pick up pipette and tip
- Pipette 0.2N NaOH into pooled libraries tube
- Discard pipette tip and place pipette on pipette rack
- Close 0.2N NaOH tube and pooled libraries tube

d. Vortex pooled libraries tube and then centrifuge briefly

- Pick up pooled libraries tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

e. Incubate pooled libraries at room temperature for 5 seconds

f. Dilute pooled libraries with HT1 buffer (tube 22)

- Open pooled libraries and HT1 buffer tubes
- Pick up pipette and tip

- Pipette HT1 buffer into pooled libraries tube
- Discard pipette tip and place pipette on pipette rack
- Close pooled libraries and HT1 buffer tubes

g. Vortex pooled libraries tube and then centrifuge briefly

- Pick up pooled libraries tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

5. Prepare the PhiX control in tube 23

a. Stock PhiX Control, 10 nM (tube 24)

- Open PhiX control and stock PhiX control tubes
- Pick up pipette and tip
- Pipette stock PhiX control into PhiX control tube
- Discard pipette tip and place pipette on pipette rack
- Discard stock PhiX control tube

b. Tris-HCl + 0.1% Tween 20 (tube 18)

- Open Tris-HCl + 0.1% Tween 20 tube
- Pick up pipette and tip
- Pipette Tris-HCl + 0.1% Tween 20 into PhiX control tube
- Discard pipette tip and place pipette on pipette rack
- Discard Tris-HCl + 0.1% Tween 20 tube

c. 0.2N NaOH (tube 19)

- Open 0.2N NaOH tube
- Pick up pipette and tip
- Pipette 0.2N NaOH into PhiX control tube
- Discard pipette tip and place pipette on pipette rack
- Close PhiX control tube
- Discard 0.2N NaOH tube

d. Vortex PhiX control tube and then centrifuge briefly

- Pick up PhiX control tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

e. Incubate PhiX control at room temperature for 5 seconds

f. Add HT1 buffer (tube 22) to PhiX control tube

- Open HT1 buffer and PhiX control tubes
- Pick up pipette and tip
- Pipette HT1 buffer into PhiX control tube

- Discard pipette tip and place pipette on pipette rack
- Close HT1 buffer and PhiX control tubes

g. Vortex PhiX control tube and then centrifuge briefly

- Pick up PhiX control tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

6. Prepare the sequencing dilution in tube 25

a. HT1 buffer (tube 22)

- Open HT1 buffer and sequencing dilution tubes
- Pick up pipette and tip
- Pipette HT1 buffer into sequencing dilution tube
- Discard pipette tip and place pipette on pipette rack
- Discard HT1 buffer tube

b. Pooled libraries (tube 21)

- Open pooled libraries tube
- Pick up pipette and tip
- Pipette pooled libraries into sequencing dilution tube
- Discard pipette tip and place pipette on pipette rack
- Discard pooled libraries tube

c. PhiX control (tube 23)

- Open PhiX control tube
- Pick up pipette and tip
- Pipette PhiX control into sequencing dilution tube
- Discard pipette tip and place pipette on pipette rack
- Close sequencing dilution tube
- Discard PhiX control tube

d. Vortex sequencing dilution tube and then centrifuge briefly

- Pick up sequencing dilution tube and vortex for 5 seconds
- Place tube in minifuge and close lid
- After 5 seconds, minifuge lid will automatically open
- Remove tube from minifuge and place in working tube rack

7. Load libraries onto the MiSeq reagent cartridge

a. Open sequencing dilution tube
b. Pick up pipette and tip
c. Pipette libraries onto the MiSeq reagent cartridge
d. Discard pipette tip and place pipette on pipette rack
e. Discard sequencing dilution tube; vignette will play automatically
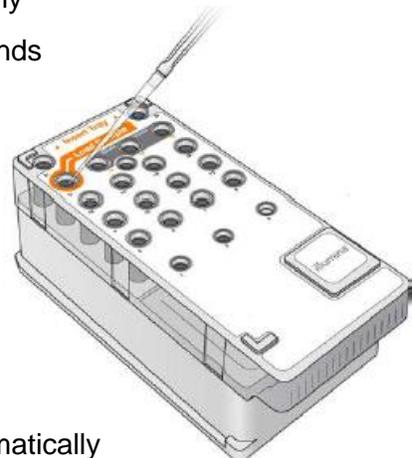
Part 4 – Start Sequencing Run

In this process, the reagent cartridge and flow cell are loaded into the MiSeq, and the run is started.

Consumables

MiSeq Reagent Cartridge
MiSeq Flow Cell

Procedure

1. Open flow cell compartment door and press flow cell latch release button
2. Load flow cell, then close flow cell latch and flow cell compartment door
3. Open reagent compartment door and reagent chiller door
4. Load reagent cartridge, then close reagent chiller door and reagent compartment door
5. Press "Start Run"; vignette will play automatically

MiSeq Components
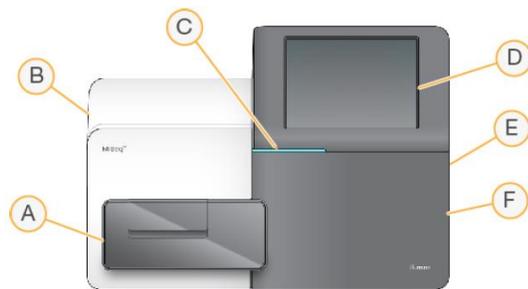
G **Flow cell compartment** – Contains the flow cell stage that houses the flow cell throughout the run.

H **Enclosed optics module** – Contains optical components that enable imaging of the flow cell.

I **Status bar** – Indicates instrument status. Green indicates the instruments is ready to begin the next run, blue indicates the instrument is processing & orange indicates the instruments needs attention.

J **Touch screen monitor**
K **External USB ports**
L **Reagent compartment** – Holds reagents, wash solutions & the waste bottle

Flow Cell Compartment
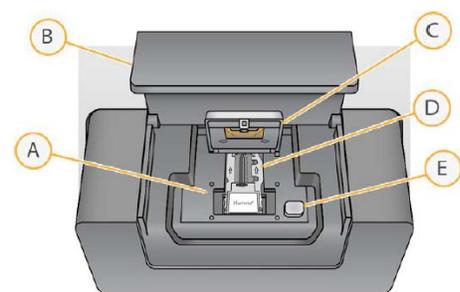
F Flow Cell Stage
G Flow Cell Compartment Door
H Flow Cell Latch
I Flow Cell
J Flow Cell Latch Release Button

Reagent Compartment
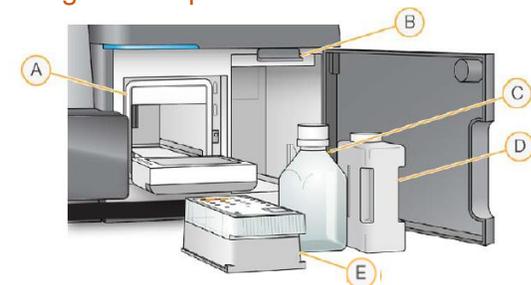
F Reagent Chiller
G Sipper Handle
H Wash Buffer Bottle
I Waste Bottle
J Reagent Cartridge

Bioinformatics

The Genomic Identification Lab @ NC State

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Bioinformatics

for the Forensic Scientist

"the collection, classification, storage, and analysis of biochemical and biological information using computers especially as applied to molecular genetics and genomics"

Data Formats

Seqence data can come in a variety of formats.

They are categorized by level or step from raw data to interpreted data.

See the flow diagram below and click each data type to explore further.

Primary Analysis
- Sequencer sensing is converted to nucleotide sequence data
- Quality scores included
- Samples are demultiplexed
- Instrument run statistics included

FASTA

FASTQ

Secondary Analysis
- QA, trimming, filtering
- Alignment
- String (sequence) analysis
- Variant calling
- Recalibration
- Possible coversion to computer friendly file format

BAM

VCF

STR

Teriary Analysis
- Interpretation
  - Filtered STR data, identity, identity, phenotype predictions, haplotyping, kinship, mixtures, etc.
- "Sense Making"
- Combining datasets/samples
- Report writing

Varied

FASTA

FASTA Sequence Format

FASTA is a DNA and protein sequence alignment software package first described (as FASTP) by David J. Lipman and William R. Pearson (Science 227 (4693): 1435-41.1985)
- "FAST-A" = FAST-All
- "FAST-P" = protein
- "FAST-N" = nucleotide
Format
- ">" character defines the beginning of the description line
- Line feed (LF) or carriage return (CR) defines end of description line
- Nucleotides or amino acid sequence (string) follows LF

>chr5:149455852-149456180 5q33.1; human c-fms proto-oncogene for CSF-1 receptor gene, 6th intron349bp(LF)
gagaaagaataactgcatcttaacctattgggaggtcattgtaaagaggagagtgatggggtcagattgtacaga
ggaggcacttcgtggtggtcaggagcacacactccagggcagtgttccaacctgagtctgccaaggactagcag
gttgctaaccaccctgtgtctcagttttcctacctgtaaaatgaagatattaacagtaactgccttcatagatagaaga
tagatagattagatagatagatagatagatagatagatagatagatagatagatagatagatagatagataggaagtacttag

5' unique flank region

core repeat
[AGAT]$_{13}$

3' unique flank region

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FASTQ

<u>FASTQ Sequence Format</u>

FASTA format, plus quality scores for each nucleotide

Line1: Read Identifier Line with Metadata. Begins with '@'
Line2: Raw Sequence
Line3: Read Identifier Line. Begins with '+'
Line4: ASCII encoded quality scores.
    Sanger format: Phred+33, raw reads typically (0, 40)

Instrument Name   RunID   Flowcell ID   Lane#   Tile#   X-coord   Y-coord   Pair#   Fail Filter

```
@@M01020:23:000000000-A3VY6:1:1102:19425:16540 1:N:0:1
GTTCATGCCTACATCCCTAGTACCTAGCATGGTACCTGCAGGTGGCCCATAATCATGAG      ← Sequence
+
BBBBBFFFFFFFFGGGGGGFGGHHHHCHHHHGFFGHHHGHHH23FHHHGHHFFHBFGDH      ← Quality
```
Illumina MiSeq Example

Example of a read from locus CSF1P0

```
@M01020:23:000000000-A3VY6:1:1102:19425:16540 1:N:0:1
TCCAACCTGAGTCTGCCAAGGACTAGCAGGTTGCTAACCACCCTGTGTCTCAGT
TTTCCTACCTGTAAAATGAAGATATTAACAGTAACTGCCTTCATAGATAGAAGA
TAGATAGATT**AGATAGATAGATAGATAGATAGATAGATAGATAGATAGATAGAT
AGATAGAT**AGGAAGTACTTAG                                      ← Core repeat [AGAT]$_{13}$
+
BBBBBFFFFFFFFGGGGGGFGGHHHHCHHHHGFFGHHHGHHH23FHHHGHHFFH
BFGDHFFGGGGGGFGGHHHHCHHHHGFFGHHHGHHH^7FFGGGGGGFGGHHHHC
HHHHGFFGHHHGHHH83FFGGGGGGFGGHHHHCHHHHGFFGHHHGHHH23          ← Quality scores
```

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BAM


```
00000AC0   00 00 00 00 00 00 00 BC  94 4D 6E 83 30 10 85 F7
00000AD0   95 7A 07 E4 7D 31 90 36  FD 51 48 36 55 A5 2C 2A
00000AE0   55 4D 7A 00 07 26 C4 2A  D8 96 67 9A 36 B7 AF 45
00000AF0   10 10 94 D0 8D D5 0D D2  3C DB CF 9F C6 F3 98 2D
00000B00   7E AA 32 D8 83 45 A9 55  CA E2 30 62 01 A8 4C E7
00000B10   52 15 29 FB 58 BF DC 3C  B0 00 49 A8 5C 94 5A 41
00000B20   CA 0E 80 6C 31 BF BE 9A  BD 43 29 C8 1D C2 9D 34
00000B30   18 38 17 85 29 DB 11 99  27 CE 31 DB 41 25 30 D4
00000B40   06 94 5B D9 6A 5B 09 72  A5 2D B8 11 D9 A7 28 80
00000B50   27 51 34 E5 B6 EF C1 E6  27 9E C1 32 4F 99 5D E6
00000B60   13 16 AC 0F C6 DD FC B7  B7 DE 6E 65 06 CF 3A FB
00000B70   AA 40 D1 99 2B 38 96 32  07 67 28 6C 01 94 B2 BA
00000B80   C4 A3 9A 84 8E 94 F1 F3  10 B7 FF 04 31 19 83 B8
00000B90   F3 09 61 AC 54 04 76 05  44 EE A9 B1 EB C9 60 81
00000BA0   0F EA 38 DC 48 75 A9 4D  53 BF 84 80 6F 56 9B 13
00000BB0   B6 46 1A 6B D3 BD 4F 88  BD 84 EF 01 44 2B 8D 41
00000BC0   B8 D4 F8 9B 5A 72 69 EA  4D 6D 5D F2 FA 1B 8F 31
00000BD0   3C 7A 65 10 9B 12 56 74  28 5D FE DB FC 50 27 8E
00000BE0   81 C4 3E 41 EA CC BE 0A  74 C3 DB 81 F4 C4 26 CE
```

Binary file format
  • Derivative of the SAM file format (Sequence Aligned/Mapped)

Is not (by itself) human readable

Smaller size and easily compressed for storage

Similar in concept to .hid or .abi file

Must be interpreted by a software program

Data in the BAM file
- DNA sequence and length
- Sequence identifiers
- Quality per nucloetide
- If alignment was conducted:
  • location (index) of match region to reference genome
  • alignment match (can be a sequence match or mismatch)
  • insertion to the reference
  • deletion from the reference
  • skipped region from the reference
  • soft clipping (clipped sequences present in SEQ)
  • hard clipping (clipped sequences NOT present in SEQ)
  • padding (silent deletion from padded reference)
- If alignment was not done it is a UBAM (unaligned) binary
file lacking the alignment data.

More documentation can be found here
(https://samtools.github.io/hts-specs/SAMv1.pdf)

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The Genomic Identification Lab @ NC State

Variant Call Format (.vcf)

Common in many genomic fields

Can be read by humans (MSExcel type format)

The top portion (header) displays metadata about how the sample was processed and what is contained in the columns

The columns typically show:
A) The chromosome
B) The vairant position on the chromosome
C) Identifier, such as an rs# the references dbSNP at NCBI
D) The nucleotide in the standard reference
E) The variant discovered in that position (ie., SNP, indel)
F-J) Information on the quality, format and other bioinformatics data

Mito Example
Whole mtGenome 2800M

The SWGDAM Mitochondrial interpretation guidelines (July 2013) state that that HV1 (positions 16024-16365) and HV2 (positions 73-340) are a minimum for mito interpretation.

Can you list the variants in HV1 and HV2 for the 2800M sample for the SWGDAM minimal regions in HV1 and HV2?
   *Remember there is a homopolymeric stretch of C's (311-315) in the rCRS reference.

```
##fileformat=VCFv4.1
##basecallerVersion="4.4-11/b4969eb"
##contig=<ID=chrM,length=16649>
##fileDate=20160415
##fileUTCtime=2016-04-15T07:19:44
##parametersDetails="custom, TS version: 4.4"
##parametersName="Custom based on Generic - PGM - Germ Line - Low Stringency"
##phasing=none
##reference=/results/referenceLibrary/tmap-f3/rCRS_plus_80/rCRS_plus_80.fasta
##reference=file:///results/referenceLibrary/tmap-f3/rCRS_plus_80/rCRS_plus_80.fasta
##source="tvc 4.4-11 (b4969eb) - Torrent Variant Caller"
##tmapVersion="4.4.11 (b4969eb) (201504131844)"
##INFO=<ID=OID,Number=.,Type=String,Description="List of original Hotspot IDs">
##INFO=<ID=OPOS,Number=.,Type=Integer,Description="List of original allele positions">
##INFO=<ID=OREF,Number=.,Type=String,Description="List of original reference bases">
##INFO=<ID=OALT,Number=.,Type=String,Description="List of original variant bases">
##INFO=<ID=OMAPALT,Number=.,Type=String,Description="Maps OID,OPOS,OREF,OALT entries to specific ALT alleles">
##deamination_metric=0.272418990203
```

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	2800M
chrM	152	.	T	C	15278.1	PASS	AF=1;AO=1594;DP=1602;	GT:GQ:DP:FDP:RO	1/1:99:1602:1601:3:0:1594:1601:1:735:859:1:2:737:864:0:0
chrM	263	.	A	G	11396.4	PASS	AF=1;AO=1189;DP=1195;	GT:GQ:DP:FDP:RO	1/1:99:1195:1189:2:0:1189:1189:1:539:650:2:0:539:650:0:0
chrM	315	.	C	CC	7996.22	PASS	AF=0,1;AO=157,753;DP=	GT:GQ:DP:FDP:RO	2/2:99:1014:1055:27:0:157,753:0,1055:0,1:156,748:1,5:3:24:0,1046:0,9:0:0
chrM	477	.	T	C	14293	PASS	AF=1;AO=1492;DP=1495;	GT:GQ:DP:FDP:RO	1/1:99:1495:1494:2:0:1492:1494:1:974:518:0:2:976:518:0:0
chrM	750	.	A	G	15661.9	PASS	AF=1;AO=1669;DP=1686;	GT:GQ:DP:FDP:RO	1/1:99:1686:1667:17:0:1669:1667:1:910:759:9:8:909:758:0:0
chrM	1438	.	A	G	16715	PASS	AF=0.979426;AO=1807;D	GT:GQ:DP:FDP:RO	1/1:99:1847:1847:31:38:1807:1809:0.979426:817:990:11:20:819:990:11:27
chrM	3010	.	G	A	14646.2	PASS	AF=1;AO=1529;DP=1535;	GT:GQ:DP:FDP:RO	1/1:99:1535:1534:4:0:1529:1534:1:733:796:0:4:735:799:0:0
chrM	4769	.	A	G	14096.4	PASS	AF=1;AO=1472;DP=1475;	GT:GQ:DP:FDP:RO	1/1:99:1475:1473:1:0:1472:1473:1:788:684:1:0:789:684:0:0
chrM	8860	.	A	G	15671.9	PASS	AF=1;AO=1641;DP=1654;	GT:GQ:DP:FDP:RO	1/1:99:1654:1643:7:0:1641:1643:1:948:693:1:6:950:693:0:0
chrM	14761	.	CA	C	3991.09	PASS	AF=1;AO=237;DP=489;FA	GT:AF:AO:DP:FAO	1/1:1:237:489:487:487:0:13:474:0:0:99:209:4:233:9:200
chrM	15326	.	A	G	8025.87	PASS	AF=1;AO=837;DP=839;FA	GT:GQ:DP:FDP:RO	1/1:99:839:839:1:0:837:839:1:819:18:0:1:821:18:0:0
chrM	16519	.	T	C	13877.3	PASS	AF=1;AO=1450;DP=1457;	GT:GQ:DP:FDP:RO	1/1:99:1457:1451:4:0:1450:1451:1:678:772:4:0:678:773:0:0

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STR Sequence Data

There is no unified file format for sequence based STR data

Below is one representation that contains data as given per ISFG recommendations (Parson et al., FSI:Gen 2016)
- Locus name
- Sequence of the core repeat (reported as top or forward strand of the human GRCh38 reference)
- Number of reads
- The chromosome
- The coordinates (start, stop) on the chromosome for the STR

Note: some information is not present in this example

What have you learned today that you would possibly include in this data?

Locus	allele	Sequence	seq_Len	Reads	allele_ratio	total_ratio	Allele	chromo	pos_start	pos_end
AMEL	AUTOSOMAL	tgttgattctttatcccagatgtttctcaagtggtcctgattttacagttcctacctc	58	12	0.00	0.00000	X	X	11296898	11296955
AMEL	AUTOSOMAL	tgttgattctttatcccagatgtttctcaagtggtcctgattttacagttcctcccac	58	13	0.00	0.00000	X	X	11296898	11296955
AMEL	AUTOSOMAL	tgttgattctttatcccagatgtttctcaagtggtcctgattttacagttcctaccac	58	9152	0.41	0.02150	X	X	11296898	11296955
AMEL	AUTOSOMAL	tgttgattctttatcccagatgtttctcaagtggtcctgattttacagttcctaccgc	58	14	0.00	0.00000	X	X	11296898	11296955
AMEL	AUTOSOMAL	ggtggattcttcatcccaaataaagtggtttctcaagtggtcccaattttacagttcctaccat	64	1713	0.08	0.00400	Y	Y	6869871	6869934
CSF1PO	AUTOSOMAL	atctatctatctatctatctatctatctatct	32	15	0.00	0.00000	8	5	150076324	150076375
CSF1PO	AUTOSOMAL	atctatctatctatctatctatctatctatctatct	36	455	0.03	0.00110	9	5	150076324	150076375
CSF1PO	AUTOSOMAL	atctatctatctatctatctatctatctatctatctatct	40	6911	0.41	0.01630	10	5	150076324	150076375
CSF1PO	AUTOSOMAL	atctatctatctatctatctatctatctatctatctatctatct	44	246	0.01	0.00060	11	5	150076324	150076375
CSF1PO	AUTOSOMAL	atctatctatctatctatctatctatctatctatctatctatctatct	48	728	0.04	0.00170	12	5	150076324	150076375
D10S1248	AUTOSOMAL	ggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaa	44	82	0.01	0.00020	11	10	129294244	129294295
D10S1248	AUTOSOMAL	ggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaa	48	806	0.06	0.00190	12	10	129294244	129294295
D10S1248	AUTOSOMAL	ggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaa	52	69	0.01	0.00020	13	10	129294244	129294295
D10S1248	AUTOSOMAL	ggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaa	56	534	0.04	0.00130	14	10	129294244	129294295
D10S1248	AUTOSOMAL	ggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaa	60	2836	0.21	0.00670	15	10	129294244	129294295
D10S1248	AUTOSOMAL	ggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaaggaa	64	2267	0.16	0.00530	16	10	129294244	129294295
D12S391	AUTOSOMAL	agatgatagatagatagatagatagatagatagacagacagacagacagacagacagat	67	20	0.00	0.00000	16.3	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagacagacagacagacagacagat	68	26	0.00	0.00010	17	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	68	64	0.00	0.00020	17	12	12297020	12297095
D12S391	AUTOSOMAL	agatgatagatagatagatagatagatagatagatagacagacagacagacagacagat	71	97	0.00	0.00020	17.3	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	71	186	0.01	0.00040	17.3	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagacagacagacagacagacagat	72	20	0.00	0.00000	18	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	72	515	0.02	0.00120	18	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagat	72	183	0.01	0.00040	18	12	12297020	12297095
D12S391	AUTOSOMAL	agatgatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	75	17	0.00	0.00000	18.3	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	75	3396	0.16	0.00800	18.3	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagat	75	36	0.00	0.00010	18.3	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagat	76	15	0.00	0.00000	19	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagat	76	1200	0.06	0.00280	19	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	76	412	0.02	0.00100	19	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	80	29	0.00	0.00010	20	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagacagat	80	19	0.00	0.00000	20	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagacagacagat	84	409	0.02	0.00100	21	12	12297020	12297095
D12S391	AUTOSOMAL	agatagatagatagatagatagatagatagatagatagatagatagacagacagacagacagacagacagacagat	84	135	0.01	0.00030	21	12	12297020	12297095

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Quality in Sequence Data

Low quality sequence data can affect the bioinformatic processing downstream, resulting in drop-out/-in, low confidence in variants and other issues that would impact interpretation.

Generally, each nucleotide in a sequence file is assigned a confidence score that we can use to quality check the data before analysis.

PHRED quality scoring

Phred quality scores (Q) are defined as a property which is logarithmically related to the base-calling error probabilities (P).

$$Q = -10 \log_{10} P$$

For example:
Q40 = probability of error 1 in 10,000 or 99.99% accuracy
Q30 = probability of error 1 in 1,000 or 99.9% accuracy
Q20 = probability of error 1 in 100 or 99% accuracy
Q10 = probability of error 1 in 10 or 90% accuracy

This PHRED lookup table shows the conversion of FASTQ quality (ASCII) to Q-score (Q) and the probability of error (P).

Q	ASCII	P	Q	ASCII	P
1	"	0.79433	21	6	0.00794
2	#	0.63096	22	7	0.00631
3	$	0.50119	23	8	0.00501
4	%	0.39811	24	9	0.00398
5	&	0.31623	25	:	0.00316
6	'	0.25119	26	;	0.00251
7	(	0.19953	27	<	0.00200
8	)	0.15849	28	=	0.00158
9	*	0.12589	29	>	0.00126
10	+	0.10000	30	?	0.00100
11	,	0.07943	31	@	0.00079
12	-	0.06310	32	A	0.00063
13	.	0.05012	33	B	0.00050
14	/	0.03981	34	C	0.00040
15	0	0.03162	35	D	0.00032
16	1	0.02512	36	E	0.00025
17	2	0.01995	37	F	0.00020
18	3	0.01585	38	G	0.00016
19	4	0.01259	39	H	0.00013
20	5	0.01000	40	I	0.00010
PHRED_ASCII_BASE=33			41	J	0.00008

Below are quality boxplots for two samples from 1 - 300 bps of sequencing. The line shows the average Q-score, the yellow boxes show to first and third quartile distributions, the black vertical bars represent the min and max values.

Quality scores across all bases (Sanger / Illumina 1.9 encoding)

Sample 1

Position in read (bp)

Quality scores across all bases (Sanger / Illumina 1.9 encoding)

Sample 2

Position in read (bp)

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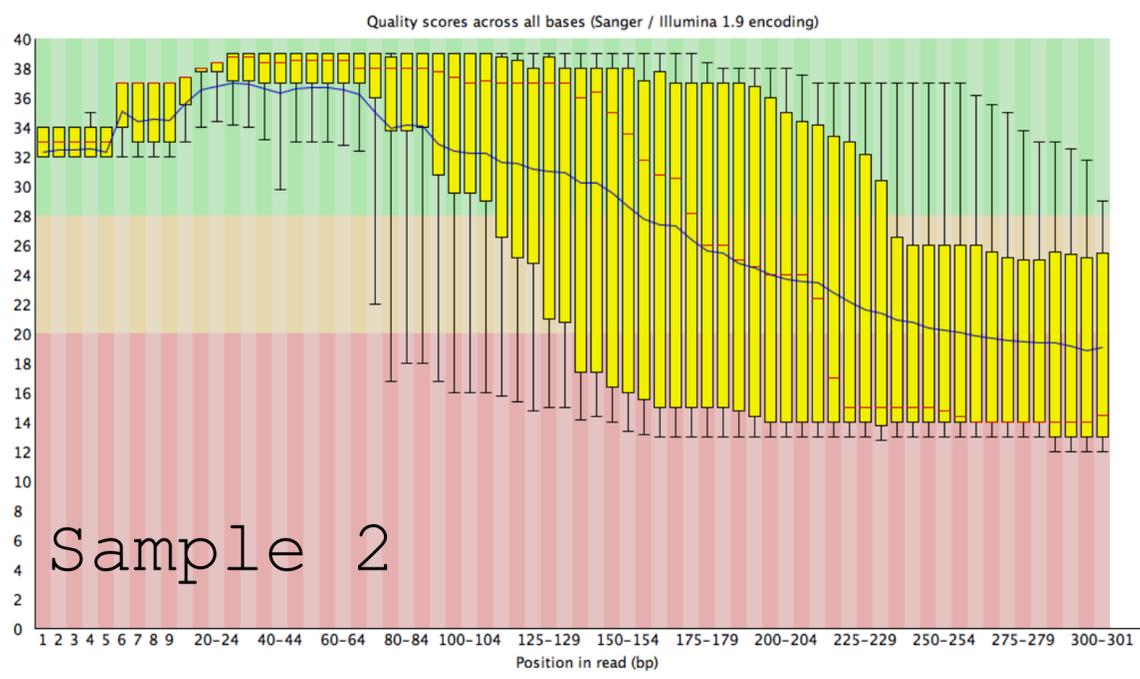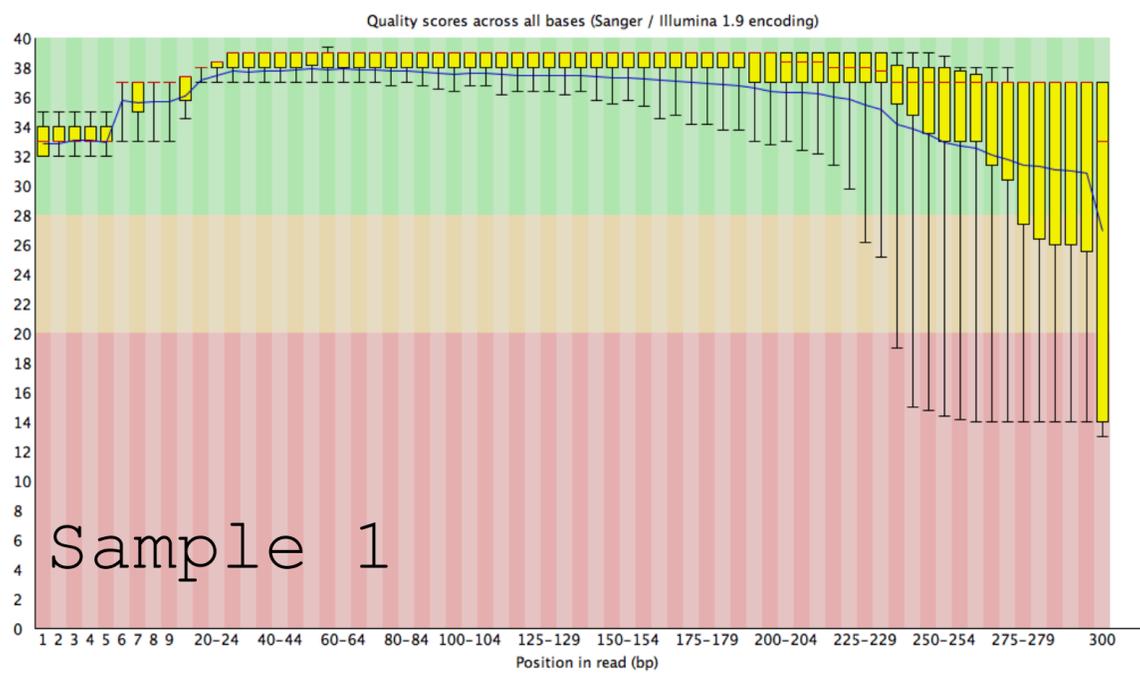
STR Analysis by Sequence

We have built a demo tool for you to explore STR sequence data.

The range slider can be moved to filter the low and high end of your reads.

The table can be scrolled to see all data and clicking the column headers will sort by column.

The two panels of bar charts can be used to visualize any locus in the data set or mutiple loci in combination. Select each locus from the drop down menu and deselect by clicking the 'x' next to the locus.

Follow the in-class exercise to begin interpretting this sample.

Phenotype Prediction

Single nuclotide polymorphisms (SNPs) have the capability to predict certain physical features (e.g., hair and eye color), ancestry, and may be also used to determine kinship and identity.

In this exercise we'll predict the phentoype of two first-degree relatives, Sample 1 and Sample 2, for sequencing data that has been processed by a genotype panel called the HIrisPlex.

Follow the link below to use the HIrisPlex tool developed by Eramus MC to predict eye color and hair color for these samples. Instructions for use are provided on the HIrisPlex webpage.

Link to HIrisPlex

1. Can you differentiate the individuals based on their Phenotype prediction?

Sample 1

HIrisPlex System Example Data		
Gene	SNP	Allele
MC1R	rs86insA	n/a
MC1R	rs11547464	GG
MC1R	rs885479	CC
MC1R	rs1805008	CC
MC1R	rs1805005	GG
MC1R	rs1805006	CC
MC1R	rs1805007	GT
MC1R	rs1805009	AA
MC1R	Y152OCH	n/a
MC1R	rs2228479	GG
MC1R	rs1110400	TT
SLC45A2	rs28777	AC
SLC45A2	rs16891982	CG
KITLG	rs12821256	AA
EXOC2	rs4959270	AC
IRF4	rs12203592	TT
TYR	rs1042602	GG
OCA2	rs1800407	AA
SLC24A4	rs2402130	AG
HERC2	rs12913832	TC
ASIP	rs2378249	TC
SLC24A4	rs12896399	GG
TYR	rs1393350	TT
TYRP1	rs683	TT

Sample 2

HIrisPlex System Example Data		
Gene	SNP	Allele
MC1R	rs86insA	n/a
MC1R	rs11547464	GG
MC1R	rs885479	TC
MC1R	rs1805008	CC
MC1R	rs1805005	GG
MC1R	rs1805006	CC
MC1R	rs1805007	GT
MC1R	rs1805009	AA
MC1R	Y152OCH	n/a
MC1R	rs2228479	GG
MC1R	rs1110400	TT
SLC45A2	rs28777	CC
SLC45A2	rs16891982	CC
KITLG	rs12821256	AA
EXOC2	rs4959270	AC
IRF4	rs12203592	CT
TYR	rs1042602	GG
OCA2	rs1800407	AG
SLC24A4	rs2402130	AG
HERC2	rs12913832	TT
ASIP	rs2378249	TT
SLC24A4	rs12896399	GG
TYR	rs1393350	TT
TYRP1	rs683	TT

Glossary

ASCII - A standard way of representing numbers and letters in a computer file so that they can be read by most computers.

Bioinformatics -A method to process and analyze biological data produced by a sequencing system using combined tools such as computer science, statistics, and mathematics. Alt: the collection, classification, storage, and analysis of biochemical and biological information using computers especially as applied to molecular genetics and genomics.

Coverage - A measurement of a genetic dataset often expressed as the number of reads or instances a single nucleotide, genetic locus, region of a genome, or whole genome is observed.

Demultiplex - A process of separating multiplexed genetic data using molecular barcodes/indexes.

Index - A molecular barcode, typically consisting of DNA sequence(s) covalently bound to genetic material from a sequencing library that provides sample identity within a multiplex system and allows for multiple samples to be analyzed in tandem.

Library Preparation - A sequencing system specific process of modifying genetic material, typically by addition of oligonucleotide sequences, such as adaptors and indexes, for the purposes of sequencing.

Read - A string of nucleotide data produced by a sequencing system, also called "sequence read."
Sequencing library - A work product consisting of genetic material prepared for analysis by a sequencing system.

Sequencing - A process of analyzing the nucleotide composition of a genetic sample or sequencing library using a sequencing system.

Single nucleotide polymorphism (SNP) - a DNA sequence variation occurring commonly within a population (e.g. 1%) in which a single nucleotide in the genome differs between members of a biological species or paired chromosomes.

String - A continuous nucleotide sequence derived from a genetic marker, locus, or gene contained within raw and analyzed data.

Quiz

Data Formats

Q1: Which of the following are in FASTQ format?

a.
>read1_20150909_SAF
TTAGGATTCTTAGGGCTTTGGGGGGGGATTTATAGATCTTAAAAGGGGAAACCCCCAG

b.

@M02089:95:000000000-AC6RN:1:1101:16305:1431 1:N:0:1
AAGTGCCAGATGCTCGTTGTGCACAAATCTAAATGCAGAAAAGCACTGAAAGAAGAATCCAGAAAACCACAGTTCCCATTTTTATATG
GGAGCAAACAAAGGCAGATCCCAAGCTCTTCCTCTTCCCTAGATCAATACAGACAGACAGACAGGTGGATAGATAGATAGATAGATAG
ATAGATAGATAGATATCATTGAAAGACAAACCAGAGATGGAT
+
AAAAAF1FFFFFGGGCAFG1EGFGHHHHGHFGHHHFHHFHHHBGHHGFHGHFHHHHHHFHHHHHHFGHGHHEGDFEBFGDGBGGHFH
HAEFGHHHHAGHHGHHGFHFHHHHHEHHGGHHGHDHHHHHHHHHHHHHHFHHHHFHHGHHHFGGGGHHGHFDFHHHHHGHHHHGHF
FHHHHHBHFGHFHHFBDHFGGHHHHHHHHG.<GHGHHHDGGH

c.

>CSF1POflankSNP_20150909_SAF
ATTTTTTTCCCTTTTAAACCCTTTTTTACCCATTACCCAAAATTTTTTTACATACCCC

d.

@M02089:95:000000000-AC6RN:1:1101:15017:1367 1:N:0:1
ATTAACTCAAGTCCAAAAAATGAGGTATGTCTCATAGAAAAGACATACATATCTATCTATCTATCTATCTATCTATCTATCTATCTAT
CTATCTATCTATCTGTATTGACACAAGTAGAAGAC
+
CCCDDFFFFFFFGGGGGGGGGGGHHHGGHHHHHHHHHGHHHHHGHHGHGHHHHHHHHHHHHHHHHHHHHHHHHHGHHHHHHEHHH
HHGHGHHHGHHHHGHHHHHHHHHGHHHHHHGGH

Q2: The SWGDAM Mitochondrial interpretation guidelines ([July 2013](#)) state that
that HV1 (positions 16024-16365) and HV2 (positions 73-340) are a minimum for mito
interpretation.

Can you list the variants in HV1 and HV2 for the 2800M sample for the SWGDAM minimal
regions in HV1 and HV2?
    *Remember there is a homopolymeric stretch of C's (311-315) in the rCRS reference.

Quality

Q4: What is the probability of error for the forth nucleotide in this FASTQ sequence?

@M02089:95:000000000-AC6RN:1:1101:15017:1367 1:N:0:1
ATTAACTCAAGTCCAAAAAATGAGGTATGTCTCATAGAAAAGACATACATATCTAAA
+
CCCDDFFFFFFFGGGGGGGGGGGHHHGGHHHHHHHHHGHHHHHGHHGHGHHHHHH

Q5: On the "Quality" page there are two Q-score plots.
    a. What is the average Q-score for each sample at 100 and 200bps?
    b. Would you say one of the samples is of generally LOW quality?

STR Analysis

Q6: Is this sample a mixture?
    a. If so, how many contributors?
    b. How many male contributors? Why?

Q7: Take a closer look at the D2S441 locus.
   a. How many isoalleles (same size, different sequence) are observed?
   b. Can you attribute the alleles to the major and minor contributors?

Phenotype

Q8. Can you differentiate the individuals based on their Phenotype prediction?
   a. Are there predicted hair color differences?
   b. Are there predicted eye color differences?

All presentations will be made available at www.forensiccoe.org

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