SRSLY Library Preparation for Forensic Samples

1. Purpose

This procedure outlines the steps required for library preparation of low-input forensic DNA samples for whole genome sequencing (WGS) using the Single Reaction Single Stranded Library (SRSLY) preparation at the Intermountain Forensics - Astrea Santa Cruz laboratory. This method was originally described in Troll et al 2020 with modifications by Dr. Joshua Kapp.

2. Summary

The Single Reaction Single Stranded Library (SRSLY) library preparation protocol is used to generate libraries to be run on any Illumina platform, e.g. NovaSeq 6000, for whole genome sequencing. This describes the reagent preparation, preparation of samples, and the Quality Control Evaluation of the libraries and pools generated.

3. Procedure

Personal Protective Equipment (PPE)

- Tyvek Coveralls
- Hair net/surgical cap
- Facemask
- Close toed shoes
- Double gloves
- Eye protection

Equipment List

- ThermoMixer
- Microcentrifuge
- Minicentrifuge
- Vortex
- Thermocycler
- 0.2 mL PCR strip magnet separator
- Qubit Fluorometer
- Tapestation
- Covaris M220

Reagent/Consumables List

- Sodium Hypochlorite, at least 5%
- NEBNext® Multiplex Oligos for Illumina E6446
- UltraPure Water
- Pre-validated ClaretBio SRSLY® NGS Library Preparation Reagents: SSE-002, SRX-002, ADA-002, ADB-002
- 1M Tris-HCL, pH 8.0

- 0.5M EDTA, pH 8.0
- 5M NaCl
- Tween 20 Solution
- 90-100% Ethanol
- 100% Isopropanol
- KAPA HiFi U+ Hotstart ReadyMix
- BD-001 Pre-Validated ClaretBio Clarefy DNA Purification Beads
- Qubit™ 1x dsDNA HS Assay kit
- Qubit™ Assay Tubes
- Tapestation D1000 (or D1000 High Sensitivity) Ladder
- Tapestation D1000 (or D1000 High Sensitivity) Sample Buffer
- Tapestation D1000 (or D1000 High Sensitivity) ScreenTape
- Covaris microTUBE-15 AFA Beads Screw-Cap

Buffers and Solutions

Low TE solution (50 mL stock)		
Reagent	**Volume Per Sample**	**Final Concentration**
1M Tris-HCl, pH 8.0	500 µL	10 mM
0.5M EDTA, pH 8.0	10 µL	0.1 mM
UltraPure Water	49.49 mL	

Aliquot into smaller working dilutions for benchtop use.

EBT Buffer (50 mL)		
Reagent	**Volume**	**Final Concentration**
1M Tris-HCl, pH 8.0	500 µL	10mM
UltraPure Water	49.25 mL (to 50mL line)	
Tween 20 Solution	25µL	0.05%

To make EB buffer, follow the instructions for EBT but omit Tween and replace volume with 25µl water.

Pre-Prep

- **Use DOC-337 template to record the batch of DNA extracts that will be undergo DNA library preparation.**
  - Include a minimum of one non-template control per library batch
  - A positive control is optional; notate in the worksheet when a positive control is used, including DNA type and concentration.
- **Determine how much DNA will be used per extract to build libraries**: This library prep protocol can use *up to* 5 nanograms of DNA mass within 9 µL of volume.
  - Calculate the concentration of DNA of the extract using a Qubit 3.0 and the Qubit™ 1x dsDNA HS Assay.
  - The void volume can be filled with water, EB, or EBT buffer.

- **Select indices**: Select a unique combination of i5 and i7 indices per sample from NEB E6446 plate.
- Using at least 0.5% sodium hypochlorite dilution, bleach all work surfaces, pipettes, and pipette tip boxes.
- Use validated SRSLY reagents SSE-002, SRX-002, ADA-002, ADB-002 from the ClaretBio Production Lab.

Reconstituting Extracts

When extracts are received in a dried down state, reconstitute the extract by adding 30 µL of EBT to the extract tube and lightly vortex.

Note: Refer to worksheet for detailed steps taken for process.

Record Valid Library Preparation Reagents*

*All SRSLY reagents used are lot tested and have passed QC according to metrics set forth by Claret Bioscience's QMS system and Certificate of Analysis (Kit manufacturer).

1. In the library prep worksheet, document each reagent's lot number and confirm expiration dates of each reagent and buffer used in the protocol

Covaris Shearing Protocol

Illumina sequencers require fragmented DNA below approximately 1kb in length. When DNA extracts are expected to contain high molecular weight DNA above 1kb, use the Covaris M220 ultrasonicator to shear the sample.

1. Label all shearing tubes with the extract sample identification numbers.
2. Preparing DNA for Covaris shearing:
    a. Follow Library Prep worksheet for calculations; dilutions may be necessary
    b. The microTUBE-15 must fill to 15 µl; Add no more than 1 µg of DNA.
    c. If DNA extract volume is below 15 µl, fill the MicroTube-15 tube to 15 µl. The new concentration is auto-calculated in Library Prep worksheet.
3. Once sample is added to MicroTube-15, spin down briefly.
4. Load the microTUBE into the Covaris instrument as described in the manufacturer's instructions.
5. Verify the following shearing conditions:
    a. Duration:140
    b. Peak power: 50
    c. Duty factor: 30
    d. Cycles/Burst: 50
    e. Average power: 15
    f. Temperature: 20°C
6. Once the shearing cycle is completed, retrieve the sheared sample from the tube with a pipette tip and transfer into a fresh tube.
    a. It may be necessary to push the beads aside for full recovery.

b. Label with the DNA extract sample identification plus the word “Sheared”

7. Store sheared DNA extracts in 4°C FL freezer until ready to prepare into DNA libraries.

Prepare DNA libraries:

Denature DNA and add SRSLY SSE-002

1. Pre-heat thermocycler blocks to 98°C and 37°C.
2. Vortex and spin all reagents.
3. Add 1µL of SSE-002 to a 0.2 mL strip tube.
4. Add up to 9 µL of template DNA to each tube. If necessary, fill up to 9 µL with water, EB or EBT buffer. Total volume of the mix should be 10 µL.
5. Mix with vortex and then centrifuge.
6. Denature at 98°C in the thermocycler for 3 minutes. Make sure the lid is at 105°C.
7. Immediately cool reactions for 2 minutes on a cold block.

Prepare the DNA library

1. Thaw library prep reagents by setting on bench at room temperature.
2. Vortex and spin all reagents.
3. Leaving the reactions on the cold block add the following:
   a. Add 1 µL ADA-002 and 1µl of ADB-002 to the denatured DNA.
   b. Add 13 µL SRSLY SRX-002 to the denatured DNA.
4. Mix vigorously by vortexing and then centrifuging 2-3 times.
5. Incubate reactions in the thermocycler block for 1 hour at 37°C. Make sure the lid is at 50°C.

Purify the DNA library

1. Add 40 µL of EBT and 10 µL of 100% Isopropanol to each reaction.
2. Add 72.6 µL Clarefy Purification Beads to each reaction.
3. Mix and incubate at RT for 10 minutes. Check them after 5 minutes and if necessary, vortex and centrifuge briefly.
4. Place tubes on magnet and remove supernatant.
5. Wash beads twice with 190 µL of 80% EtOH.
6. Remove the EtOH and let beads dry with open lids, approximately 5 minutes or until the beads appear cracked.
7. When beads are dry, remove from magnet, add 20 µL EBT, wait 5 minutes.
8. Transfer eluate directly to the indexing PCR reaction.

Prepare and run indexing PCR in a 50 µL final volume

1. Prepare a PCR reaction with the following conditions:

reagent	Lot #	volume (µL) per sample	volume for 0.4 samples
KAPA HiFi U+ Hotstart ReadyMix	XXXX	25	10
sum		*25*	*10*

i5/i7 Indexing Primers: NEB Multiplex Oligo (5uM each)	XXXX	5	2
Template DNA		20	8
Total		**50**	**20**

2. Set the reaction in the thermocycler with the following conditions:
    a. 98 °C for 3 minutes
    b. 98 °C for 20 seconds
    c. 65 °C for 30 seconds
    d. 72 °C for 30 seconds
    e. Return to step b for a total of 13 cycles.
    f. 72 °C for 1 minutes
    g. Keep at 4°C or 12°C overnight

Purify the indexed DNA library (

1. Add 60 µL Clarefy Purification Beads to each reaction.
2. Mix and incubate for 10 minutes. Check them after 5 minutes and if necessary, vortex and centrifuge briefly.
3. Place tubes on magnet and remove supernatant.
4. Wash beads twice with 190 µL of 80% EtOH.
5. Remove the EtOH and let beads dry at 28°C with open lids for 5 minutes.
6. Once beads are dry, add 20 µL of Low TE, wait 5 minutes and then transfer eluate to a clean 0.2 mL tube. Make sure the tubes are clearly labeled for final storage at -20°C. Use LIMFXXXXX for IMF-Astrea Santa Cruz libraries.

QC the indexed DNA library

For post-indexing library QC, you can use either the Qubit 1x dsDNA HS Assay or the Quant-iT 1x dsDNA HS Assay. Both Assays can be performed on a Qubit 3.0 in the post-PCR space. **The reagents for all three of these assays should be at room temperature before taking the reading.**

Qubit 1x dsDNA HS Assay

1. Set up required number of 0.5 mL **Thin Wall PCR tubes** (such as Axygen). The number of tubes required should correspond to the number DNA libraries needing quantification (1 tube/library) as well as two standards (low and high).
2. Aliquot 190 µL of the 1x dsDNA Buffer into (2) tubes (for the standards). Aliquot 199 µL of the buffer into the appropriate number of tubes for DNA libraries.
    a. NOTE: the final volume with the buffer and DNA library should be 200 µL.
3. Place tubes in a drawer and allow reagents to come to room temperature.
4. Add 10 µL of standards 1 and 2 into the tubes with 190 µL of the buffer.
5. Add 1µL of DNA library into the appropriate tube (with 199 µL of buffer).
6. Vortex the samples for 15 seconds.

7. Let the tubes sit at room temperature for 2 minutes in a drawer or covered with a box to shield from the light. This step can be done in a thermocycler at 25C if the lab is colder than room temp (below 23°C).
8. Make sure the Qubit fluorometer is set to 1x dsDNA Assay. Read and note the Standards values to make sure the fluorometer is calibrated.
9. Once the standards are read, set µL to "1" and read the remaining tubes.
10. Record the values in the corresponding MolecularQC excel sheet
11. Have a second individual verify the values were transcribed accurately.
    a. If another person in unavailable to perform the verification, save the data from the Qubit run and place in the Batch folder for verification during the technical review.

Quant-iT 1x dsDNA Assay

1. Set up required number of 0.5 mL **Thin Wall PCR tubes** (such as Axygen). The number of tubes required should correspond to the number DNA libraries needing quantification (1 tube/library) as well as two standards (low and high).
2. Aliquot 190 µL of Working Solution to (2) tubes (for the standards). Aliquot 199 µL of the Working Solution into the appropriate number of tubes for DNA libraries.
    a. Note: The final volume of Working Solution and DNA library should be 200 µL
3. Place tubes in a drawer and allow Working Solution to come to room temp.
4. Add 10 µL of the 0 ng/µL and 10 ng/µL Quant-iT 1x dsDNA HS Standards to the tubes containing 190 µL of the Working Solution.
5. Add 1 µL of DNA library to the appropriate tube (with 199 µL of Working Solution).
6. Vortex the samples for 15 seconds.
7. Let the tubes sit at room temperature for 2 minutes in a drawer or covered with a box to shield from the light. This step can be done in a thermocycler at 25°C if the lab is colder than room temp (below 23°C).
8. Make sure the Qubit fluorometer is set to 1x dsDNA Assay. Read the Standards to make sure the fluorometer is calibrated.
9. Once the standards are read, set µL to "1" and read the remaining tubes.
10. Record the values in the corresponding MolecularQC excel sheet.

D1000 Tapestation

1. Allow the reagents to come to room temperature before use. Vortex and spin down the reagents before aliquoting them into tubes. Place ScreenTape in the Tapestation so that it equilibrates to the machine temperature.
2. Set up required number of 0.5 mL **Thin Wall PCR tubes**. The number of tubes required should correspond to the number DNA libraries needing quantification (1 tube/library) as well a ladder.
    a. Note: This can be performed in strip tubes or on a plate. Astrea usually uses the strip tubes.
3. Aliquot 3 µL of D1000 Sample Buffer into the appropriate number of tubes.
4. Aliquot 1 µL of D1000 Ladder into one of the tubes.
5. Aliquot 1 µL DNA library into the remaining tubes.
6. Vortex for 3-5 seconds and spin down. Make sure there are NO BUBBLES.

7. Set up Tapestation Controller software and run the samples.
8. Once analysis is completed, add regions to the Tapestation traces to get the average length and amount of dimers. Save this to the Tapestation folder on OneDrive. Additionally, "Create Report" so that a .pdf is saved. Finally, "Export Data" -> Images to the appropriate folder on OneDrive. These images will be uploaded to the ShareFile Folder under the corresponding library.

** If using DNA D1000 High Sensitivity reagents, follow manufacturer's instructions for loading. D1000 High Sensitivity reagents are used when DNA concentrations are so low they fail to visualize using D1000 reagents.*

Preparing the pool of libraries

1. Refer to the PoolingSheet tab of the DOC-337 IMF_LibPrep_Batch spreadsheet
2. Combine the volumes in Column R, which based on the quantity and molecular weight values
    a. The bump factor may be utilized which is a correction value to allow for volumes that are within the range of the pipettes.
    b. A dilution of the sample might be necessary so that the library is not consumed when preparing the pool.
3. Perform a quantitation (Qubit) and QC TapeStation) of the pool using the methods described above to verify that proper preparation of the pool.
4. Dilute the pool according to the values for a 310 uL pool at 1.5 nM in Column T of the PoolingSheet tab of the spreadsheet

4. References

Covaris M Series User Manual. Publication P/N 010157

Agilent Technologies 2200 TapeStation User Manual

5. Definitions

None