SOP for Library Prep using KAPA HyperPlus Kit for WGS on Illumina platforms

(adapted from here[1] by Silvia A. Justi, PhD. Genomics Research Associate)

Before Starting:

a) Use the Illumina LibPrep spreadsheet to keep track of the samples. Make sure to change the plate name according to the Illumina plate logbook in the laboratory. Save the latest copy of the spreadsheet in the Library prep folder, on Dropbox. To request access email justis@si.edu.

b) Use the Lib_Prep_Calculator to prepare all your mixes prior to the start of the experiment. With exception of the 80% ethanol and the fragmentation mix (that need to be prepared on the same day of use), all other mixes can be prepared up to 4 weeks prior to use, if kept at -15°C to -25°C.

c) Have all your DNA quantified using Qubit. Alternatively (not advised), use a fixed volume of the stock DNA and quantify the DNA while running the fragmentation. If the DNA is eluted in buffer containing EDTA, perform a 3X bead cleanup and then quantify the DNA, prior to fragmentation. Alternatively, If the exact concentration of EDTA is known, follow the instructions (page 4) for the use of conditioning solution.

d) Pre-dilute adapters, to a final volume of 6ul each, according to DNA input. If using DNA from a collection specimen, use the fragment size as 75bp, if following the fragmentation outlined below, use 150bp. Always use the adapter in the corresponding well. E.g. if your sample is in well A01, use adapter from well A01. THIS IS THE PRIMARY INFORMATION FOR POOLING THE SAMPLES!

[1] Most of the wording and tables on this protocol were copied from the original Kapa technical datasheet, and formatted for simplicity. My additions and comments to the original protocol will be italicized for reference.

Checklist for library prep:

- *Timer*
- *Ice and/or cold rack*
- *KAPA Pure Beads fully resuspended and at room temperature (140ul per sample – after EDTA cleanup)*
- *Agencourt AMPure beads fully resuspended and at room temperature (37ul per sample)*
- *Magnetic plate*
- *Freshly diluted Ethanol 80% (1.2ml per sample – if the whole library prep is done the same day)*
- *Elution buffer -10 mM Tris-HCl (pH 8.0 – 8.5)*
- *Fragmentation mix (FRESH!)*
- *ER &A-tailing mix*
- *Ligation mix*
- *Library Amplification Mix*
- *Thermocycler*
- *Vortex*
- *Centrifuge (to spin down solutions)*
- *Pre-diluted adapters, according to DNA input (see Illumina LibPrep spreadsheet)*
- *3 extra 0.2ul tubes (per sample) or plates per plate*
- *Tips:*
  - *At least 15 2-20ul tips per sample*
  - *At least 40 20-200ul tips per sample*
  - *Other size tips will be less frequently used, but good to have in hand*
- *Pencil*
- *Lab notes/notebook*
- *Sharpie*
- *Plate cover*
- *Tube labeled (if not using a plate)/plate map*
- *Enough Tapestation tapes and reagents to check each sample twice*

1. Fragmentation (If DNA was extracted from collection specimens, go straight to step 2)

1.1. Assemble each fragmentation reaction on ice by adding:

Component	Volume
Double-stranded DNA (with Conditioning Solution, if needed)	35 µL
KAPA Frag Buffer (10X)*	5 µL
KAPA Frag Enzyme*	10 µL
Total volume:	50 µL

*or 15ul of freshly mixed fragmentation mix

1.2. Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.

1.3. Incubate in a thermocycler, pre-cooled to 4°C and programmed as outlined below (*or just remove from ice straight to a thermocycler previously set to 37°C*). A heated lid is not required for this step. If used, set the temperature of the heated lid to ≤50°C.

Step	Temp	Time
Pre-cool block	4°C	N/A
Fragmentation	37°C	40 min
HOLD	4°C	∞

1.4. Transfer reactions to ice, and proceed immediately to End Repair and A-tailing (step 2).

2. End Repair and A-tailing

2.1. In the same plate/tube assemble each end repair and A-tailing reaction as follows:

Component	Volume
Fragmented, double-stranded DNA	50 µL
End Repair & A-Tailing Buffer*	7 µL
HyperPrep/HyperPlus ERAT Enzyme Mix**	3 µL
Total volume:	60 µL

*or 10ul of pre-mixed ER & A-Tail mix

2.2. Vortex gently and spin down briefly. Return the reaction plate/tube(s) to ice. Proceed immediately to the next step.

2.3. Incubate in a thermocycler programmed as outlined below. A heated lid is required for this step. If possible, set the temperature of the heated lid to ~85°C (instead of the usual 105°C).

Step	Temp	Time
End repair and A-tailing	65°C*	30 min
HOLD	4°C**	∞

2.4. Proceed immediately to Adapter Ligation

3. Adapter Ligation

3.1. Dilute adapter stocks to the appropriate concentration using the sample spreadsheet.

3.2. In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

Component	Volume
End repair and A-tailing reaction product	60 µL
Adapter stock (diluted)	5 µL
PCR-grade water*	5 µL
Ligation Buffer*	30 µL
DNA Ligase*	10 µL
Total volume:	110 µL

*or 45ul of pre-mixed ligation mix

3.3. Mix thoroughly by vortexing and centrifuge briefly.

3.4. Incubate at 20°C for 15 min.

3.5. Proceed immediately to Post-ligation Cleanup.

4. Post-ligation Cleanup

4.1. In the same plate/tube(s), perform a 0.8X bead- based cleanup by combining the following:

Component	Volume
Adapter ligation reaction product	110 µL
KAPA cleanup beads	88 µL
Total volume:	198 µL

4.2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.

4.3. Incubate the plate/tube(s) at room temperature for 15 min to bind DNA to the beads.

4.4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

4.5. Carefully remove and discard the supernatant (*removing half the supernatant at a time, reduces the loss of beads*).

4.6. Keeping the plate/tube(s) on the magnet, *slowly* add 200 µL of 80% ethanol.

4.7. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4.8. Carefully remove and discard the ethanol (*removing half the supernatant at a time, reduces the loss of beads*).

4.9. Keeping the plate/tube(s) on the magnet, *slowly* add 200 µL of 80% ethanol.

4.10. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

4.11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads (*removing half the supernatant at a time, reduces the loss of beads. Spinning down the plate/tube helps collect the remaining ethanol. Use a 1-10ul pipette to remove that with tubes/plate on the magnet, for minimum loss*).

4.12. Dry the beads at room temperature for 3 – 5 min (*observe beads, when the bead smear on the tube wall starts to "crack" it is time to add the buffer*), or until all of the ethanol has evaporated. Caution: over-drying the beads may result in reduced yield.

4.13. Remove the plate/tube(s) from the magnet.

4.14. Resuspend the beads in 25 µL of elution buffer to proceed with Library Amplification

4.15. Vortex/mix well and spin down samples

4.16. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

4.17. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

4.18. Transfer 20 µL of the clear supernatant to a new plate/tube(s)

----------SAFE STOP POINT----------

If stopped here, label tubes/plates as Ligated DNA.

5. Library Amplification

5.1. Assemble each library amplification reaction as follows:

Component	Volume
KAPA HiFi HotStart ReadyMix (2X)	25 µL
Library Amplification Primer Mix (10X)*	5 µL
Adapter-ligated library	20 µL
Total volume:	50 µL

*or 30ul of pre-mixed amplification mix

5.2. Mix thoroughly and centrifuge briefly.

5.3. Amplify using the following cycling protocol:

Step	Temp	Duration	Cycles
Initial denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	*Defined according to the input DNA on the Illumina LibPrep spreadsheet*
Annealing*	60°C	30 sec	
Extension	72°C	30 sec	
Final extension	72°C	1 min	1
HOLD	4°C	∞	1

5.4. Proceed directly to Post-amplification Cleanup

6. Post-amplification Cleanup

6.1. In the library amplification plate/tube(s), perform a 1X bead-based cleanup by combining the following:

Component	Volume
Library amplification reaction product	50 µL
KAPA cleanup beads	50 µL
Total volume:	100 µL

6.2. Mix thoroughly by vortexing and/or pipetting up and down multiple times.

6.3. Incubate the plate/tube(s) at room temperature for 15 min to bind DNA to the beads.

6.4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

6.5. Carefully remove and discard the supernatant (*removing half the supernatant at a time, reduces the loss of beads*).

6.6. Keeping the plate/tube(s) on the magnet, *slowly* add 200 µL of 80% ethanol.

6.7. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

6.8. Carefully remove and discard the ethanol (*removing half the supernatant at a time, reduces the loss of beads*).

6.9. Keeping the plate/tube(s) on the magnet, *slowly* add 200 µL of 80% ethanol.

6.10. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

6.11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads (*removing half the supernatant at a time, reduces the loss of beads. Spinning down the plate/tube helps collect the remaining ethanol. Use a 1-10ul pipette to remove that with tubes/plate on the magnet, for minimum loss*).

6.12. Dry the beads at room temperature for 3 – 5 min (*observe beads, when the bead smear on the tube wall starts to "crack" it is time to add the buffer*), or until all of the ethanol has evaporated. Caution: over-drying the beads may result in reduced yield.

6.13. Remove the plate/tube(s) from the magnet.

6.14. Thoroughly resuspend the beads (*ideally, use a vortex*) in 50ul volume of elution buffer

6.15. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

6.16. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

6.17. Transfer the clear supernatant to a new plate/ tube(s) and proceed adapter-dimer cleanup.

----------SAFE STOP POINT----------

If stopped here, label tubes/plates as Amplified library.

7. **Tapestation check libraries** (reagents and tapes must be at room temperature)
   7.1. Add 2ul of High Sensitivity D1000 Buffer and 2ul of amplified library to each tube/plate well
   7.2. Add 2ul of ladder and High Sensitivity D1000 per tape to A1 on the strip, as indicated by the software (*the software will always tell you to use 15ul of ladder + 15ul of buffer. I found that this is wasteful, and that using 2ul of ladder + 2ul of buffer per sample works just as well*).
   7.3. Vortex for 2 minutes (*the plate vortex next to the Tapestation is automatically set to the time. Just use the max spin speed and press start. It will stop on its own*)
   7.4. Spin down and load to Tapestation
   7.5. Load Tapestation tips
   7.6. Load Tape, with QR code facing right and down. Load extra tapes on the same position.
   7.7. On the TapeStation control Software select wells with samples
   7.8. Add sample names on "Sample Descriptions" (*use intelligible names, that will allow tracking back samples to the Tapestation run*) and click start.

8. **Adapter-dimer Cleanup**

   If Tapestation results show adapter dimer peaks (~150bp or less), adapter dimer cleanup is necessary.

   Use Agencourt AMPure beads at ROOM TEMPERATURE! (Seriously! Room temperature and freshly diluted - immediately before starting cleanup - ethanol).

   8.1. In the library amplification plate/tube(s), perform a 0.8X bead-based cleanup by combining the following:

Component	Volume
Library amplification reaction product	45 µL
Agencourt AMPure beads	36 µL
Total volume:	81µL

*Use the 0.8X beads for 1X Library amplification product

8.2. Mix thoroughly by *gently* pipetting up and down at least 12 times.

8.3. Incubate the plate/tube(s) at room temperature for **5** min to bind DNA to the beads.

8.4. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

8.5. Carefully remove and discard the supernatant (*removing half the supernatant at a time, reduces the loss of beads*).

8.6. Keeping the plate/tube(s) on the magnet, *slowly* add 200 µL of freshly diluted 80% ethanol.

8.7. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

8.8. Carefully remove and discard the ethanol (*removing half the supernatant at a time, reduces the loss of beads*).

8.9. Keeping the plate/tube(s) on the magnet, add 200 µL of freshly diluted 80% ethanol.

8.10. Incubate the plate/tube(s) on the magnet at room temperature for ≥30 sec.

8.11. Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads (*removing half the supernatant at a time, reduces the loss of beads. Spinning down the plate/tube helps collect the remaining ethanol. Use a 1-10ul pipette to remove that with tubes/plate on the magnet, for minimum loss*).

8.12. Dry the beads at room temperature for 2 min, or until all of the ethanol has evaporated (*observe beads, when the bead smear on the tube wall starts to "crack" it is time to add the buffer*). Caution: over-drying the beads may result in reduced yield.

8.13. Remove the plate/tube(s) from the magnet.

8.14. Thoroughly resuspend the beads in in 50ul volume of elution buffer

8.15. Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

8.16. Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

8.17. Transfer the clear supernatant to a new plate/ tube(s).

8.18. Repeat the Tapestation check (step 7) to see if adapter dimers were discarded

8.19. Store purified, amplified libraries at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C.

8.20. Label tubes/plates as Library ready.

9. Pooling the libraries (figures will be added when possible)

9.1. Open the Tapestation results on the analysis software

9.2. For each sample, select the region containing the library fragments

*9.3. Export the results to csv and close the software. This will create 3 files: *_peakTable.csv, *_regionTable.csv and *_sampleTable.csv. We want to look at the *_regionTable.csv*

9.4. In the Illumina sample spreadsheet set the number of molecules to 40 for Anophelinae (~300 Mb genomes) and 80 for Culicinae (~500Mb genomes). If you are working with other Diptera families, of unknown genome size, use 80 or higher for large genome bugs.

9.5. *The *_regionTable.csv file generated by the TapeStation software is not straightforward to copy from one spreadsheet to another, since the sample name and results are not in the same row, So you will need to copy the corresponding columns (A – E) to the Illumina sample spreadsheet one by one (I am working on a script to automate this step).*

	A	B	C	D	E	F	G	H	I	J
1	A1									
2	From [bp]	To [bp]	Average Size	Conc. [pg/µl]	Region Mola	% of Total	Area	Region Comr	Automatic Region	
3	246	1318	542	12600	38900	92.85	50.258		FALSE	
4										
5	B1									
6	From [bp]	To [bp]	Average Size	Conc. [pg/µl]	Region Mola	% of Total	Area	Region Comr	Automatic Region	
7	170	804	382	361	1680	78.64	1.445		FALSE	
8										
9	C1									
10	From [bp]	To [bp]	Average Size	Conc. [pg/µl]	Region Mola	% of Total	Area	Region Comr	Automatic Region	
11	170	577	275	993	6030	64.38	3.972		FALSE	
12										
13	D1									

9.6. The volume for pooling the sample should automatically show on Illumina sample spreadsheet. If you don't have enough volume, re-amplify the sample before pooling. Follow steps 5 through 8 of this protocol using the Illumina primers and the Kapa Amplification Mix purchased separately from the kit.

9.7. Library needs to be a minimum of 4nM as indicated on the calculations that will show in cell F2 of the Illumina sample spreadsheet.