BGE Protocol for low volume library preparation using NEBNext Ultra II kit for Illumina sequencing

Genome skimming of Museum Collections

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Workflow

❶ Scope

This protocol is meant for genomic library preparation using degraded (ancient) DNA samples. This is a low volume and altered version of the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina, in order to prepare libraries as cheaply as possible. This protocol includes three different volume options ($1/8^{th}$, $1/12^{th}$ of $1/16^{th}$ of the original protocol).

❷ Reagents & Equipment

2.1 Reagents

- NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®
- NucleoMag NGS Cleanup and Size Select beads
- Indexing primers
- Fragment Analyzer Genomic DNA 50Kb kit (DNF-467-0500)
- TapeStation D5000 High Sensitivity kit
- Fragment Analyzer dsDNA 910 Kit 35-1500bp (DNF-910-K0500) or HS Small Fragment Kit (DNF-477-0500) kit

2.2 Equipment

- PCR machine
- Eppendorf Concentrator Centrifuge (SpeedVac)
- I.DOT Mini Dispensing Robot
- Magnetic bead separator stamp
- Fragment Analyzer System
- TapeStation
- Qiagility pipetting robot
- Centrifuge
- Vortexer

❸ DNA quantification and normalisation

Preparation

- After the DNA extraction, take forward **30 µl** of eluate into a skirted PCR working plate.

- Transfer the remaining eluate in Eppendorf twin.tec® PCR skirted plates (blue, 125 µl) and seal using Flat PCR Caps strips. Store the plates in a **-20 °C** freezer.

Procedure

- Concentrate the working plate using the Eppendorf Concentrator Centrifuge for **25 min** at **30 °C.**

- Use **2 µl** to measure DNA concentrations and fragment size using the Fragment Analyzer Genomic DNA 50Kb kit (DNF-467-0500).

- Normalise the plate based on results. Keep in mind that all samples need the same amount of cycles during the PCR amplification step.
    - Based on previous results, plates are normalised to **1 – 1,5 ng** total DNA input.

❹ Library preparation

4.1 End-repair

Thaw the **O NEBNext Ultra II End Prep Enzyme Mix** and **O Reaction Buffer**, place the Enzyme Mix on ice. Gently mix and spin down the components before use.

	Volume option 1 (1:8)	**Volume option 2 (1:12)**	**Volume option 3 (1:16)**
O NEBNext Ultra II End Prep Enzyme Mix	0,375 µl	0,25 µl	0,15 µl
O NEBNext Ultra II End Prep Reaction Buffer	0,875 µl	0,58 µl	0,35 µl
Fragmented DNA (can be adjust with 0,1 xTE)	6,26 µl	4,17 µl	2,5 µl
Total volume	7,5 µl	5 µl	3 µl

1. Prepare a mastermix of the O NEBNext Ultra II End Prep Enzyme Mix and O Reaction Buffer based on the chosen volume option.

2. Let the **I.DOT Mini** distribute the appropriate volume of the master mix:
   a. Option 1: **1,25 µl**, option 2: **0,83 µl**, and option 3: **0,5 µl**.
   b. I.DOT Mini Liquid class: **H_20**.

3. Add the appropriate amount of mastermix using a P10 Multichannel pipette and pipette the entire volume up and down 10 times. Spin down shortly.

4. Seal the plate and place in a thermocycler with the heated lid set to ≥ **75°C**, and run the following program:
   - **30 minutes @ 20°C**
   - **30 minutes @ 65°C**
   - **Hold at 4°C**

If necessary, samples can be stored at –20°C; however, a slight loss in yield (~20%) may be observed. Continuing with adaptor ligation before stopping is recommended.

4.2 Adaptor Ligation

Preparation

Before starting the Adapter Ligation, determine whether adaptor dilution is necessary using the following table:

Input	**ADAPTOR DILUTION (VOLUME OF ADAPTOR: TOTAL VOLUME)**	**WORKING ADAPTOR CONCENTRATION**
1 µg–101 ng	No Dilution	15 µM
100 ng–5 ng	10-Fold (1:10)	1.5 µM

less than 5 ng	25-Fold (1:25)	0.6 µM

- If needed, dilute the **NEBNext Adaptor for Illumina** in Tris/NaCl, pH 8.0 (10 mM Tris, 10mM NaCl) as indicated in the table.
    - Always make a **fresh dilution** of the NEBNext Adaptor to guarantee good performance.

Thaw the **O** **NEBNext Ultra II Ligation Master Mix**, **O** **Ligation Enhancer** & (diluted) **O** Adaptor for Illumina.

	Volume option 1 (1:8)	**Volume option 2 (1:12)**	**Volume option 3 (1:16)**
End Prep Reaction Mixture from step 4.1	7,5 µl	5 µl	3 µl
O NEBNext Ultra II Ligation Master Mix	3,75 µl	2,5 µl	1,5 µl
O NEBNext Ligation Enhancer	0,125 µl	0,083 µl	0,05 µl
O NEBNext Adaptor for Illumina	0,31 µl	0,208 µl	0,125 µl
Total Volume	11,41 µl	7,79 µl	4,56 µl

1. Prepare a master mix of the **O** Ligation Master Mix and **O** Ligation Enhancer.
    a. This mixture is stable for at least 8 hours at **4°C**.

2. Divide the **Master Mix** over an 8-well capped strip and add the appropriate volume to the **end prepped reaction mixture** from 4.1 using a P10 multichannel.
    a. Option 1: **3,875 µl**, option **2**: **2,583 µl**, and option 3: **1,55 µl**.

3. Let the **I.DOT Mini** distribute the appropriate volume of the NEBNext Adaptor for Illumina.
    a. Liquid class: **H_20**.
    b. Seal the plate and spin down immediately.
    c. Gently vortex the plate on a low speed and spin down again.

4. Incubate at **20°C** for **15 minutes** in a thermocycler with the **heated lid off**.

Remove the **O** USER™ Enzyme from the freezer and place on ice.

O USER™ Enzyme	0,5 µl	0,35 µl	0,2 µl

5. The **I.DOT Mini** is not able to dispense the **O** **USER™ Enzyme** correctly. To use the I.DOT, add an equal amount of UltraPure Water (1:1) and double the volume to be dispensed per sample (e.g. 0,5 µl > 1 µl).
    a. **Liquid class:** Glycerol 10%.
    b. Seal the plate and spin down immediately.

    c. Gently vortex the plate on a low speed and spin down again.

6. Mix well and incubate at **37°C** for **15 minutes** with the **heated lid set to ≥ 47°C**.

7. Bring the total reaction volume to 10 µl if the 2nd or 3rd volume option has been chosen before proceeding to the next step.
    a. Mix well by gently pipetting the entire volume up and down 10 times.

THIS IS A SAFE STOPPING POINT. Samples can be stored overnight at –20°C.

4.3 Clean-up of adaptor-ligated DNA

This bead clean-up method uses a magnetic bead separator stamp in combination with NucleoMag NGS Cleanup and Size Select beads. Make sure to use Corning® 3788 Round Bottom Assay Microplate with Lid.

Preparations

- Let the MN beads get to room temperature (remove from fridge 15 min before use).
- Prepare fresh **80% EtOH** (e.g. 41.7ml EtOH 96% + 8.3 ml MQ in a 50 ML Falcon tube).
- The amount of beads to be added is dependent on the fragment size. Make sure to check the bead ratio you have to work with.
    - For fragments between **75 - 350 bp**, a **1,2X clean-up** is recommended.

1. Mix the **MN beads** well by vortexing the tube and spin down shortly.

2. Fill a 96- well round bottom plate with the appropriate amount of magnetic beads, based on the volume of adaptor ligated DNA.

3. Add the Adaptor ligated DNA using a P10 Multichannel and mix by pipetting the volume up and down at least 10 times.
    a. Incubate for at least **5 minutes.**

4. Fill two round bottom plates with **50 µl** of **80% EtOH**.

5. Fill one round bottom plate with **12 µl of UltraPure or MiliQ Water**.

6. Load the **magnetic stamp** with a **cover plate** and make sure the plate is locked into place.

7. Place the **magnetic stamp** in the sample / beads plate and wait for at least **1 minute** until all beads are covering the magnet.

8. Bring the stamp to the first **wash plate** and leave for **30 seconds**.

9. Bring the stamp to the second **wash plate** and leave for **30 seconds.**

10. Take out the stamp from the wash plate and let the beads dry for **30 seconds** by holding it in the air.

11. Place the stamp in the **elution plate** and release the cover plate in a **very quick snapping motion** to prevent beads from following the magnetic pins up the sides of the cover plate.

12. Swirl the **cover plate** in the elution plate to release all beads. It takes some effort to swirl carefully and thoroughly. Keep checking if all the beads are released in the eluate.

13. Leave the beads in the eluate for at least **5 minutes** before attaching the cover plate to the stamp again. Wait another **5 minutes** until all beads are attached to the magnetic stamp.

14. Remove the magnetic stamp and dispose of the cover plate.

15. Seal the plate and store in a **-20 °C** freezer or move the appropriate volume of purified Adaptor ligated DNA to a **0,2 ml skirted PCR plate** when proceeding with the **PCR Enrichment Step.**

NOTE: The plate can be concentrated again using the Eppendorf Concentrator if volume option 2 or volume option 3 is chosen and low concentrations are expected.

4.4 PCR Enrichment of Adaptor-ligated DNA

Remove the ONEBNext Ultra II Q5 Master Mix 2X and PCR indexes from the freezer and place on ice.

	Volume option 1 (1:8)	**Volume option 2 (1:12)**	**Volume option 3 (1:16)**
Adaptor Ligated DNA Fragments	5,25 µl	3,15 µl	2,1 µl
ONEBNext Ultra II Q5 Master Mix 2X	6,25 µl	3.75 µl	2,5 µl
O Illumina i7 Primer*	0,5 µl	0,3 µl	0,2 µl
O Illumina i5 Primer*	0,5 µl	0,3 µl	0,2 µl
Total volume	12,5 µl	7,5 µl	5 µl

1. Distribute the ONEBNext Ultra II Q5 Master Mix 2X with the chosen volume option into a 0,2 ml skirted PCR plate containing the i5 and i7 primer mixture.*

2. Add the Adaptor Ligated DNA Fragments to the mixture using a P10 Multichannel and pipette the entire volume up and down at least 10 times.

3. Place the plate in a thermocycler and perform PCR amplification using the following PCR cycling conditions:

Cycle Step	**Temp**	**Time**	**Cycles**
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	3–22
Annealing/Extension	65°C	75 seconds	
Final Extension	65°C	5 minutes	1
Hold	4°C	∞	

The number of PCR cycles should be chosen based on input amount and sample type. Thus, samples prepared with a different method prior to library prep may require re-optimization of the number of PCR cycles. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low

enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on TapeStation or Fragment Analyzer). Use the table below for applications requiring high library yields (~1 µg).

Cycle number table

INPUT DNA IN THE END PREP REACTION	# OF CYCLES REQUIRED FOR ENRICHMENT LIBRARY PREP (~1 µg):
1 µg*	3–4*, **
500 ng*	4–5*
100 ng*	6–7*
50 ng	7–8
10 ng	9–10
5 ng	10–11
1 ng	12–13
0.5 ng	14–15

* Cycle number was determined for size selected libraries. ** NEBNext adaptors contain a unique truncated design. Libraries constructed with NEBNext adaptors require a minimum of 3 amplification cycles to add the complete adaptor sequences for downstream processes.

4.4 Check Amplification success

1. Quantify 2-3 amplified samples on the **TapeStation** using the **D5000 High Sensitivity kit.**

2. If the amplification was not sufficient you can add additional cycles.

3. If **overcycling** has occurred, you have to redo the amplification with the remaining sample from step 4.3 with fewer cycles.

4. Bring the volume of the PCR reaction to at least **10 µl** when the enrichment step was successful before proceeding to the next step.

4.5 Clean-up of PCR reaction

This bead clean-up method uses a magnetic bead separator stamp in combination with NucleoMag NGS Cleanup and Size Select beads. Make sure to use Corning® 3788 Round Bottom Assay Microplate with Lid.

Preparation

- Let the MN beads get to room temperature (remove from fridge 15 min before use).
- Prepare fresh **80% EtOH** (e.g. 41.7ml EtOH 96% + 8.3 ml MQ in a 50 ML Falcon tube).
- The amount of beads to be added is dependent on the fragment size. Make sure to check the bead ratio you have to work with.
    - For library clean-up, a **0,9X clean-up** is recommended to wash away primer/adaptor dimers.

1. Mix the **MN beads** well by vortexing the tube.

2. Fill a 96- well round bottom plate with the appropriate amount of magnetic beads (0,9X), based on the volume of the PCR reaction.

3. Add the Amplified libraries using a P10 Multichannel and mix by pipetting the volume up and down at least 10 times.
    a. Incubate for at least **5 minutes.**

4. Fill two round bottom plates with **50 µl** of **80% EtOH**.

5. Fill one round bottom plate with **17 µl of UltraPure Water**.

6. Load the **magnetic stamp** with a **cover plate** and make sure the plate is locked into place.

7. Place the **magnetic stamp** in the sample / beads plate and wait for at least **1 minute** until all beads are covering the magnet.

8. Bring the stamp to the first **wash plate** and leave for **30 seconds**.

9. Bring the stamp to the second **wash plate** and leave for **30 seconds.**

10. Take out the stamp from the wash plate and let the beads dry for **30 seconds** by holding it in the air.

11. Place the stamp in the **elution plate** and release the cover plate in a **very quick snapping motion** to prevent beads from following the magnetic pins up the sides of the cover plate.

12. Swirl the **cover plate** in the elution plate to release all beads. It takes some effort to swirl carefully and thoroughly. Keep checking if all the beads are released in the eluate.

13. Leave the beads in the eluate for at least **5 minutes** before attaching the cover plate to the stamp again. Wait another **5 minutes** until all beads are attached to the magnetic stamp.

14. Remove the magnetic stamp and dispose of the cover plate.

15. Transfer the eluate to a **0,2 ml skirted PCR plate** and store in a **-20 °C** freezer.

❺ Library quantification

1. When working with a high number of samples, measure the concentration of all samples on the Fragment Analyzer using either the **dsDNA 910 Kit 35-1500bp (DNF-910-K0500)** or **HS Small Fragment Kit (DNF-477-0500)** kit.
    - The kit to use depends on the concentration you would expect for your libraries. Use the **HS Small Fragment kit (DNF-477-0500)** if your starting concentrations were really low (e.g. under 2 ng).

2. When working with a low number of samples, measure the concentration of all samples on the TapeStation using the **D5000 High Sensitivity kit** using a 1:10 dilution.

❻ Normalisation and pooling

1. Normalise the libraries based on the Fragment Analyzer or TapeStation Results using **the Qiagility equimolar pooling robot**.

2. Manually pool all samples in a **1,5 ml LoBind tube**.

3. Measure the pool in duplicate using the **TapeStation High Sensitivity D5000 kit**.

4. **Optional:** if adaptor or primer dimers are observed, perform a 0,9X bead clean-up on the pool. If the pool contains a lot of bigger fragments, try a **size selection** on the pool.
    a. Repeat step 3 if this step is followed.

5. If equimolar pooling of subpools is required, use the average molar concentration of the measured subpools for pooling of these pools.
    a. This should be calculated based on the lowest pool concentration.

6. **SNP&SEQ Technology Platform Sequencing** needs between **25 µl** and **50 µl** of end pool for sequencing on their Illumina system.