Library preparation for Hyb-Seq using NEBNext ultra DNA Library Prep Kit for Illumina, half reactions, *Crataegus* Prague protocol 2024

Department of Botany, Charles University, Prague, Czech Republic

Department of Forest Growth, Silviculture & Genetics,

Austrian Research Centre for Forests (BFW), Vienna, Austria

Extraction – Invisorb Spin Plant Mini Kit, 250 purifications (Stratec)

The best yield suitable for preparation of libraries was obtained from Invisorb Spin Plant Mini Kit. The samples are extracted according the protocol with following changes: short centrifugation of lysis solution before using Prefilters, adding 5µl of RNase A (100 mg/ml) to the filtrate. The samples can get gely or slimy during the extraction. The chunk of slime that usually appears especially after mixing with the binding buffer can be carefully removed from liquid with a tip to avoid blocking the elution membranes. Elution for 30 minutes in 60 µl.

Sonication – Covaris M220 sonicator, tubes 50µl micro TUBE AFA Fiber - Screw-Cap (520166)

Sample preparation:

We need (250) – 500 – ideally **1000 ng DNA** (measured on Qubit). Samples should be diluted with ddH_2O to the total volume of **50 µl**. During sonication we aim for fragments of length 500 to 700bp. We use program with following parameters: **50W, 20% duty factor, 200 cycles, t 20°C, duration 32 sec.** (program: Lenka-1000bp for whole non-fragmented DNA) or between 5s to 25 sec for partly fragmented samples). Very fragmented samples with the majority of fragments around 500bp and less are not sonicated at all.

We check fragment length on **0.8%** agarose gel with Gel Red. Load: **2µl** DNA +**1µl** dye 6x Orange LD, **0.7 µl** ladder 1kb, and **0.4 µl** ladder 100bp.

If we found on the gel that DNA is not fragmented properly sonication has to be repeated.

End repair and A-tailing

NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645L).

Master Mix preparation: *on ice*

- **3.5 µl** Ultra II End Repair Reaction Buffer *vortex* * *samples =* ***µl***
- **1.5 µl** Ultra II End Prep Enzyme Mix *only spin down* * *samples =* ***µl***

We prepare **25 µl** of fragmented DNA to strips, than prepare Master Mix – gently vortex and spin down and add **5 µl** to the each sample and thoroughly mix by pipetting.

Put the samples to thermocycler, program:

20°C - 30 min - enzyme repairs the ends to have both DNA strands of the same length

65°C - 30 min - put A to the ends

4°C hold

Adaptor ligation

- **15 µl** Ultra II Ligation Master Mix kit E7645L * = **µl**
- **1.25 µl** NEBNext Adaptor for Illumina kit E6440S * = **µl**
- **0.5 µl** Ligation Enhancer kit E7645L * = **µl**

Mix this master mix and add **16,75 µl** of MM to the DNA mix with ligated as from the previous step. Mix by pipetting.

Incubate in thermocycler at **20°C - 15 mins**

USER enzym

Add **1.5 µl** USER enzyme (E6440S) to each sample, mix well by pipetting, centrifuge. Incubate at **37°C - 15 min**

Cleanup – cleaning product using - **QIAquick PCR Purification Kit**

Add ca. **5x** of sample volume, ca **48 µl** * 5 = **240 µl** of Buffer PB. Mix by pipetting and put to the column.

Centrifuge at 13,000 rpm - **1 min** and discard the flow-through. Product is bound at the column.

Add **740** µl washing buffer PE.

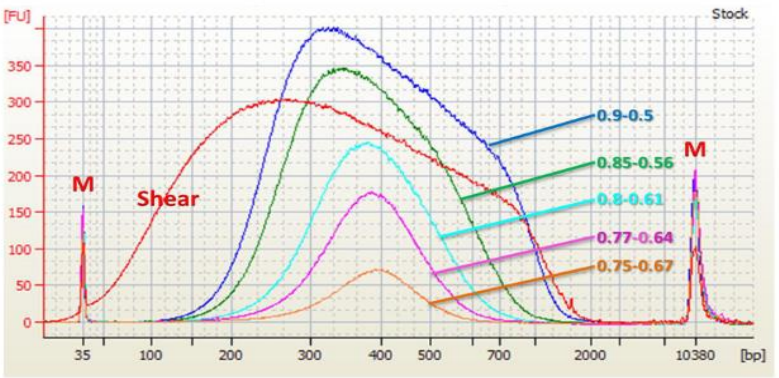
Centrifuge at 13,000 rpm - **1 min** and discard the flow-through. Again centrifuge at 13,000 rpm - **1 min** to remove all EtOH and discard the flow-through.

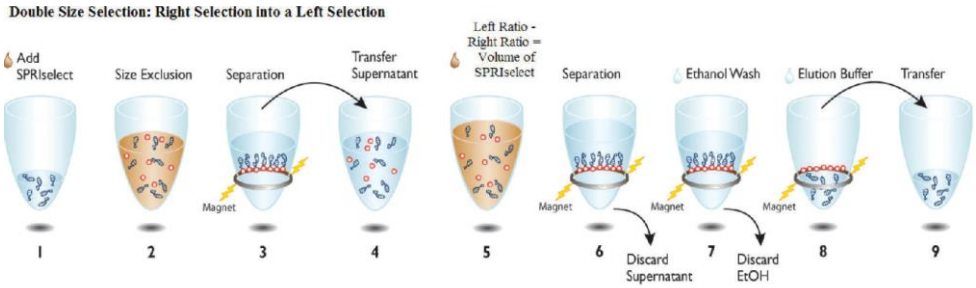
Move the column to a new 1.5 ml tube. Pipette **51.5 µl** of ddH_2O onto the column (this volume is OK to load it on the gel) – **do not pre-heat!!!** Centrifuge 1min at 13,000rpm.

Elution **30 min for Crataegus.** Usually 5 min.

Double Size selection with SPRIselect beads

Figure 5 Agilent High Sensitivity DNA chip Electropherogram.





We will choose the beads ratio 0.75-0.56 (product length 280-700bp) according to experience and sonicated DNA. We will use 50µl of purified DNA.

Right size selection: 50µl x 0.56= **28µl** of SPRI beads

Put 28µl of SPRI into wells in the plate and add the 50µl of DNA to each of them (the different samples) mix by pipetting 10x. Incubate for **1 min**. and put on the magnetic plate (MP), and wait for about **2 minutes**.

Transfer the clear supernatant to new wells (pipet water into the remaining beads and pipet that to the original tube as backup).

Left size selection: calculate the difference between ratios 0.75-0.56 = x0.19; 50µl DNA x 0.19= **9.5µl** SPRI

Add 9.5 µl of SPRI to the supernatant in the wells, mixing by pipetting 10x. Incubate 1 min. Put on the MP Discard the supernatant.

Washing the magnetics beads: add **180 µl-200µl 85% ethanol**, leave it for 30 seconds. Put on MP. Pipet and discard the supernatant. Repeat the wash.

Remove the plate from the MP and allow the beads to dry, max 3'.

Add water ddH_2O: **17µl**, mix 10x. Incubate 1 min., put the plate in the MP. Pipet the clear supernatant into new strips.

Checking of size selected fragments with electrophoresis,1.2 ul DNA – gel 1% TAE

PCR Enrichment (kit 6440S)

- **12.5 µl** NEBNext **Q5** Master Mix
- **5 µl** Unique Index Primers (**10µM**)
- **7.5 µl** DNA

mix by pipetting (be careful – it foams). Work on ice – big box.

PCR cycle:

(1) 98°C - 30 sec

(2) 98°C - 10 sec

(3) 65°C – 1 min 15 sec

step 2, 3 – 10-12 cycles according to concentrations of input DNA

(4) 65°C - **5 min**

7°C - hold

Cleanup – using SPRI kit – magnetic beads

Mix thoroughly the SPRI bottle.

Pipette appropriate amount of the kit towards DNA (**ratio 0.78** kit **: 1** DNA (19.5 µl kit : 25 µl DNA) and mix 10x by pipetting. Incubate **1 mins** at room temperature. Put the samples to the magnetic stand – **2 mins** (until the solution get clear and transparent). Remove carefully the clear supernatant – the beads must not whirl. Pipette **180 µl 85**% EtOH (**freshly prepared**!!!), incubate **30s**. Carefully remove EtOH. Repeat this step again. Thoroughly remove all the ethanol and let it stand for max. 3 min to evaporate residual ethanol. *WARNING! The beads must not overdry – i.e., the pellet must not start to crumble – otherwise you get less DNA.* Move the samples off the magnetic stand and add **25µl** ddH2O and 10x mixed by pipetting. Put the samples back to the magnetic stand – for **1 min**. Pipette out the solution with DNA to the new tube.

II. Cleaning: ratio 0.78=**18.72µl** SPRI beads**, elution** – only with **22 µl** ddH2O.

Check the cleaning quality on 1% agarose gel:

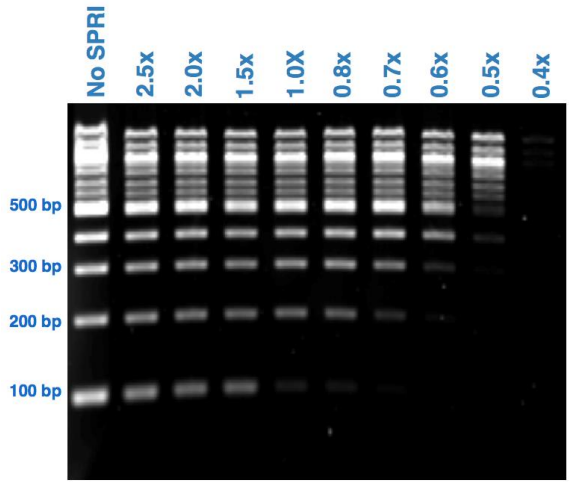
1%TAE gel, **Gel Red** – the amount according to the size of the chamber - **1ul/60ml gel**, **2 µl** DNA, **1 µl** 6x Orange Loading Dye, **0.4 µl** ladder **100bp** and **0.7 µl** ladder **1kb**

According to the gel visualization we can repeat SPRI cleaning step. We only want products **500 bp** and longer.

If we have fragments of unwanted size use following ratios:

300bp – **0.65 kit : 1 DNA**.

100bp – **0.75 – 0.8 kit : 1 DNA**



Check again the cleaning quality on 1% agarose gel:

1%TAE gel, **Gel Red** – the amount according to the size of the chamber - **1ul/100ml gel**, **2 µl** DNA, **1 µl** 6x Orange Loading Dye, **0.4 µl** ladder **100bp** and **0.7 µl** ladder **1kb**

Library pooling

Measure concentration of all library samples on Qubit. All the samples are pooled into single Low - bind 1.5 ml tube in a such way that in the total volume there is equal amount of each sample (in ng). The total amount of DNA in the pool of 24-sample should be 100 – 500ng, **for *Crataegus* 29 ng of each library, a pool of 12 samples ~ 350 ng**. **Two pools are prepared, the one for a hybridization reaction and the second that would not be enriched.** The resulting volume in the tube is different each time but the pool for hybridization should be reduced to **7 µl** using concentrator – this is the volume we need for hybridization enrichment.

Hybridization enrichment

(see the original MYcroarray manual v.5.00, 2020)

…

Hybridization temperature – 60°C up to 65°C for 26 hours.

Add 15 µl of Nuclease-free H_2O (Gibco) (instead of 30 µl Buffer E in the manual) to a pellet of beads in the end.

Post - Capture Amplification

The goal is to amplify rather small amount of DNA obtained in the previous step to get enough material for Illumina sequencing. It is important to limit the number of cycles to minimum: get enough material but minimize the number of duplications.

Prepare **PCR mix 50 µl** to **0.2 ml** Lo-bind tubes, mix by pipetting.

Nuclease-free H_2O (Gibco)..... **5 µl**
2x KAPA HiFi HotStart ReadyMix.....**25 µl**
PCR primer mix (10µM each)…….....2x **2.5 µl**
Our library …….... **15 µl**
50 µl

Illumina P5 – 5´- AATGATACGGCGACCACCGA 3´
Illumina P7 – 5´-CAAGCAGAAGACGGCATACGA 3´
The stock primer solution is 100pmol. We want a primer mixture of final concentration 10pmol: **1µl** P5 + **1µl** P7 + **8µl** H_2O = **10ul 10pmol** primer mix

Cycle: (cca 35 min)
(1) 98°C – 2 min.
(2) 98°C – 20 s
(3) 62°C – 30 s step 2, 3, 4 - **12 cycles**
(4) 72°C – 45 s
(5) 72°C – 5 min
(6) 8°C – hold

Extension length 72°C depends on the fragment size. Fragments shorter than 500bp -30 s, 500 - 700bp - 45 s, 700bp - 1 kb - 1 min. Number of cycles 8 – 14, optimize in order to get enough material for sequencing but minimize PCR duplicates and errors

PCR product clean up – QIAquick PCR Purification kit

Use **25 µl** Gibco (RNa-free) water for elution 5 min.

Additional 1x Cleanup – using AM-Pure kit – magnetic beads

with ratio x0.8 to cut out residual primers

Bianalyzer, mixing for sequencing

Measure sample concentrations of all hybridized and non-hybridized pools on Qubit and dilute for Bianalyzer measurements. Maximum 5 ng/ul should be used. Resulting concentrations and fragment lengths obtained from the Bioanalyzer will be used to equally mix all the hybridized pools and spike in 1/3 of the non-hybridized pools. Alternatively Qubit concentrations and the fragment lengths from agarose gel can be used.

Conversion of ng/µl to nM:

1bp (1pair of nucleotides) weighs 660pg **1bp = 660pg**

If we have DNA fragments of ca **700 bp * 660pg = 462 000 g/mol = 462mg/mmol = 0.462 µg/µmol**

If library concentration is **16ng/µl = 0,016 µg**

0,462 µg.....1 µmol

↕0,016 µg.....x µmol↕

x / 1 = 0,016 / 0,462

x = 0,0346 µmol

x = 34,6 nM