

An efficient method to prepare barcoded cDNA libraries from plant callus for long-read sequencing

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Method Article

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Abstract

Background

Long-read sequencing methods allow a comprehensive analysis of transcriptomes in identifying full-length transcripts. This revolutionary method represents a considerable breakthrough for non-model species since it allows enhanced gene annotation and gene expression studies. However, some plant tissues are recalcitrant to the successful preparation of cDNA libraries thus impairing further analyses.

Results

A detailed protocol for preparing cDNA libraries suitable for high throughput RNA sequencing using Oxford Nanopore Technologies® was developed. Through this method, 8 barcoded cDNA libraries from two tamarillo (*Solanum betaceum* Cav.) types of callus (friable and compact) and with distinct embryogenic competencies (embryogenic non-embryogenic) have been successfully prepared and sequenced.

Conclusions

An efficient method for preparing barcoded cDNA libraries from plant callus is presented.

Long-read sequencing using these libraries allows a comprehensive analysis of plant transcriptomes.

Background

In recent years, transcriptome sequencing has been an invaluable tool in gene expression profiling. By unravelling gene regulatory mechanisms, high-throughput sequencing approaches have shown widespread applications in many fields of biology, including plant physiology and developmental analysis [1–3].

Nanopore sequencing from Oxford Nanopore Technologies® (ONT) is a high-throughput sequencing technology that detects ionic current changes as DNA or RNA molecules pass through nanopores [4]. Compared to other next-generation sequencing methods, Nanopore offers native and long-read sequencing (> 4Mb in a single read), which, together with the template-switching method, allows the detection of complete full-length transcripts [5]. Moreover, barcoding kits are available allowing cDNA tagging. Multiple barcoded libraries can be pooled and sequenced in a single run optimising the assay for high-throughput. With Nanopore, it is also possible to sequence cDNA directly using a PCR-free protocol, thus avoiding amplification bias and preserving samples' quantitative information [6].

Despite the rapid advances in the development and optimization of sequencing technologies, some methods are not so straightforward applicable to particular organisms. Due to the richness of secondary metabolites and high ribonucleases (RNases) content, plant tissues have proven to be a complicated challenge for the application of cutting-edge sequencing technologies and to obtain high-quality RNA

yields for subsequent construction of good-quality cDNA libraries [7]. Although several kits are available for total RNA extraction and poly(A) RNA isolation, they have proved to be somewhat inefficient for some plant tissues, making it difficult to reproduce results between different laboratories, which is still a limitation to gene expression studies, particularly in non-model species [8]. Furthermore, RNA extraction yields widely vary according to species, genotypes, sample type, age and oxidative state [9–12]. For instance, *in vitro* growing callus, rich in starch, pectins and other polysaccharides (e.g., arabinoxylan, β -glucan) [13, 14], has shown to be a particularly difficult tissue to achieve high yield and quality RNA extractions for further processing methods [9]. Finally, the polyadenylated transcripts (such as mRNAs and some long non-coding (lncRNAs)), the most targeted in transcriptome analysis studies, comprise only a small part of the total RNA in eukaryotes [15, 16].

Because of its great morphogenic plasticity, callus samples are valuable experimental models for fundamental research as well as for industrial and bioengineering applications [17]. However, straightforward optimized procedures for high-throughput analysis are still often required. Here, a detailed methodology that combines poly(A) RNA isolation and barcoded cDNA library preparation is described for plant callus samples. To develop this protocol Dynabeads™ mRNA DIRECT™ Kit from Invitrogen™ and Direct cDNA Sequencing Kit (SQK-DCS109) with Native Barcoding Expansion 1–12 (EXP-NBD104) from ONT were used to prepare barcoded cDNA libraries from two tamarillo (solanaceous tree) callus lines with different cytological (compact and friable) and morphogenic competencies (embryogenic and non-embryogenic).

Materials And Methods

Plant material

Two tamarillo (*Solanum betaceum*) callus lines were used: a compact embryogenic callus (EC, Fig. 1a) and a friable non-embryogenic (NEC, Fig. 1b). Both were induced from leaf explants of *in vitro* growing shoots and had been maintained in Murashige and Skoog medium [18] supplemented with 20 μ M Picloram plus 9% (w/v) sucrose, solidified with 0.25% (w/v) Phytigel™ (Sigma-Aldrich) at pH 5.7 and incubated at 24 ± 1 °C under dark conditions, following a procedure previously described [19]. For each callus line, EC and NEC, four biological replicates were used.

Reagents

Agencourt AMPure XP beads (Beckman Coulter, Inc, Cat. No. A63880)

Blunt/TA Ligase Master Mix (New England Biolabs (NEB), Cat. No. M0367)

Direct cDNA Sequencing Kit (ONT, Cat. No. SQK-DCS109)

dNTPs NZYMix (NZYTech, Lda. - Genes and Enzymes(NZY), Cat. No. MB086)

Dynabeads™ mRNA DIRECT™ Kit (Invitrogen™, Cat. No. 61011)

Ethanol for molecular biology (Sigma-Aldrich[®], Cat. No. 51976)

Flow Cell R9.4.1 (ONT, Cat. No. FLO-MIN106)

LongAmp[®] *Taq* 2X Master Mix (NEB, Cat. No. M0287)

Maxima H Minus Reverse Transcriptase (Thermo Scientific[™], Cat. No. EP0751)

Native Barcoding Expansion 1-12 (ONT, Cat. No. EXP-NBD104)

NEBNext[®] Quick Ligation Module (NEB, Cat. No. E6056)

NEBNext[®] Ultra[™] II End Repair/dA-Tailing Module (NEB, Cat. No. E7546)

Nuclease-free water (PanReac AppliChem ITW Reagents, Cat. No. A7398)

NZYRibonuclease Inhibitor (NZY, Cat. No. MB084)

Qubit[™] dsDNA HS Assay Kit (Invitrogen[™], Cat. No. Q32851)

Qubit[™] RNA High Sensitivity (HS) Assay Kit (Invitrogen[™], Cat. No. Q32852)

RNase Cleaner (NZY, Cat. No. MB16001)

RNase Cocktail[™] Enzyme Mix (Invitrogen[™], Cat. No. AM2286)

Supplies

0.2 mL PCR tubes

1.5 mL microcentrifuge tubes

21-gauge needles, sterile

Liquid nitrogen

Low-binding P10 barrier tips, sterile

Low-binding P1000 barrier tips, sterile

Low-binding P200 barrier tips, sterile

Mortars

Pestles

Qubit[™] Assay Tubes (Invitrogen[™], Cat. No. Q32856)

Spatulas

Sterilized syringues

Equipment

DynaMag™-2 magnet (Invitrogen™, Cat. No. 12321D)

Heating block

Microspin

MinION (ONT, MK1C)

NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, Cat. No. ND-2000)

Qubit™ 4 fluorometer (Invitrogen™, Cat. No. Q33238)

Rotator mixer

Thermo Scientific™ Arktik™ thermal cycler (Thermo Scientific™, Cat. No. 11999984)

Vacuum concentrator

Vortex mixer

Software

MinKnow (ONT, version 21.10.8)

-

PROTOCOL

Setup

Autoclave twice all no nuclease-free materials.

Clean mortars, pestles and spatulas with ethanol and RNase Cleaner before autoclaving.

Work in RNase-free environment, use RNase Cleaner and wear gloves.

Bring Agencourt AMPure XP beads to room temperature 30 min before use.

Prepare fresh 70% (v/v) ethanol before each Agencourt AMPure XP beads purification step.

Poly(A) RNA purification

Poly(A) RNA was purified directly from callus samples using the Dynabeads™ mRNA DIRECT™ Kit (Invitrogen™) (Fig. 2).

1. Sample preparation

- a. Weight 100 mg of fresh callus samples and flash-freeze in liquid nitrogen.
- b. Grind the frozen material in liquid nitrogen, using mortar and pestle, and transfer the fine powder to a microcentrifuge tube with a spatula.
Maintain tubes in liquid nitrogen while all samples are processed.
- c. Add 1250 µL of lysis/binding buffer and vortex for 2 min.
Don't let the samples heat up, place tubes on ice.
- d. Force the lysate through a 21-gauge needle 3–5 times using a 1 mL syringe to shear the DNA.
Spin down briefly.

2. Dynabeads preparation

- a. Bring lysis/binding buffer and washing buffers A and B to room temperature before use. Keep 10 mM Tris-HCl at 4 °C.
- b. Resuspend Dynabeads™ Oligo(dT)25 thoroughly.
- c. Transfer 250 µL of beads to a 1.5 mL microcentrifuge tube and place the tube on the DynaMag™-2 magnet.
- d. When the suspension is clear discard the supernatant.
- e. Remove the tube from the magnet and wash the beads by resuspending in 250 µL of fresh lysis/binding buffer.

3. Poly(A) RNA isolation

- a. Place the washed beads on the magnet and discard the supernatant when the suspension is clear.
- b. Remove the tube from the magnet and add the sample lysate from step 1.d., pipetting to resuspend the beads completely.
- c. Incubate the mixture on a mixer for 15 min at room temperature.
- d. Place the tube on the magnet for 5 min. Rotate the tube slowly 90° to the right and then back to the starting position. Do the same for the left and hold for more 5 min. When the suspension is clear, discard the supernatant.
- e. Remove the tube from the magnet and wash the RNA-beads complex twice with 1 mL of washing buffer A by pipetting until beads are thoroughly resuspended.
Use the magnet to remove completely all traces between each washing step.
- f. Wash the RNA-beads complex twice with 1 mL of washing buffer B by pipetting until beads are thoroughly resuspended.
Use the magnet to remove completely all traces between each washing step.

- g. Elute the poly(A) RNA from the beads by adding 12 μL of 10 mM Tris-HCl (pH 7.5). Pipet until beads are thoroughly resuspended and keep the tube at 80 $^{\circ}\text{C}$ for 2 min.
- h. Immediately place the tube on the magnet and transfer the supernatant containing the poly(A) RNA to a new RNase-free tube. Place poly(A) RNA on ice.
- i. Analyse 1 μL of the poly(A) RNA using Qubit[™] RNA High Sensitivity (HS) Assay Kit and 1 μL using a NanoDrop[™] spectrophotometer.
Use only samples with A260/A280 ratio between 1.8 and 2.1 and A260/A230 ratio higher than 1.8 for cDNA synthesis.

Reverse transcription and strand-switching

Barcoded libraries were prepared following the Direct cDNA Native Barcoding protocol from ONT with some modifications (Fig. 2).

1. Transfer 100 ng poly(A) RNA into a 0.2 mL PCR tube and adjust the volume to 7.5 μL with nuclease-free water.
2. Add 2.5 μL of VN primer and 1 μL of dNTPs (10mM).
3. Mix thoroughly by pipetting and spin down briefly.
4. Incubate at 65 $^{\circ}\text{C}$ for 5 min and then snap cool on a pre-chilled freezer block.
5. Prepare a master mix with the following reagents:
 - μL 5x RT Buffer
 - μL NZYRibonuclease Inhibitor
 - μL nuclease-free water
 - μL Strand-Switching Primer
6. Mix thoroughly by pipetting and spin down briefly.
7. Add 8 μL of the master mix to the snap-cooled RNA. Mix by flicking the tube and spin down briefly.
8. Incubate at 50 $^{\circ}\text{C}$ for 2 min.
9. Add 1 μL of Maxima H Minus Reverse Transcriptase. Mix gently by flicking the tube and spin down briefly.
10. Incubate at 50 $^{\circ}\text{C}$ for 120 min and at 85 $^{\circ}\text{C}$ for 5 min. Then hold at 4 $^{\circ}\text{C}$.

RNA degradation

1. Add 1 μL RNase Cocktail[™] Enzyme Mix to the reverse transcription reaction.
2. Incubate the reaction at 37 $^{\circ}\text{C}$ for 10 min.
3. Resuspend the beads by vortexing and transfer 36 μL to a new 1.5 mL microcentrifuge tube.

4. Add the reaction to the beads and mix thoroughly by pipetting.
5. Incubate on a mixer for 15 min at room temperature.
6. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant when the suspension is clear.
7. Keep the tube on the magnet and wash the beads twice with 200 μ L of freshly prepared 70% ethanol without disturbing the pellet. After 1 min remove the ethanol using a pipette and discard.
8. Spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.
9. Remove the tube from the magnet and resuspend the pellet in 40 μ L nuclease-free water.
10. Incubate on a mixer for 15 min at room temperature.
11. Pellet beads on magnet until the eluate is clear and colourless.
12. Remove and retain 40 μ L of eluate into a new 1.5 mL microcentrifuge tube.
13. Let the tubes cold down on ice and take them open to a vacuum concentrator to reduce the sample volume up to 23 μ L.

Second strand synthesis

1. Transfer the samples to a 0.2 mL PCR tube and adjust the volume to 23 μ L with nuclease-free water.
2. Add 25 μ L of LongAmp[®] *Taq* 2X Master Mix and 2 μ L of PR2 primer.
3. Mix thoroughly by pipetting and spin down briefly.
4. Incubate at 94 °C for 1 min, 50 °C for 1 min and 65 °C for 20 min. Then hold at 4 °C.
5. Resuspend the beads by vortexing and transfer 90 μ L to a new 1.5 mL microcentrifuge tube.
6. Repeat steps 4-12 from “RNA degradation”.
7. Analyse 1 μ L of the strand-switched DNA using Qubit[™] dsDNA HS Assay Kit from Invitrogen[™].
8. If necessary, pool cDNA samples to reach 70-200 ng in a maximum volume of 50 μ L. Use a vacuum concentrator to reduce the sample volume.

End-prep

1. Transfer the samples to a 0.2 mL PCR tube and adjust the volume to 50 μ L with nuclease-free water.
2. Add 7 μ L of NEBNext Ultra II End Prep Reaction Buffer and 3 μ L of NEBNext Ultra II End Prep Enzyme Mix.
3. Mix thoroughly by pipetting and spin down briefly.
4. Incubate at 20 °C for 30 min and 65 °C for 30 min.

5. Resuspend the beads by vortexing and transfer 108 μL to a new 1.5 mL microcentrifuge tube.
6. Repeat steps 4-12 from "RNA degradation".
7. Let the tubes cold down on ice and take them open to a vacuum concentrator to reduce the sample volume up to 22.5 μL .

Barcode ligation

1. Transfer Blunt/TA Ligase Master Mix to ice before reaction set up. Mix tube by finger flicking before use.
2. Adjust the volume sample to 22.5 μL with nuclease-free water.
3. Add 2.5 μL of a native barcode to each sample and 25 μL of Blunt/TA Ligase Master Mix.
4. Mix thoroughly by pipetting and spin down briefly.
5. Incubate at 25 °C for 15 min.
6. Resuspend the beads by vortexing and transfer 90 μL to the reaction. Mix thoroughly by pipetting.
7. Repeat steps 5-12 from "RNA degradation".
8. Analyse 1 μL of the eluted sample using QubitTM dsDNA HS Assay Kit from InvitrogenTM.
9. Estimate cDNA sequences' average length by electropherogram analysis (e.g., Agilent's Bioanalyzer) or from previously obtained transcriptomic data analysis.

Adapter ligation

1. Based on the predicted average length of sequences and not exceeding 200 fmol, calculate the maximum cDNA concentration to use. For example, if the average length of sequences is 1000 bp, use a maximum of 130 ng.
2. Calculate the volume to be used from each barcoded sample to do a pool with the same concentration for all samples.
3. Pool barcoded samples to get the maximum cDNA concentration in a 1.5 mL microcentrifuge tube. Adjust the volume to 65 μL by adding nuclease-free water or using a Vacuum Concentrator to reduce the sample volume.
4. Thaw Wash Buffer (WSB), Elution Buffer (EB) and NEBNext Quick Ligation Reaction Buffer (5x) at room temperature. Mix by vortexing, spin down and place on ice. Check if the contents of each tube are clear of any precipitate. Spin down the T4 Ligase and the Adapter Mix II (AMII) and place them on ice.
5. Add 5 μL of Adapter Mix II (AMII), 20 μL of NEBNext Quick Ligation Reaction Buffer (5X) and 10 μL of Quick T4 DNA Ligase to the pooled and barcoded DNA, mixing by flicking the tube between each sequential addition.
6. Spin down and incubate at 20 °C for 15 min.

7. Resuspend the beads by vortexing and transfer 50 μ L to the reaction.
8. Mix thoroughly by pipetting.
9. Incubate on a mixer for 15 min at room temperature.
10. Pellet beads on magnet and pipette off the supernatant when the suspension is clear.
11. Wash beads-DNA complex twice with 140 μ L of the Wash Buffer (WSB). Close the tube lid and resuspend the beads by flicking the tube. Return the tube to the magnet, allow beads to pellet and pipette off the supernatant.
12. Spin down and place the tube back on the magnet. Pipette off any residual supernatant.
13. Remove the tube from the magnet and resuspend the pellet in 13 μ L of Elution Buffer (EB).
14. Incubate on a mixer for 15 min at room temperature.
15. Pellet beads on magnet until the eluate is clear and colourless.
16. Remove and retain 13 μ L of eluate into a clean 1.5 mL microcentrifuge tube.
17. Quantify 1 μ L of the eluted sample using QubitTM dsDNA HS Assay Kit from InvitrogenTM.

Quality assessment of cDNA libraries and ONT sequencing

1. Fragment size and purity of cDNA libraries can be assessed using an Agilent Bioanalyzer High Sensitivity DNA chip.
2. High throughput ONT sequencing.

Results And Discussion

Aiming to perform ONT sequencing in difficult plant tissues, an optimized protocol for preparing barcoded cDNA libraries from callus samples with very distinct histological and morphogenic features was developed. The protocol combines high yield and quality poly(A) RNA isolation and barcoded cDNA library preparation.

Poly(A) RNA isolation was performed from total RNA using the DynabeadsTM mRNA Purification Kit (InvitrogenTM), at the beginning of this assay. Total RNA from tamarillo EC and NEC was extracted using NZYol (NZY, Cat. No. MB18501) and the Direct-zolTM RNA MicroPrep from Zymo[©] Research Corporation. As recommended by the manufacturer's protocol, sample pools with 75 μ g of total RNA were used as starting material. Nevertheless, even starting with 85–100 μ g of total RNA and following the manufacturer's protocol, it was only possible to obtain the required 13 ng/ μ L of poly(A) RNA for the Direct cDNA Native Barcoding protocol in less than 50% of the NEC samples used (Table 1). This approach thus proved ineffective to reach the required quantity of total RNA (75–100 μ g), entailing significant losses of tissue samples and reagents due to the need to pool total RNA extractions from three replicates of $\sim$ 100 mg fresh callus samples. Therefore, another kit for poly(A) RNA isolation was used, DynabeadsTM mRNA

DIRECT™ Kit (Invitrogen™). With this kit, poly(A) RNA was isolated directly from callus samples, which enabled to get 100 ng of poly(A) RNA (Table 2) with much less tissue, extractions and time.

Table 1
Poly(A) RNA yield from embryogenic (EC) and non-embryogenic (NEC) callus using Dynabeads™ mRNA Purification Kit.

EC		NEC	
Total RNA (µg)	Poly(A) RNA (ng/µL)	Total RNA (µg)	Poly(A) RNA (ng µL)
75	17.3	61	4.0
75	14.7	86	6.2
75	10.5	88	15.0
75	26.8	91	14.2
88	4.2	93	24.8
97	4.4	107	0
98	18.9	100	0

Table 2
Poly(A) RNA yield from embryogenic (EC) and non-embryogenic (NEC) callus using Dynabeads™ mRNA DIRECT™ Kit.

EC	NEC
Poly(A) RNA (ng)	Poly(A) RNA (ng)
40.4	11.6
58.0	14.1
72.2	21.8
94.4	27.0

For the cDNA libraries preparation, the Direct cDNA Native Barcoding from ONT protocol was first followed. However, DNA losses were verified. In fact, several hard-to-remove contaminants inherent to plant tissues (e.g., phenolic compounds and polysaccharides) inhibit enzymes in library preparation processes for nucleic acid sequencing [10, 12, 20]. Therefore, optimizations were also needed. First, the incubation time of all reactions was prolonged. Also, the incubation temperature of the Maxima H Minus Reverse Transcriptase (Thermo Scientific™) was 50 °C (and not 42 °C, as suggested by ONT protocol), since this was the optimal reaction temperature described for this enzyme by the manufacturer. Finally, to guarantee that all DNA was recovered in the Agencourt AMPure XP beads purification steps, adequate beads and elution volume were used. As recommended by the manufacturer's protocol, 1.8 µL AMPure XP were added per 1.0 µL of sample (almost twice the beads volume suggested by ONT protocol). Withal,

after ethanol wash, beads were always eluted in 40 µL, since, as indicated by the manufacturer, recovery decreases with a decrease in elution volume. In several cases, to overcome the problem of too much volume a Vacuum Concentrator was used.

With this protocol, we have prepared and sequenced 8 barcoded cDNA libraries from two tamarillo cell lines with different histological and embryogenic competencies. Libraries were pooled and sequenced using one flow cell. Direct cDNA Sequencing was carried out using MinION equipment.

In a single run, 4.07 M reads with N50 ~ 1.2 kb were generated. Per base sequence quality of reads from each barcoded cDNA library was verified using the standard Phred-scaled quality score (Fig. 3). Reads showed an average quality score across all bases over 15 and all above 7 as recommended by Nanopore. This indicates an accuracy higher than 96.8% [21], which is considered as assigning confidence for long reads with little effect on downstream analysis [22, 23]. Therefore, barcoded libraries prepared using this protocol are suitable for further analysis.

Conclusion

An optimized and detailed protocol that combines poly(A) RNA isolation using Dynabeads™ mRNA DIRECT™ Kit (Invitrogen™) and cDNA library preparation using Direct cDNA Sequencing Kit (ONT, Cat. No. SQK-DCS109) with Native Barcoding Expansion 1–12 (ONT, Cat. No. EXP-NBD104) was developed. Barcoded cDNA libraries prepared following this method can be used for long-read sequencing methods. Moreover, this protocol can be applied to other plant species, overcoming the recalcitrance of some plant tissues to these molecular biology methods that remain a limitation of gene expression studies.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DC, SC and CS conceived the idea and designed the protocol described. DC and AP isolated total RNA. DC and SC performed mRNA purification. DC prepared cDNA. DC, AC and CS performed end-prep, barcode ligation, adaptor ligation and sequencing. SC, CS, JC managed resources and funding acquisition. DC wrote the manuscript and the other authors reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Figures

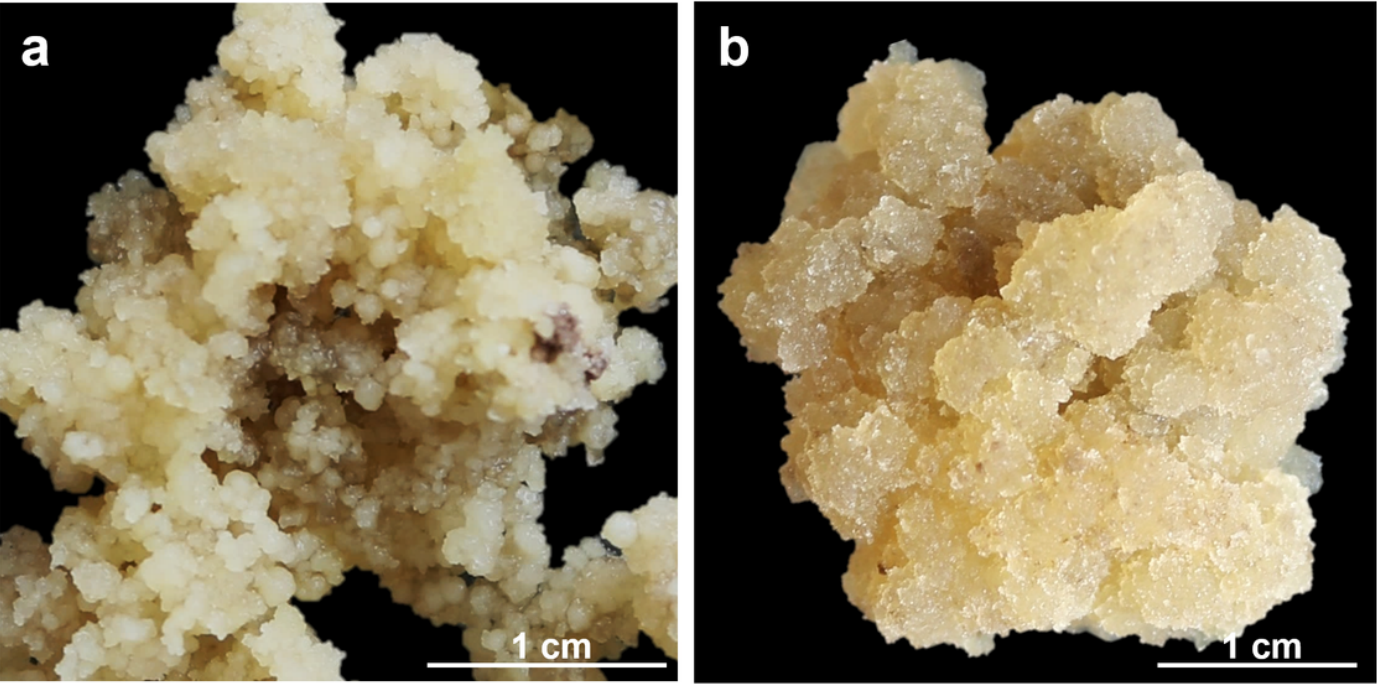


Figure 1

a - Tamarillo embryogenic (EC) and b - non-embryogenic callus (NEC).

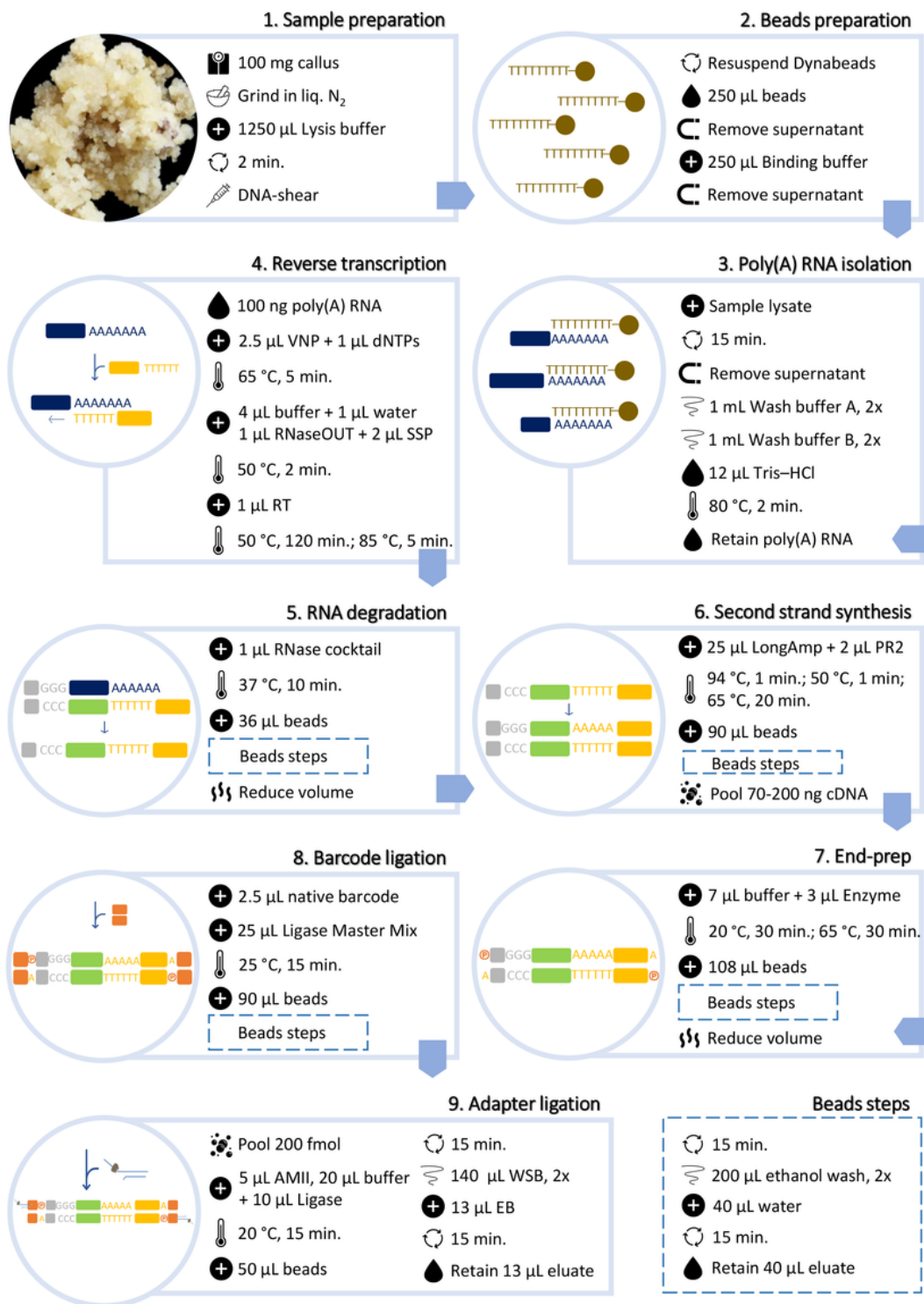


Figure 2

Schematic workflow for preparing barcoded cDNA libraries from plant callus for long-read sequencing, using DynabeadsTMmRNA DIRECTTM Kit (InvitrogenTM) and Direct cDNA Sequencing Kit (ONT, Cat. No. SQK-DCS109) with Native Barcoding Expansion 1-12 (ONT, Cat. No. EXP-NBD104).

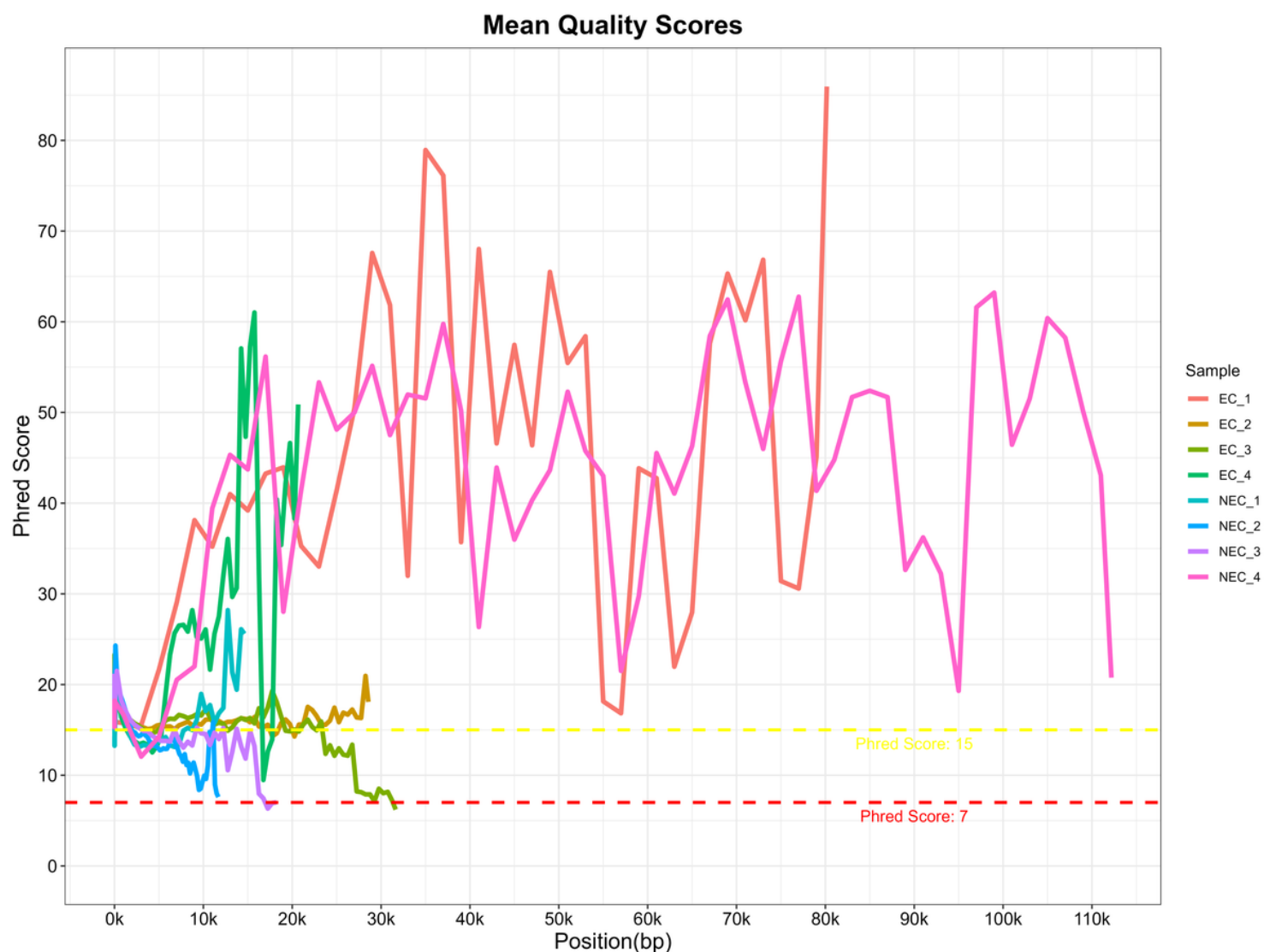


Figure 3

Mean quality scores of long reads for eight barcoded cDNA libraries. Libraries were prepared from compact embryogenic (EC) and friable non-embryogenic (NEC) *S. betaceum* callus, using DynabeadsTMmRNA DIRECTTM Kit (InvitrogenTM) and Direct cDNA Sequencing Kit (ONT, Cat. No. SQK-DCS109) with Native Barcoding Expansion 1-12 (ONT, Cat. No. EXP-NBD104).