Research

Standard Operating Procedure for Next Generation Sequencing of RNA Isolated form Extracellular Vesicles within the Nanostructurome Methods Pipeline

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Abstract:
The standard operating procedure (SOP) for next generation sequencing (NGS) of RNA from extracellular vesicles is presented. The SOP enables purification of small or total RNAs from isolated extracellular vesicles. Next, the SOP describes the sequencing of RNAs using NGS sequencing platform IonTorrent. In the presented protocol we define all the steps from isolation of RNA to sequencing data management, together with the cautions and checkpoints crucial for obtaining reliable high-quality sequencing reads, suitable for further analysis. NGS enables high-throughput DNA and RNA sequencing, offering accuracy, scalability, and cost-efficiency. RNA sequencing is widely used for analysing gene expression, alternative splicing, and non-coding RNAs. NGS also facilitates the study of extracellular vesicles (EVs), which play a key role in intercellular communication, especially in plant-pathogen interactions. Insights gained could inform novel disease management strategies, such as Spray-Induced Gene Silencing, for improved crop protection.

Keywords: Next generation sequencing; Extracellular vesicles; RNA-seq; Total RNA; Small RNA; IonTorrent.

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1. Definitions

NGS: next generation sequencing
EVs: extracellular vesicles
RNA-seq: sequencing of RNA molecules
SOP: standard operating procedure

2. Background

2.1. Next Generation Sequencing

Next Generation Sequencing (NGS) refers to a high-throughput DNA sequencing technology that enables the rapid and parallel sequencing of millions to billions of DNA fragments. Unlike traditional Sanger sequencing, NGS employs massively parallel processing, allowing for comprehensive genomic, transcriptomic, and epigenomic analyses with high accuracy, scalability, and cost efficiency (Goodwin et al., 2016). This technology is widely utilized in various fields, including agronomy, where NGS enables the genomic and transcriptomic analysis of crops, aiding in the identification of traits related to yield, stress resistance, and genetic diversity for improved breeding strategies. NGS facilitates the study of plant-pathogen interactions by uncovering resistance genes, defence-related small RNAs, and regulatory networks, enhancing disease resistance and crop protection efforts (Ashraf et al., 2022).

In transcriptome analysis, NGS, particularly RNA sequencing (RNA-Seq), allows for the in-depth characterization of gene expression profiles, alternative splicing events, and non-coding RNA populations, providing critical insights into cellular function, disease mechanisms, and developmental processes (Jiang et al., 2015; Krishanpal et al., 2015). In extracellular vesicle (EV) research, NGS is used to analyse the DNA and RNA cargo of EVs, their roles in intercellular communication, with emphasis on plant-pathogen interactions, where EVs play essential roles by transferring broad spectrum of biological molecules across different kingdoms (Zhang et al., 2024).

2.2. Extracellular Vesicles

Extracellular vesicles are spherical, bilayer nanovesicles, released by cells from prokaryotes, eukaryotes and archaea alike, that are primarily involved in intracellular communication (Ullah et al., 2023). Extracellular vesicles (EVs) play a crucial role in the secretory pathways of fungi, facilitating the transport of proteins, carbohydrates, lipids, different classes of RNA, nucleic acids, toxins and other macromolecules to the extracellular environment (Clos-Sanslavadore et al., 2022). Fungal pathogens, especially phytopathogens, exploit these secretory vesicles to transport a variety of effectors and secondary metabolites that aid in host manipulation and contribute to their pathogenicity (Rutter et al., 2021). On the other hand, plants secrete RNA-loaded EVs targeting pathogenicity genes, thus enhancing defence upon pathogen infection (Cheng et al., 2023). These host-pathogen inter-actions and the critical role that EVs play in them make the study of the EVs transcriptome highly coveted.

3. Purpose, Scope and Applicability

Extracellular vesicles are recently being studied as one of the key players in host-pathogen interactions, where they mediate the exchange of different biological molecules, serving as virulence/defence factors. Small RNAs are one of the classes of RNA molecules, present in the extracellular transcriptome in plant-pathogen interactions, that are transported via EVs to promote virulence in host plants upon infection with fungal pathogens. NGS RNA-seq of purified RNAs from isolated EVs and transcriptomic studies are crucial for identification of key aspects in plant-pathogen interaction. Understanding of the extracellular transcriptome is the foundation to development of novel disease management strategies, such as Spray-induced gene silencing, utilizing double-stranded RNAs. The presented SOP de-fines the key steps in NGS RNA-seq protocol, from EVs samples to bioinformatic analysis.

4. Health and Safety Warning

The SOP involves certain risks, particularly due to the use of hazardous chemicals such as phenol, chloroform, and guanidine salts for nucleic acid isolation, which are toxic, volatile, and pose risks of inhalation, skin absorption, and environmental contamination. Phenol is corrosive and causes severe chemical burns on contact. Chloroform is carcinogenic and toxic if inhaled, causes skin irritation and eye irritation.

5. Cautions

To minimize exposure, all work involving these reagents must be conducted in a properly functioning fume hood with adequate ventilation, and personnel should wear appropriate personal protective equipment, including lab coats, gloves, and safety goggles. The disposal of used chemicals must be performed according to regulative for hazardous chemicals. Additionally, the use of ultracentrifuge requires strict adherence to safety guidelines to prevent mechanical hazards, sample imbalance, and rotor failure. Users must ensure that tubes are properly balanced, rotors are securely fastened, and speed limits specified by the manufacturer are not exceeded. The centrifuge lid must remain closed during operation, and samples should be handled with caution to avoid aerosol formation and contamination. Regular maintenance and inspection of the equipment are essential to ensure safe and efficient operation.

6. Personnel Qualifications / Responsibilities

The sequencing infrastructure (Ion S5 sequencer, Ion Chef) should be used by trained personnel to avoid to prevent improper use and device deterioration. The staff who uses SOP should be trained in handling hazardous chemicals supplied with the commercial kits for nucleic acids isolation.

7. Materials, Equipment and Supplies

Materials: Monarch® Total RNA Miniprep Kit (New England Biolabs), mirVana™ miRNA Isolation Kit (Invitrogen), Ion Total RNA-Seq Kit v2 (IonTorrent, ThermoFisher), Ion 540™ Kit – Chef (IonTorrent, ThermoFisher), Agilent Small RNA kit

(Agilent), Agilent RNA 6000 Nano Kit (Agilent), Agilent RNA 6000 Pico Kit (Agilent), Agilent High Sensitivity DNA Kit (Agilent)

Devices: Agilent 2100 Bioanalyzer (Agilent), Ion Chef™ Instrument (IonTorrent, ThermoFisher), Ion GeneStudio™ S5 Prime System (IonTorrent, ThermoFisher), Qubit 4 Fluorometer (ThermoFisher)

Other equipment: microcentrifuge tubes (0.2 ml, 0.5 ml, 1.5 ml, 2 ml), micropipettes, heat block, ethanol, nuclease-free water.

8. Computer Hardware & Software

The S5 Prime system is supplied with server Dell PowerEdge T640 (2x Intel Xeon Gold 6140 Processor, 251 Gb of RAM and 25 TB of hard drive space) with Ubuntu 18.04.6 LTS operating system. Processing of the sequencing run, reporting and data availability is performed by Torent Suite software (version 5.18.1) accessible through the IP address of the computer. for automated sequencing data analysis. Torrent Suite is intuitive, browser-based interface which makes it fast and easy to plan, monitor, and view sequencing run results. Although adapters and quality trimming is performed by Torrent Suite software additional quality check can be performed by FastQC software (current version 0.12.1) (Andrews, 2010) and additional adapter and quality trimming performed by CUTADAPT (current version 5.0) (Martin, 2011).

9. Ion Torrent Semi-conductive Technology

The Ion Torrent sequencing is a next-generation sequencing technology that detects nucleotide incorporation by measuring changes in pH rather than using fluorescence-based methods. The sequencing process relies on semiconductor technology and proton detection. When a DNA polymerase incorporates a complementary nucleotide during sequencing, a hydrogen ion (H^+) is released as a byproduct. This ion release causes a slight pH change, which is detected by a semiconductor sensor. The intensity of the signal corresponds to the number of nucleotides added in a single cycle. This real-time, label-free sequencing approach enables rapid and cost-effective DNA sequencing with high throughput (ThermoFisher; https://www.thermofisher.com/si/en/home/life-science/sequencing/next-generation-sequencing/ion-torrent-next-generation-sequencing-technology.html).

10. Step by Step Procedure

10.1. Total RNA isolation from EVs

The Monarch Total RNA Miniprep kit is used for total RNA purification from sample of isolated EVs, according to manufacturers protocol. Briefly, the isolation of RNA is performed in two steps. Firstly, the EVs must be pelleted by centrifugation and resuspended in Lysis buffer. Next, the lysate homogenate must be loaded to the gDNA Removal Column and the flow-trough stored, as it contains RNA. The absolute ethanol is added and the suspension is loaded to the RNA Pruficiation Column. Flow-trough is discarged and the column, containing total RNA, is washed with RNA Wash buffer.

10.2. Small RNA Isolation from EVs

Enrichment of small RNAs (< 200 nt) is achieved by using the mirVana miRNA Isolation Kit, folowing manufacturers protocol. Briefly, sample of EVs is mixed with Lysis/Binding Solution to disrupt the EVs. Next, the organic extraction with Phenol:Chloroform is used to remove organic compounds. The aqueus phase is mixed with absolute ethanol and filtered trough the Filter Cartridge. Small RNAs are present in the flow-through filtrate. The filtrate is mixed with absolute ethanol and loaded to the second Filter Cartridge. After centrifugation, small RNAs are captured in the filter. The filter is washed with miRNA Wash Solution and small RNAs are eluted with nuclease-free water.

10.3. Library Preparation and Sequencing

The Ion Total RNA-Seq Kit v2 is used for the total/small RNA libraries preparation, following the manufacturer's instructions. The protocol must be performed by

authorized and trained personnel, and all essential safety guidelines must be followed as it is outlined in the user guide. The starting material for the library preparation is isolated total or small RNA. After RNA purification, the quality of the RNA must be evaluated with the Agilent 2100 Bioanalyzer to ensure that it contains the suitable fractions of small or total RNAs. If small RNAs will be sequenced, the quality and quantity of the miRNA needs to be assessed again with the Agilent 2100 Bioanalyzer. Next, appropriate input amount of RNA is to be determined for the construction of the RNA library.

Briefly, the library construction consists of hybridization and ligation, reverse transcription, purification and size selection, followed by amplification. Following the amplification, the DNA needs to be purified and size-selected by magnetic-based purification. The quality (yield and size distribution of the purified DNA) are assesed. If preparing multiple libraries, each barcoded library needst to be diluted to an equimolar concentration before mixing equal volumes to prepare a combined library.

Next, the Ion Chef Instrument is used for the amplification of the sequencing library and the sequencing. The sequencing plan and workflow needs to be created in the coresponding sequencing software.

After the planned run is created, the Ion Chef run for the preparation of the sequencing chip. The Ion 540™ Kit is used for library enrichment, chip loading and sequencing with the Ion GeneStudio S5 System, following the manufactur-er's instructions. Before starting the Ion Chef™ run, it is important to do one final review to ensure that all the settings are correct. The run needs to be monitored closely and all issues need to be addressed as they arise. After completion of the run, the data is retrieved on the correspodnig software.

10.4. Data Quality Check and Raw Data Processing

The deafult output format of the S5 prime system is UBAM format (unaligned BAM), which can be converted to standard FASTQ format inside Torrent Suite software with FileExporter plugin. TorrentSuite software reports basic QC statistics of sequencing run and barcoded samples like number of sequences, number of total bases, number of bases with quality score above 20. Standard QC software is FastQC (CITAT), which reports several standard quality related reports like basic statistics, per base sequence quality, per sequence quality scores, per base sequence content, per sequence GC content, per base N content, sequence length distribution, sequence duplication levels, overrepresented sequences and adapter content. Based o the report, decision can be made either your data has any problems of which you should be aware before doing any further analysis. The use of the software is from the bash shell like:

fastqc -t number_of_threads name_of_the_sequencing_file.fastq

The results is HTML file, which is easily viewed in any internet browser. In case additional adapter trimmin or quality trimming is required, tools like CUTADAPT can be used. Refer to manual for use, available at: https://cutadapt.readthedocs.io/en/stable/#.

11. Data Management

All sequencing data is stored in UBAM and FASTQ format on local server. The raw and processed data are archived in appropriate repositories (e.g. Sequence Read Archive - SRA).

12. Waste Management

The wase in this SOP is produced from nucleic acids isolation and sequencing protocol using commercial kits. The waste should be disposed according to manufacturers instructions and according to applicable laws. The gloves used by the staff should be disposed according to good research laboratory practices.

13. Related protocols or SOPs

The SOP specifies the use of commercially available protocols for efficient RNA isolation and sequencing, in accordance with best practices to ensure quality and accuracy of results.

14. Quality Control and Quality Assurance

14.1. Instrument Calibration

All instruments, used in the SOP, are regularly maintained and calibrated by trained service technicians and personnel.

14.2. Critical Steps and Checkpoints

The quality of RNA is essential for obtaining reliable sequencing data, as the success of the entire library preparation process hinges on the quality and integrity of the starting RNA material. Therfore, the quality of RNA must be determined before library preparation procedure. The isolated RNA must be stored at – 80 °C to prevent degradation.

Multiplexing of barcoded libryries of equimolar concentration is crucial for maximizing throughput and reducing costs, by allowing multiple samples to be sequenced in a single run, while maintaining quality and consistency. It is critical to ensure that all generated data can be attributed back to corresponding material via barcodes.

A critical step of sequencing procedure is creating the planned run, as it forms the foundation for the sequencing workflow. Selecting the correct sequencing application is vital, as it determines the entire setup based on goals, sample types and desired outputs. Accurately documenting and inputting the run details is essential for the effective analysis of the data. Proper documentation of barcodes for samples ensures that all data can be accurately linked back to corresponding libraries.

15. Data on Procedures and Samples

Data on procedures and samples are given in **Table 1**.

Table 1. Data on procedures and samples

	RNA isolation	Sequencing
Description of the sample	Purified EVs, stored at – 80 °C	Purified total/small RNA, stored at – 80 °C
Aliquots needed	1	1
Total volume of the sample	150 – 300 μL	10 – 100 μL isolated RNA
Estimated content needed	10^{10}/ml EVs	100 – 500 ng RNA, RIN 7
Time required to obtain results	1 day	1 week
Manpower	Highly skilled researcher	Highly skilled researcher
Estimated cost per sample without manpower	17 EUR	2500 EUR
Contact person	Jernej Jakše, jernej.jakse@bf.uni-lj.si	

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