Streamlining Nanopore Sequencing Protocols for Untargeted RNA Virus Detection

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Abstract

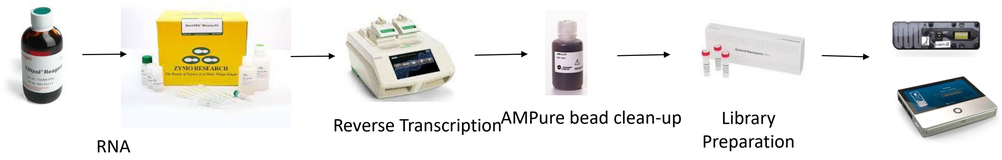
Sequencing technology has improved in leaps and bounds in the recent past; however, RNA can still prove difficult and time consuming to sequence. Oftentimes, RNA extraction requires hazardous reagents, a multitude of consumables, and a lengthy cDNA conversion step. To make RNA virus sequencing more accessible, and potentially field-deployable, these obstacles need to be overcome. We evaluated numerous workflows with various kits, reagents, and consumables to find an optimal solution. In testing, we found that RNA-binding magnetic bead workflows could be employed for RNA extraction, with modifications to replace the more hazardous reagents from the waste-stream, reduce incubation times, and number of steps. Using magnetic-bead based extraction also eliminates the need for a microcentrifuge. The RNA yield and quality were sufficient for reverse transcription and final preparation of the cDNA with the Rapid Sequencing Kit. Total preparation time was under 90 minutes and it was possible to generate up to 913,440 total reads, 810,457 passing reads, and a Q Score of 12 after 20h sequencing with high accuracy base-calling. Centrifuge-or Minimap2-based algorithms were used to map reads and identify the organism. This decrease in time, removal of hazardous reagents, and use of Oxford Nanopore Technology sequencing allows for a more fieldable workflow to identify RNA viruses.

Introduction

- There are numerous extraction and purification methods on the market for RNA
- RNA extraction and purification methods are often highly involved, involve hazardous reagents, necessitate a skilled worker, and require numerous hours
- All of these obstacles reduce the ease with which we can move RNA methodology into the field

Methods

- Our initial extraction and purification approach used for RNA is shown below, utilizing Trizol and a Zymo Direct-zol spin column kit



- Focus on replacing commercial, spin column based-approach with DNA-binding magnetic beads
- Approach 1: RNA extraction and purification utilizing lysis buffers from commercial kits and AMPure XP beads, tested with MS2 bacteriophage
  - Tested with 4 different lysis buffers:
    1. Qiagen RNeasy - RLT Buffer
    2. Applied Biosystems PicoPure RNA Isolation – Extraction Buffer
    3. Invitrogen PureLink - Lysis Buffer
    4. GE Illustra RNAspin - Lysis Solution
  - Modifications tested to decrease total preparation time
- Approach 2: Zymo DirectZol Magbead Kit
  - Incorporated modifications to decrease preparation time
  - Replaced hazardous TRI reagent used with this kit

Results

Table 1: Representative Analysis using Centrifuge Classification Tool

Total Reads	Organism	Reads	Unique Reads	% Mapped of Total Reads	Conc (ng/uL)
810457	Enterobacteria phage MS2	755012	755012	93.158798	0.83
	Enterobacteriaceae	12068	12068	1.4890364	
	Escherichia coli KO11FL	8450	8450	1.0426216	
	root	6301	6301	0.7774626	
	Escherichia coli BW2952	5318	5318	0.656173	
	Escherichia coli	4585	4585	0.5657302	
	Shigella boydii	3821	3821	0.4714624	
	Shigella flexneri	475	475	0.0586089	
	MT121215.1 Severe acute respiratory syndrome coronavirus 2 isolate SARS-CoV-2/SH01/human/2020/CHN complete genome	295	295	0.0363992	
	Escherichia coli SE11	252	252	0.0310936	

Table 2: Representative Analysis using Minimap2 Alignment Tool with RefSeq database

Organism	Genome Size	Reads Mapped	Unique Reads	% Unique Reads
phage MS2 genome	3,569	763,962	763,962	100.0
Plantactinospora sp. BB1 chromosome complete genome	8,386,853	305	114	37.4
Leishmania major strain Friedlin complete genome chromosome 23	772,565	229	199	86.9
Plantactinospora sp. BC1 chromosome complete genome	8,333,965	111	10	9.0
Ostreococcus lucimarinus CCE9901 chromosome 1 complete sequence	1,152,508	77	75	97.4
Leishmania major strain Friedlin complete genome chromosome 35	2,090,474	23	19	82.6
Leishmania major strain Friedlin complete genome chromosome 12	675,346	19	14	73.7
Leishmania major strain Friedlin complete genome chromosome 6	516,869	17	1	5.9
Escherichia coli strain RM13752 chromosome complete genome	5,264,517	16	7	43.8

Results Continued

Table 3: Approach 1 - RNA Extraction and Purification utilizing Commercial Lysis Buffers + AMPure XP Beads

	RLT	Illustra	PicoPure	PureLink
Total Reads	979.04k	1.21M	67.92k	754.05k
Passed Reads	854,472	1,071,017	42,601	654,236
N50	598	687	267	677
Q Score	11-10	12-11	Jumpy 6	11-10
MS2 Reads - Centrifuge	778196	990356	3476	607118
MS2 Reads - Minimap2/RefSeq	801471	1015989	3505	626998

- Total time for extraction/purification, reverse transcription, Ampure bead cleanup, and sequencing prep took approximately 2 hours and 15 minutes

Table 4: Approach 2 – Zymo DirectZol/Magbead Kit + RLT buffer

	Test 1	Test 2	Test 3	Test 4
Eliminate Air Dry Step	-	+	-	+
Reduce Elution Volume	-	-	+	+
RLT buffer	-	-	-	+
Total Reads	622.67k	916.46k	689.74k	913.44k
Passed Reads	558,761	791,366	593,800	810,457
N50	386b	365b	474b	559b
Q Score	11	11-8	11-10	12
MS2 Reads - Centrifuge	506,442	691,575	536,606	755,012
MS2 Reads - Minimap2/RefSeq	518,352	724,469	546,893	763,962

- Total time for extraction/purification, reverse transcription, AMPure XP bead cleanup, and sequencing prep took approximately 1 hour and 30 minutes

Conclusion

- The best approaches based on output and metrics use of RLT or Illustra lysis buffers with AmpureXP bead purification, or RLT with the Zymo Magbead Kit
- Using RLT with the modified Zymo Magbead Kit significantly reduces sample preparation time; offers both time and ease of use improvements

Future Work

- Further optimization to decrease workflow time
- Additional RNA extraction and purification kits will be tested
- Will test if cDNA clean-up steps can be eliminated to reduce more time

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