

Streamlining Nanopore Sequencing Protocols for Untargeted RNA Virus Identification in the Field

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ABSTRACT

RNA sequencing in the field remains difficult and time-consuming process despite recent leaps and bounds in portable sequencing technology. RNA extraction requires the use of hazardous reagents, a large number of consumables, and lengthy protocols for cDNA conversion. To improve accessibility of RNA virus sequencing, and potentially allow for field-ready capabilities, we need to circumvent these roadblocks. Previous work in our laboratory demonstrated an alternative protocol that decreased total preparation time to under 90 minutes and utilized safer reagents. This new approach utilizes a magnetic bead-based process for RNA extraction, with modifications to replace more hazardous reagents, and reduce incubation times and steps. This adjusted protocol also eliminates the need for a microcentrifuge that is required for spin column-based RNA extraction kits. To make RNA sequencing more field-friendly, even to inexperienced users, we modified the protocol to utilize syringes and remove the need for pipettors. With the use of the previously streamlined protocol, as well as the introduction of a syringe-based process, a more accessible option for untargeted RNA sequencing may be possible.



Portable nanopore technologies have revolutionized the ability to sequence organisms in the field, however, the ability to extract RNA and conduct library preparation in the field has been a bottleneck to rapid detection.

INTRODUCTION

Previously established extraction and purification methods for RNA that reduced preparation time to under 90 minutes and utilized safer reagents. In order to proceed with testing in the field an appropriate portable thermocycler is needed for reverse transcription, as well as ways to handle liquids without the need for pipettes. All of these obstacles reduce the ease with which we can move RNA methodology into the field with non laboratory trained users

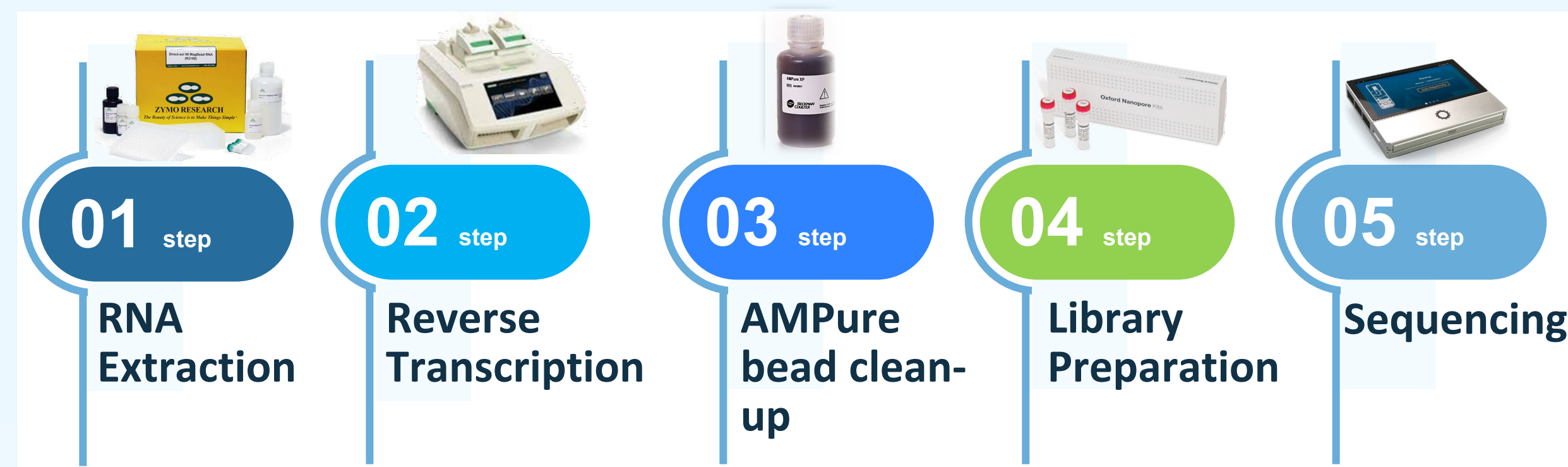


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METHODS

Our previously established extraction and purification approach used for RNA is shown below (substituting RLT buffer for Trizol and a Zymo Direct-zol Magbead kit):

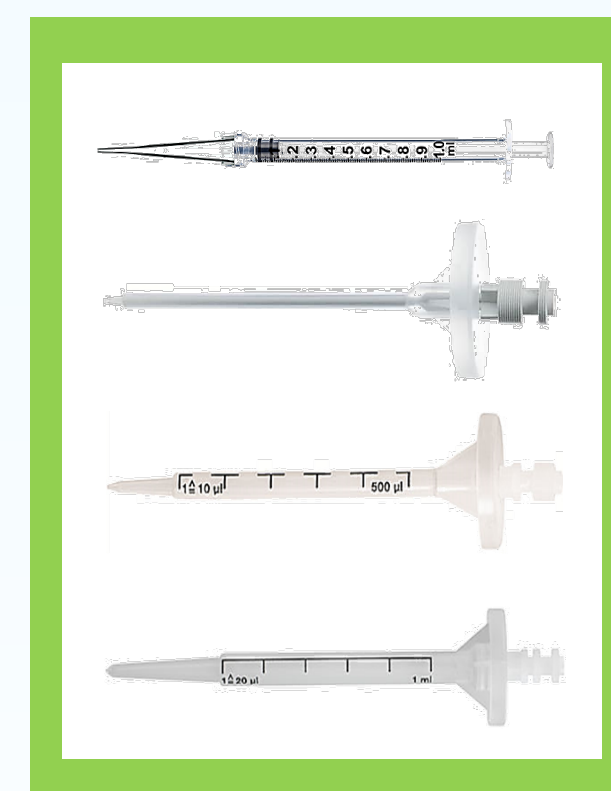


OBJECTIVE 1: Alternatives to stationary thermocycler in a non laboratory environment (3 portable thermocyclers tested).

	↓ Temp	Power
MiniOne	4°C	Wall
MiniPCR	25°C	Wall
Watson	35°C	USB

OBJECTIVE 2:

Improve ability to field with non laboratory trained users and improve output



1. Tested doubling the volume for reverse transcription, doubling both the RNA input as well as the associated LunaScript® RT SuperMix
2. Doubled the volume of *Emesvirus zinderi* (MS2) to the RNA extraction.
3. Tested syringe/tips made in house
4. Tested repeating pipette tips as sterile alternative.

	Test 1 a	Test 2 a	Test 3 a	Test 4 b	Test 5 b
Double Initial Input			x	x	x
Double RT		x	x	x	x
In house syringe				x	
Sterile syringe					x

RESULTS

Table 1: Representative Analysis using Centrifuge Classification Tool

Total Reads	Organism	Reads	Unique Reads	% Mapped of Total Reads	Conc (ng/uL)
331554	Enterobacteria phage MS2	288942	288942	87.147795	NA
	Enterobacteriaceae	11292	11292	3.4057801	
	Escherichia coli KO11FL	6164	6164	1.859124	
	root	5722	5722	1.7258124	
	Escherichia coli BW2952	3222	3222	0.9717874	
	Escherichia coli	3180	3180	0.9591198	
	Shigella boydii	1754	1754	0.5290239	
	Shigella flexneri	245	245	0.0738944	
	Escherichia coli SE11	223	223	0.067259	
	Salmonella enterica	187	187	0.0564011	

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

MiniMap2 / Refseq Database Results				
Organism	Genome Size	Reads Mapped	Unique Reads	% Unique Reads
phage MS2 genome	3,649	299,519	299,519	100.0
Plantactinospira sp. BB1 chromosome, complete genome	8,386,933	338	133	39.3
Leishmania major strain Friedlin complete genome, chromosome 23	772,645	198	197	99.5
Plantactinospira sp. BC1 chromosome, complete genome	8,334,045	102	6	5.9
Leishmania major strain Friedlin complete genome, chromosome 12	675,426	42	42	100.0
Ostreococcus lucimarinus CCE9901 chromosome 1, complete sequence	1,152,588	37	37	100.0
Leishmania major strain Friedlin complete genome, chromosome 35	2,090,554	26	26	100.0
Cutibacterium acnes strain FDAARGOS_503 chromosome, complete genome	2,494,619	24	0	0.0
Cutibacterium acnes strain PA_21_1_L1 chromosome, complete genome	2,560,434	22	1	4.5

Table 3: RNA Extraction & Purification with various volume changes and syringes

	Test 1 a	Test 2 a	Test 3 a	Test 4 b	Test 5 b
Total Reads		162.47k		105.34k	373.23k
Passed Reads	35054	48321	49887	87337	331554
N50		231b		434b	376b
Q Score		10		11-10	11-10
MS2 Reads - Centrifuge	3565	3051	5825	75827	288942
MS2 Reads - Minimap2/RefSeq	3252	2733	5128	78377	299519

a – RBK004 kit; b – RAD004 kit

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

CONCLUSION

Increasing the initial volume into the preparation as well as increasing the volume for reverse transcription allowed for easier manipulation using syringes as well as increasing the number of sequencing reads seen mapping to MS2



FUTURE WORK

Further test with updated changes in the hands of non laboratory trained users to obtain feedback on necessary changes to improve workflow and usefulness

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