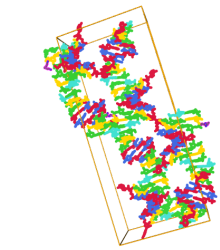
Nov 16, 2019 Version 1

Enrichment of a specific polyadenylated RNA for nanopore direct RNA sequencing (RNA SPACE) V.1



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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

This RNA Sequence Picking After Cutting Enzymatically (RNA SPACE) protocol is intended to enrich for a specific polyadenylated RNA, to be performed before the Oxford Nanopore Technologies (ONT) **direct RNA sequencing** protocol. This could be used to promote sequencing of a low abundance polyadenylated transcript in a mixture (e.g. polyadenylated viral RNA in a human clinical sample), or to elucidate the unknown 5' of a transcript (i.e. replacement for 5' RACE sequencing). This methods should become increasingly valuable as lower throughput nanopore devices such as the Flongle, Plongle and SmidgION get official support for direct RNA sequencing.

The RNA SPACE protocol takes advantage of the unusual property of six DNA restriction enzymes (**AvaII**, **AvrII**, **BanI**, **HaeIII**, **HinfI** and **Taq1**) to cut the RNA strand in RNA:DNA duplexes.

CITATION

Murray IA, Stickel SK, Roberts RJ (2010). Sequence-specific cleavage of RNA by Type II restriction enzymes.. Nucleic acids research.

LINK

https://doi.org/10.1093/nar/gkq702

This introduces a 3' end that is uniquely targetable using the Oxford Nanopore Technologies protocol's sequence-specific "RTA Oligo B" probe option, rather than the standard poly(dT) overhang version of "RTA Oligo B" which pulls down all polyadenylated transcripts.

The RNA SPACE software designs two oligonucleotide (oligo) probes for this protocol, for any given gene:

- the "RE" oligo to generate a RNA:DNA duplex in the known transcripts of interest
- the sequence-specific RTA Oligo B

Materials

MATERIALS

- CutSmart Buffer - 5.0 ml **New England Biolabs Catalog #**B7204S
- BanI - 5,000 units **New England Biolabs Catalog #**R0118S
- DNAse/RNAse free distilled water **Thermo Fisher Scientific Catalog #**10977023
- 1.5ml Eppendorf DNA LoBind tubes
- RNeasy MinElute Cleanup Kit **Qiagen Catalog #**74204
- DNA Oligo, Next-day Service fee (up to 48 oligos per order) **Thermo Fisher Catalog #**A15603
- Human Kidney Total RNA **Thermo Fisher Catalog #**AM7976
- Oxford Nanopore Direct RNA sequencing (SQK-RNA002) **Oxford Nanopore Technologies Catalog #**SQK-RNA002

Human Kidney Total RNA is a placeholder for 1ug (or more) of your total RNA sample.
BanI is a placeholder for the restriction enzyme selected in Step 1.
DNA Oligo is a placeholder for the 3 oligos designed in Step 1.

For a list of materials required for the downstream nanopore sequencing protocol, please see its checklist protocol:

https://community.nanoporetech.com/protocols/ss-direct-rna-sequencing-sqk-rna002/checklist.pdf?devices=minion

Before start

Have a few millilitres of buffered saline solution in stock: 10 mM Tris-HCl pH 7.5, 50 mM NaCl. This should be prepared with nuclease free water.

Oligonucleotide design

1 Have the three oligos synthesized (RE oligo, RTA Oligos A & B), by designing your own using the open source software and your reference data.

Software	
RNA SPACE	NAME
Paul Gordon	DEVELOPER

Targeted RNA:DNA duplex formation

2 In a 1.5 ml tube, add up to 2µg of your total RNA (in 10µl or less), and 1µl of 1µM RE oligo.

2m

RNA secondary structure reduction

3 Incubate 10 minutes at 80C.

10m

Enzymatic cleavage of target RNA

4 In the same tube, perform 3h or overnight restriction digest according to the NEB protocol for your enzyme, e.g. http://nebcloner.neb.com/#!/protocol/re/single/Taq1 (65C for Taq1, 37C for the other 5 restriction enzymes)

3h

The longer incubation is suggested due to the lower efficiency of RNA:DNA restriction compared to DNA:DNA.

Step 5 can be performed while you wait for the incubation to finish.

Sequencing adapter (oligo duplex) formation

5 **If this is the first time using these custom oligos:** In a new tube, anneal standard RTA oligo A and custom RTA oligo B 1:1 at 1.4 µM in buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl) by heating to 95º C for 2 min and letting them cool down slowly (0.1º C/sec). 2m

Otherwise: fetch the custom oligo duplex left over from a previous run.

RNA Purification

6 After the 3 hour incubation has finished, perform the RNeasy MinElute Cleanup Kit protocol (spin column) on the contents of the NEB enzyme reaction tube. 15m

Sequencing adapter competitive hybridization

7 Add 1ul of the RTA duplex to the tube of eluted RNA. Store any remainder of the RTA duplex, for future uses. 2m

8 Heat the incubation tube to 80º C for 2 min and let it cool down slowly (0.1º C/sec). 20m

Direct RNA Sequencing

9 Perform the Oxford Nanopore Technologies Direct RNA sequencing protocol (RNA-002 kit) on the RNA isolated by MinElute. 2h

https://community.nanoporetech.com/protocols/ss-direct-rna-sequencing-sqk-rna002/checklist.pdf?devices=minion

Citations

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