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Discovery of RNA and DNA viruses using next-generation sequencing: Targeted enrichment

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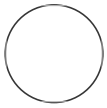
CVR Genomics



Katherine Smollett

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Protocol status: Working
We use this protocol and it's working

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ABSTRACT

Next-generation sequencing is a powerful tool for viral genomics. Viruses often constitute a very small proportion of any given sample meaning that methods that enable detection of viral nucleic acids are frequently needed for detection and characterisation. Improvement of sensitivity can be achieved by depletion of unwanted nucleic acid during sample pre-treatment or by enrichment such as PCR amplification with virus specific primers, or probe-based targeted enrichment. However, some methods for specific enrichment rely on prior knowledge of the viruses. The development of probe-capture panels targeting multiple viruses have enabled simultaneous sequencing of multiple viruses. Here we describe a highly sensitive and semi-agnostic sequencing method to identify unknown viruses using a pan-viral probe capture design (see Figure 1).

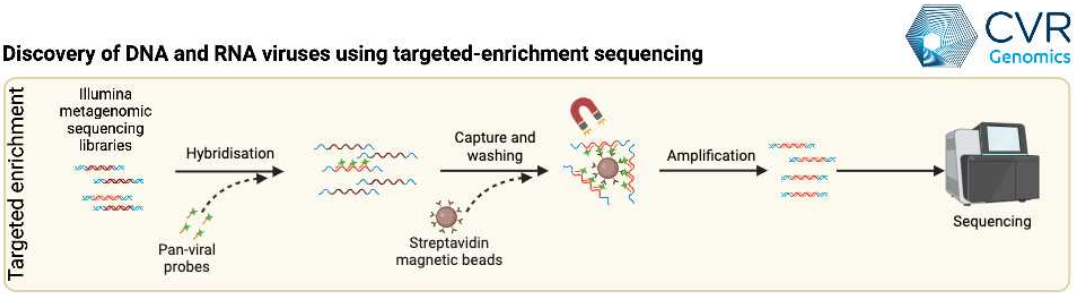

Figure 1: Discovery of DNA and RNA viruses using targeted enrichment sequencing. Image prepared using BioRender.com.

Following simultaneous extraction of RNA and DNA, samples are first split into two and subjected to non-specific enrichment treatments that improve chances of detecting RNA or DNA viruses, respectively and generate untargeted Illumina sequencing libraries as described in the accompanying protocol Discovery of RNA and DNA viruses using next-generation sequencing: Metagenomics. The same sequencing libraries can be subjected to targeted enrichment using a pan-viral probe set to achieve higher sensitivity.

We applied this approach to an outbreak of acute hepatitis of unknown aetiology in children, enabling the identification of adeno-associated virus 2 (AAV2) in all patients but not in samples from controls. This method also led to the identification of adenovirus and human herpesviruses.

This protocol describes how to perform targeted enrichment on metagenomic Illumina sequencing libraries. We enrich for unknown viruses using VirCapSeq-VERT probes, a panel of ~2 million probes that cover the genomes of members of the 207 viral taxa known to infect vertebrates.

PROTOCOL integer ID:
72942

Keywords: Viral genomics, Next-generation sequencing, Viral discovery, Illumina, DNA, RNA, Targeted-enricment

MATERIALS

Reagents:

- NG SeqCap EZ Accessory Kit V2 **Roche Catalog #7145594001**
- SeqCap EZ Hybridization and Wash Kit **Roche Catalog #5634253001**
- SEQCAP PURE CAPTURE BEAD KIT **Roche Catalog #6977952001**
- Salmon Sperm DNA Carrier **Thermofisher Catalog #15632011**
- xGen Universal Blockers TS mix **IDT Catalog #1075474**

Additional reagents required:

VirCapSeq-VERT probe pool
Absolute ethanol
Nuclease-free water
10 mM Tris pH8

BEFORE START INSTRUCTIONS

This protocol starts with DNA and RNA metagenomic Illumina sequencing libraries prepared as described in protocol Discovery of RNA and DNA viruses using next-generation sequencing: Metagenomics.

Hybridisation

3d 1h 46m 10s

1 Prepare enrichment pools from the pre-prepared Illumina metagenomic sequencing libraries. Each pool should contain 8-16 libraries equal ng of each and a total of 1 µg DNA in a 1.5 mL DNA LoBind tube.

Note

When multiplexing for targeted enrichment samples with high viral load may take over the pool. Therefore if the information is available prepare hybridisation reactions with similar viral load, or group samples into pools of similar viral load. Where no viral load information is available pool by molarity or mass.

2 Enrichment is performed with VirCapSeq-VERT probes and Roche SeqCap reagents.

- NG SeqCap EZ Accessory Kit V2 **Roche Catalog #7145594001**
- SeqCap EZ Hybridization and Wash Kit **Roche Catalog #5634253001**
- SEQCAP PURE CAPTURE BEAD KIT **Roche Catalog #6977952001**

Note

VirCapSeq-VERT is no longer commercially available but we will soon release another version of this protocol with an alternative probe set.

3 To each pool add the following blocking reagents:

	A	B
	Component	Volume (µl)
	COT DNA	5
	Salmon sperm DNA (1 mg/ml)	5
	xGen Universal blockers	2
	Total	12

NG SeqCap EZ Accessory Kit V2 **Roche Catalog #7145594001**

Salmon Sperm DNA Carrier **Thermofisher Catalog #15632011**

xGen Universal Blockers TS mix **IDT Catalog #1075474**

4 Concentrate the pool using Ampure XP.

Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

Note

Ensure Ampure XP beads are equilibrated to room temperature for 30 min and vortex well before use.

Note

Alternatively the pool can be concentrated using a speedy vac, for example if the volume is too high for Ampure clean up.

4.1 Add 2X total volume of the pool plus blocking reagent of AmpureXP.

4.2 Place on a magnetic rack until beads and solution have fully separated 00:05:00. 5m

4.3 Remove supernatant being careful not to disturb the beads.

4.4 Add 800 µL 80% Ethanol (freshly prepared) and incubate Room temperature for 00:01:00. 1m

4.5 Remove all traces of ethanol being careful not to disturb the beads.

4.6 Air-dry the beads for around 00:03:00 taking care not to over dry the beads. 3m

5 Prepare the hybridisation mix (for multiple samples prepare a master mix with 10% excess):

	A	B
	Component	Volume (µl)
	2X Hybridisation buffer	7.5
	Hybridisation component A	3
	Total	10.5

SeqCap EZ Hybridization and Wash Kit **Roche Catalog #5634253001**

6 Add 10.5 µL hybridisation mix directly to the bead-bound DNA samples, remove from magnet and mix thoroughly.

7 Incubate at Room temperature for 00:02:00 . 2m

8 Place on magnetic rack and elute the entire 10.5 µL DNA/hybridisation mix to a new 0.2 mL PCR tube tube containing 4.5 µL VirCap-VERT probe pool .

Note

It is important that all the volume is transferred, slight carry over of beads is unlikely to significantly impact results.

Note

Use single PCR tubes with caps (Applied Biosystems N8010540) as we have found these have the best lids for reducing evaporation.

9 Mix thoroughly by pipetting.

10 Incubate as follows on a PCR machine with lid set to 105 °C : 5m
95 °C for 00:05:00
cool to 47 °C

11 Quickly transfer to second PCR machine with lid set to 57 °C and incubate as follows: 3d
47 °C for 72:00:00

Note

It is important that the hybridisation reaction remains at 47 °C during the next steps so set the PCR machine to hold.

Capture and washing

12 Prepare the wash buffers per capture as follows:

	A	B	C	D	E
	Component	Tube label	Tube type	Reagent volume (µl)	Water volume (µl)
	10x stringent wash buffer	A	PCR	20	180
	10x stringent wash buffer	B	PCR	20	180
	10x wash buffer 1	C	PCR	10	90
	10x wash buffer 1	D	PCR	20	180
	10x wash buffer 2	E	PCR	20	180
	10x wash buffer 3	F	PCR	20	180
	2.5x bead wash buffer	G	1.5ml	200	300

SeqCap EZ Hybridization and Wash Kit **Roche Catalog #5634253001**

13 Transfer tubes **A** and **B** 200 µL Stringent wash buffer and tube **C** 100 µL wash buffer 1 to the PCR machine to equilabrate to 47 °C.

14 Prepare capture beads.

14.1 For each capture, place 100 µL capture beads in a 1.5 mL tube.

> **Note**
>
> Can prepare the beads for up to six captures in a single tube. Equilibrate the capture beads to room temperature for 30 min and vortex for at least 15 sec before use.

SEQCAP PURE CAPTURE BEAD KIT **Roche Catalog #6977952001**

14.2 Place tube on a magnet, remove liquid being careful not to disturb the beads.

14.3 Add 2x the initial volume of beads of bead wash buffer (tube **G**).

14.4 Remove from magnet, vortex for 00:00:10 then centrifuge briefly. 10s

14.5 Place tube on a magnet, remove liquid.

14.6 Repeat bead wash one more time (2 washes in total).

14.7 Re-suspend beads in 1x original volume of bead wash buffer (tube **G**) by vortexing.

15 Transfer 100 µL resuspended beads per capture to a fresh 0.2 mL PCR tube.

16 Place tube on a magnet, remove liquid and proceed immediately to next so that the beads do not dry out.

17 Immediately add the 15 µL probe hybridisation sample to the prepared capture beads. Mix by pipetting ten times.

18 Incubate in a PCR machine for 00:45:00 at 47 °C, with the heated lid set to 57 °C. 45m

Note

To improve binding efficiency it is recommended that you briefly mix the tubes by gentle flicking every 15 mins.

19 Add 100 µL wash buffer 1 pre-heated to 47 °C (tube **C**).

20 Mix by vortexing for 00:00:10. 10s

21 Place tube on a magnet, remove liquid.

Note

The sample has now gone from being highly concentrated but with a low proportion of viral fragments to very low concentration but high proportion of viral fragments. To prevent contamination it is recommended to move to a separate workstation at this step.

22 Add 200 µL stringent wash buffer pre-heated to 47 °C (tube **A**). Pipette 10X to mix.

23 Incubate at 47 °C for 00:05:00. 5m

24 Repeat stringent wash one more time (tube **B**, total 2 washes).

25 Transfer mixture to a fresh 1.5 mL DNA LoBind tube.

> Note
>
> The following steps require vigorous vortexing so transfer to 1.5ml tubes with more secure lids is highly recommended.

26 Place tube on a magnet, remove liquid.

27 Add 200 µL wash buffer 1 at Room temperature (tube **D**) and vortex for 00:02:00. 2m

28 Place tube on a magnet, remove liquid.

29 Add 200 µL wash buffer 2 at Room temperature (tube **E**) and vortex for 00:01:00. 1m

30 Place tube on a magnet, remove liquid.

31 Add 200 µL wash buffer 3 at Room temperature (tube **F**) and vortex for 00:00:30. 30s

32 Place tube on a magnet, remove liquid.

33 Remove from magnet, resuspend the beads in 20 µL Nuclease-free water and mix well by pipetting.

Note

Proceed directly to amplification leaving the capture beads in solution.

Amplification

3d 1h 46m 10s

34 Prepare the PCR mix (for multiple samples prepare a master mix with 10% excess):

	A	B
	Component	Volume (µl)
	2X KAPA HiFi ready mix	25
	Post-LM PCR oligos	5
	Total	30

NG SeqCap EZ Accessory Kit V2 **Roche Catalog #7145594001**

35 Set up two PCR tubes per capture and add 15 µL PCR mix to each tube.

36 Briefly vortex bead-bound captured DNA from step 33 and spin down.

37 Add 10 µL bead-bound captured DNA to each PCR reaction tube.

Note

This is an on bead PCR so include the beads in the PCR reaction and ensure the entire reaction is added to the PCR.

38 Incubate on a PCR machine as follows: 6m 5s

95 °C for 00:03:00

14 cycles of

98 °C for 00:00:20

65 °C for 00:00:15

72 °C for 00:00:30

Final cycle of

72 °C for 00:02:00

4 °C hold .

Note

Samples are now both highly concentrated and contain a higher proportion of viral fragments. If possible, the following steps should be done in a separate high viral load post-PCR room/area.

39 Briefly centrifuge PCR reactions and place on magnetic rack until the beads and solution have fully separated.

40 Transfer the 25 µL PCR reaction into fresh tubes, combining the 2 reactions from each pool to make a total volume of 50 µL per pool .

41 Pools can be cleaned up and undergo quality control as described for single libraries in protocol Library clean up and quality control for Illumina sequencing.

Pooling and sequencing

42 Using the bp size and ng/µl concentration calculate the nM concentration for each pool as follows:

Conc (nM) = \frac {sample (ng/µl)}{size (bp) * 660 (g/mol)}*1000000

43 If multiple pools are to be combined in the same sequencing run then pool by equal molarity with each pool weighted by the number of sequencing libraries contained within it as described in the protocol Library pooling and quality control for Illumina sequencing.

44 Sequence the pools on an Illumina sequencer following the manufacturer's guidelines.

Note

For targeted viral discovery sequencing we recommend sequencing at a depth of 20 million reads per sample (10 million for RNA viral discovery and 10 million for DNA viral discovery).