**TPRV Sequencing**

**Table-1: Overall Summary**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **NexSeq2k/iSeq100 Batch** | **TPRVSeq\_Batch** | **Type of Sequencing** | **Sample No.** | **Sequencing Date** |
|  | CHRF\_CSF\_2\_20May2019 |  | 1 | 31-08-2022 |
| NextSeq2000\_Run\_09 | CSF\_metagenomics\_Batch\_01 | Metagenomics | 2 | 30-05-2023 |
| NextSeq2000\_Run\_11 | TPRV\_CSF\_Batch\_01\_Metagenomics | Metagenomics | 15 | 17/4/2022 |
| NextSeq2000\_Run\_14 | TPRV\_CSF\_Batch\_02 | Amplicon-based sequencing | 8 | 31/8/2022 |
| NextSeq2000\_Run\_16 | TPRV\_CSF\_Batch\_03 | Amplicon-based sequencing | 27 | 22/11/2022 |
| NextSeq2000\_Run\_17 | TPRV\_CSF\_Batch\_04 | Amplicon-based sequencing | 7 | 24/12/2022 |
| NextSeq2000\_Run\_18\_R | TPRV\_CSF\_Batch\_05 | Amplicon-based sequencing | 12 | 02/02/2023 |

Table 1 entails the sequencing summary of all TPRV from 2009-2023

**Extraction Methods:**

|  |  |
| --- | --- |
| Batch | Kit/Methods Used for Extraction |
| CSF\_metagenomics\_Batch\_01 | Zymo Quick-DNA/RNA MiniPrep Plus Kit |
| TPRV\_CSF\_Batch\_01\_Metagenomics | Zymo Quick-DNA/RNA MiniPrep Plus Kit |
| TPRV\_CSF\_Batch\_02 | QIAamp DNA Mini Kit |
| TPRV\_CSF\_Batch\_03 | QIAamp DNA Mini Kit |
| TPRV\_CSF\_Batch\_04 | QIAamp DNA Mini Kit |
| TPRV\_CSF\_Batch\_05 | QIAamp DNA Mini Kit |

**Metagenomics Based Approach**

**Library Preparation Kit**:

1. NEBNext Ultra II FS DNA Library Prep Kit for Illumina
2. NEBNext® Ultra™ II RNA Library Prep Kit for Illumina

**Protocol**: DNA and RNA Library Preparation Protocol Shared by Biohub.

**DNA Library Preparation Steps:**

1. Fragmentation and End Prep of DNA
2. Adapter Ligation
3. SPRI Bead Clean Up and Size Selection (0.9x)
4. USER Digestion + Barcoding PCR
5. 1st SPRI Bead Clean Up and Size Selection (0.8x)
6. 2nd SPRI Bead Clean Up and Size Selection (0.75x)

**RNA Library Preparation Steps:**

1. RNA Fragmentation and Priming
2. First Strand Synthesis
3. Second Strand Synthesis
4. SPRI Bead Clean Up (1.8x)
5. End Prep of cDNA Library
6. Adapter Ligation
7. SPRI Bead Clean Up and Size Selection (0.9x)
8. USER Digestion + Barcoding PCR
9. 1st SPRI Bead Clean Up and Size Selection (0.75x)
10. 2nd SPRI Bead Clean Up and Size Selection (0.75x)

**Batch: CSF Metagenomics\_Batch 01**

**Sample Selection:**

15 CSF samples were selected for sequencing according to criteria: volume, final diagnosis and cell count.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Extraction Sl | AdmDate | Name | StudyID | TLC |
| 1 | 24-Aug-21 | salman | 12108058552 | 150 |
| 2 | 5-Nov-21 | mihan Khan | 12111012111 | 180 |
| 3 | 8-Mar-21 | Ratul | 12103021002 | 240 |
| 4 | 27-Jul-21 | jumman | 12107046352 | 430 |
| 5 | 23-Oct-21 | Sumaiya | 12110040582 | 650 |
| 6 | 6-Jun-21 | Zayra Fatema | 12106006082 | 850 |
| 7 | 22-Dec-21 | Abraham | 12112029271 | 3700 |
| 8 | 2-Dec-21 | faid | 12112003203 | 9200 |
| 9 | 7-Aug-21 | nahiyan | 12108015603 | 2 |
| 10 | 2-Sep-21 | Sayone saha | 12109004103 | 2 |
| 11 | 7-Oct-21 | Habib | 12110017531 | 210 |
| 12 | 20-Mar-21 | Esrat Jahan | 12103042671 | 1100 |
| 13 | 30-Nov-21 | pritom das | 12112001991 | 6500 |
| NC |  |  | Neg Control |  |
| PC |  |  | 12110033111 |  |
| PC |  |  | 12103007221 |  |

**Library Preparation:**

Some of the samples were found to have very low concentrations when measured with Qubit. So, we proceeded with following 5 samples, 2 Positive controls and 1 negative controls for library preparation:

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Extraction Sl.** | **AdmDate** | **CSFColDt** | **StudyID** | **TLC** | **Qubit Conc (ng/ul)** |
| 1 | 24-Aug-21 | 4-Sep-21 | 12108058552 | 150 | 0.746 |
| 4 | 27-Jul-21 | 28-Jul-21 | 12107046352 | 430 | 1.16 |
| 8 | 02-Dec-21 | 12-Dec-21 | 12112003203 | 9200 | 1.13 |
| 12 | 20-Mar-21 | 28-Mar-21 | 12103042671 | 1100 | 1.53 |
| 13 | 30-Nov-21 | 2-Dec-21 | 12112001991 | 6500 | 7.4 |
| NC |  |  | Neg Control |  | 0.204 |
| PC | 18-Oct-21 | 18-Oct-21 | 12110033111 |  | too high |
| PC | 04-Mar-21 | 7-Mar-21 | 12103007221 |  | 46 |

**Important Information of This Batch:**

1. RNA Metagenomics
2. Used 0.5rxn Volume
3. Used ERCC and FastSelect
4. Fragmentation Time: 6.5 minutes
5. Only a single 0.8x bead wash was done after barcoding PCR
6. After metagenomic analysis 2 samples were found to have PARV4.

**Batch: TPRV\_CSF\_Batch\_01\_Metagenomics**

**Sample Selection:**

Main selection criteria: <21 CT, <400 TLC, remaining volume >35 if value available

15 CSF samples were selected for this batch according to the criteria: volume, final diagnosis and cell count. 3 water controls and 1 one extracted control were also included. The table below entails the selected sample IDs.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Batch SI | StudyID | AdmDate | TLC | RemVolume | Hprv4Ct |
| 1 | 12204004541 |  | 60 | 830 | 20.57 |
| 2 | 12203026991 |  | 40 | 850 | 13.84 |
| 3 | 12203024741 |  | 20 | 2000 | 13.29 |
| 4 | 12201034831 | 17-Jan-22 | 350 | 500 | 17.5 |
| 5 | 12105004921 | 18-Apr-21 | 10 | 2000 | 19.62 |
| 6 | 12103015842 | 10-Mar-21 | 180 | 350 | 18.11 |
| 7 | 12101025462 | 23-Jan-21 | 20 | 800 | 17.58 |
| 8 | 12010030701 | 22-Oct-20 | 150 | 570 | 20.42 |
| 9 | 12010015311 | 24-Sep-20 | 60 | 800 | 19.28 |
| 10 | 12003023362 | 22-Mar-20 | 10 | 600 | 20.43 |
| 11 | 12003016171 | 23-Feb-20 | 300 | 2000 | 14.23 |
| 12 | 12002019032 | 16-Feb-20 | 10 | 750 | 18.52 |
| 13 | 12002002564 | 4-Feb-20 | 80 | 1200 | 14.62 |
| 14 | 12001029023 | 28-Jan-20 | 30 | 500 | 17.25 |
| 15 | 12001027713 | 27-Jan-20 | 20 | 400 | 19.72 |

**Library Preparation:**

This was a **DNA metagenomics** batch and we followed DNA library preparation protocol.

**Amplicon Based Approach**

**Multiplex PCR Primer Design**

The **Primalscheme** web server was used to design a primer pool for Tetraparvovirus genome. At first, we used 6 sequences from CHRF and 22 from NCBI (selected considering >=95% coverage & >=90% identity with Ref NC\_007018.1). As per the instruction, we aligned these sequences using ClustalO and uploaded them on the **Primalscheme**. Amplicon size was set to 400 bp. But due to lack of enough similarity we **Primalscheme** excluded 16 of the input sequences.

Currently we use a total of 34 primers (20 from pool 1 and 14 from pool 2) which were represented by following two reference sequences:

* CHRF\_BD0003\_CZ
* NC\_007018.1

The list of input sequences for the final primer scheme is given below:

|  |  |
| --- | --- |
| CHRF Sequences (Sequencing ID) | NCBI Sequences (Accession No.) |
| CHRF\_BD0003\_CZ | NC\_007018.1 |
| CSF\_0017\_CZ | KM390024.1 |
| CSF\_0004\_CZ | AY622943.1 |
| CSF\_0018\_CZ | EU874248.1 |
| CSF\_0019\_CZ | HQ593530.1 |
| CSF\_0023 |  |
| CSF\_0026\_CZ |  |

**Library Preparation Kit**: NEBNext Ultra II FS DNA Library Prep Kit for Illumina

**Protocol**: Modified Version of [ARTIC-NEB: SARS-CoV-2 Library Prep V.4](https://dx.doi.org/10.17504/protocols.io.br77m9rn)

**DNA Library Preparation Steps:**

1. Multiplex PCR
2. Pool and SPRI Bead Clean Up and Size Selection (1x)
3. Fragmentation and End Prep of DNA
4. Adapter Ligation
5. SPRI Bead Clean Up and Size Selection (0.9x)
6. USER Digestion + Barcoding PCR
7. 1st SPRI Bead Clean Up and Size Selection (0.8x)
8. 2nd SPRI Bead Clean Up and Size Selection (0.75x)

**Batch: TPRV\_CSF\_Batch\_02**

A total of 15 samples were selected for extraction according to criteria: volume, final diagnosis, and cell count.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sl** | **StudyID** | **Hprv4result** | **Hprv4Ct** | **Year** |
| 1 | 11905005291 | Positive | 23.52 | 2019 |
| 2 | 11905022241 | Positive | 21.42 | 2019 |
| 3 | 11906003412 | Positive | 20.25 | 2019 |
| 4 | 11804021521 | Positive | 22.47 | 2018 |
| 5 | 11806002031 | Positive | 19.2 | 2018 |
| 6 | 11806007711 | Positive | 18.4 | 2018 |
| 7 | 12004001351 | Positive | 19.91 | 2020 |
| 8 | 12009027051 | Positive | 20.34 | 2020 |
| 9 | 12102005521 | Positive | 20.9 | 2021 |
| 10 | 12103018103 | Positive | 21.63 | 2021 |
| 11 | 12104023562 | Positive | 13.08 | 2021 |
| 12 | 12202020101 | Positive | 23.33 | 2022 |
| 13 | 12203004511 | Positive | 24.3 | 2022 |
| 14 | 12204018741 | Positive | 19.96 | 2022 |

After multiplex PCR only 8 samples (sl. 2, 3, 4, 6, 8, 9, 11, 13) had proper concentrations for subsequent library preparation steps.

**Important Information of this Batch:**

1. After Multiplex PCR followed DNA Library Protocol.
2. Followed 0.5rxn volume
3. Fragmentation Time: 5 minutes
4. After Barcoding PCR, did 3 bead wash (0.8x + 0.75x + 0.75x)

**Batch: TPRV\_CSF\_Batch\_03**

**Sample Selection:**

A total of 27 samples (+ 3 EC) were extracted in this batch where CT value was less than 25.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **StudyID** | **year** | **Hprv4result** | **Hprv4Ct** | **CSF\_Tetra\_Seq.ID** |
| 12205021331 | 2022 | Positive | 23.53 | CSF\_0044\_TP4 |
| 12112010081 | 2021 | Positive | 21.83 | CSF\_0045\_TP4 |
| 12110050822 | 2021 | Positive | 18.28 | CSF\_0046\_TP4 |
| 12108055943 | 2021 | Positive | 18.67 | CSF\_0047\_TP4 |
| 12107030221 | 2021 | Positive | 23.5 | CSF\_0048\_TP4 |
| 12012026633 | 2020 | Positive | 24.22 | CSF\_0049\_TP4 |
| 12101006046 | 2021 | Positive | 23.82 | CSF\_0050\_TP4 |
| 12010032271 | 2020 | Positive | 22.69 | CSF\_0051\_TP4 |
| 12011016051 | 2020 | Positive | 23.56 | CSF\_0052\_TP4 |
| 12001031901 | 2020 | Positive | 22.77 | CSF\_0053\_TP4 |
| 12002009273 | 2020 | Positive | 18.83 | CSF\_0054\_TP4 |
| 11912026465 | 2019 | Positive | 19.15 | CSF\_0055\_TP4 |
| 11911024162 | 2019 | Positive | 20.38 | CSF\_0056\_TP4 |
| 11910033963 | 2019 | Positive | 20.77 | CSF\_0057\_TP4 |
| 11903010501 | 2019 | Positive | 13.61 | CSF\_0058\_TP4 |
| 11901019252 | 2019 | Positive | 22.33 | CSF\_0059\_TP4 |
| 11801008662 | 2018 | Positive | 22.94 | CSF\_0060\_TP4 |
| 11807028141 | 2018 | Positive | 20.61 | CSF\_0061\_TP4 |
| 11808006323 | 2018 | Positive | 22.9 | CSF\_0062\_TP4 |
| 11809018491 | 2018 | Positive | 15.21 | CSF\_0063\_TP4 |
| 11705006442 | 2017 | Positive | 17.77 | CSF\_0064\_TP4 |
| 11706015631 | 2017 | Positive | 21.89 | CSF\_0065\_TP4 |
| 12206014279 | 2022 | Positive | 23.85 | CSF\_0066\_TP4 |
| 11711014212 | 2017 | Positive | 23.46 | CSF\_0067\_TP4 |
| 11712018453 | 2017 | Positive | 21.57 | CSF\_0068\_TP4 |
| 11709007141 | 2017 | Positive | 19.12 | CSF\_0069\_TP4 |
| 1100209119 | 2017 | Positive | 19.32 | CSF\_0070\_TP4 |

As the concentration of all samples were suitable for library preparation, we considered all of them.

**Important Information of this Batch:**

1. After Multiplex PCR followed DNA Library Protocol.
2. Followed 0.5rxn volume
3. Fragmentation Time: 5 minutes
4. After Barcoding PCR, did 2 bead wash (0.8x + 0.75x)

**Batch: TPRV\_CSF\_Batch\_04**

**Sample Selection:**

Total 7 samples were selected for extraction (year: 2018-2022, CT < 25).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Extraction Sl | StudyID | CSFColDt | Hprv4result | Hprv4Ct |
| 1 | 11805018322 | 21-May-18 | Positive | 16.76 |
| 2 | 12202010981 | 6-Feb-22 | Positive | 15.86 |
| 3 | 12204021111 | 19-Apr-22 | Positive | 23.41 |
| 4 | 11901003831 | 5-Jan-19 | Positive | 18.65 |
| 5 | 11909031583 | 30-Sep-19 | Positive | 20.58 |
| 6 | 11908033342 | 24-Aug-19 | Positive | 14.74 |
| 7 | 11905005291 | 6-May-19 | Positive | 23.52 |

Then all samples were chosen for library preparation.

**Important Information of this Batch:**

1. After Multiplex PCR followed DNA Library Protocol.
2. Followed 0.5rxn volume
3. Fragmentation Time: 5 minutes
4. After Barcoding PCR, did 2 bead wash (0.8x + 0.75x)

**Batch: TPRV\_CSF\_Batch\_05**

A total of 12 samples were extracted in this batch where some samples were from CMOSH and some samples were from the year of 2009-2011.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen\_I** | **CSF\_Tetra\_Seq ID** | **CSFColDt** | **Hprv4** | **Remark** |
| 1 | 3010008785 | CSF\_0078\_TP4 | Nov-22 | 23.09 | CMOSH |
| 2 | 3010008924 | CSF\_0079\_TP4 | 16-Nov-22 | 23.38 | CMOSH |
| 3 | 3020004887 | CSF\_0080\_TP4 | 4-Apr-22 | 25.46 | CMOSH |
| 4 | 3010008978 | CSF\_0081\_TP4 | 16-Nov-22 | 28.14 | CMOSH |
| 5 | 10606 | CSF\_0082\_TP4 | 13-Apr-09 | 26.6 | OLD |
| 6 | 10815 | CSF\_0083\_TP4 | 17-Aug-09 | 23.87 | OLD |
| 7 | 11274 | CSF\_0084\_TP4 | 1-Aug-10 | 18.52 | OLD |
| 8 | 11531 | CSF\_0085\_TP4 | 17-Feb-11 | 28.56 | OLD |
| 9 | 10475 | CSF\_0086\_TP4 | 3-Jan-09 | 28.8 | OLD |
| 10 | 10771 | CSF\_0087\_TP4 | 29-Jul-09 | 26.42 | OLD |
| 11 | 10562 | CSF\_0088\_TP4 | 18-Mar-09 | 28.21 | OLD |
| 12 | 10836 | CSF\_0089\_TP4 | 6-Sep-09 | 20.84 | OLD |

Then all samples were selected for library preparation.

**Important Information of this Batch:**

1. After Multiplex PCR followed DNA Library Protocol.
2. Followed 0.5rxn volume
3. Fragmentation Time: 5 minutes
4. After Barcoding PCR, did 2 bead wash (0.8x + 0.75x)