**Tetraparvo positive CSF\_DNA libraries working outline**

**Date-24.08.2022**

**Name of the experiment**:

1. CSF sample extraction (n=15, water controls).

**1. CSF samples Extraction (n=15, water controls)**

15 samples were selected for sequencing 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 |

We followed the protocol for extraction:

**QIAamp DNA mini kit**

We did spin the serum samples first at **4k rpm for 10 mins** before starting the extraction.

**Date-27.08.2022 tgttg**

**Name of the experiment**:

1. Tetra primer dilution (10uM)
2. MULTIPLEX PCR

**Note:** We selected 8samples for the next steps in this batch and excluded 6 because of too low conc.

1. **Primer dilution (10uM)**

We diluted tetra pool-1 and pool-2 by 10uM.

1. **PCR amplification using Multiplex PCR NEBNext® Ultra™ II Q5® Master Mix\_set 1**

After DNA extrcation, Multiplex PCR was done using pool-1 and pool-2 primers. NEBNext® Ultra™ II Q5® Master Mix (M0544) was used for amplification.

We followed the protocol for pool PCR and prepared the reaction mix accordingly. Pool products were kept at –80 after completing the PCR <https://www.protocols.io/view/artic-neb-sars-cov-2-library-prep-bp2l6n69rgqe/v4>.

The table below was used to prepare separate master mixes for Pool 1 and Pool 2 primers respectively.

|  |  |  |  |
| --- | --- | --- | --- |
| **Component** | **Pool 1** | **Pool 2** | **10** |
| Hot star (2x) | 6.25 | 6.25 | 62.5 |
| Primer pool | 2 | 2 | 20 |
| Water | 0 | 0 | 0 |
| Template | 4.25 | 4.25 |  |
| Total | 12.5uL | 12.5uL |  |

**PCR profile:**

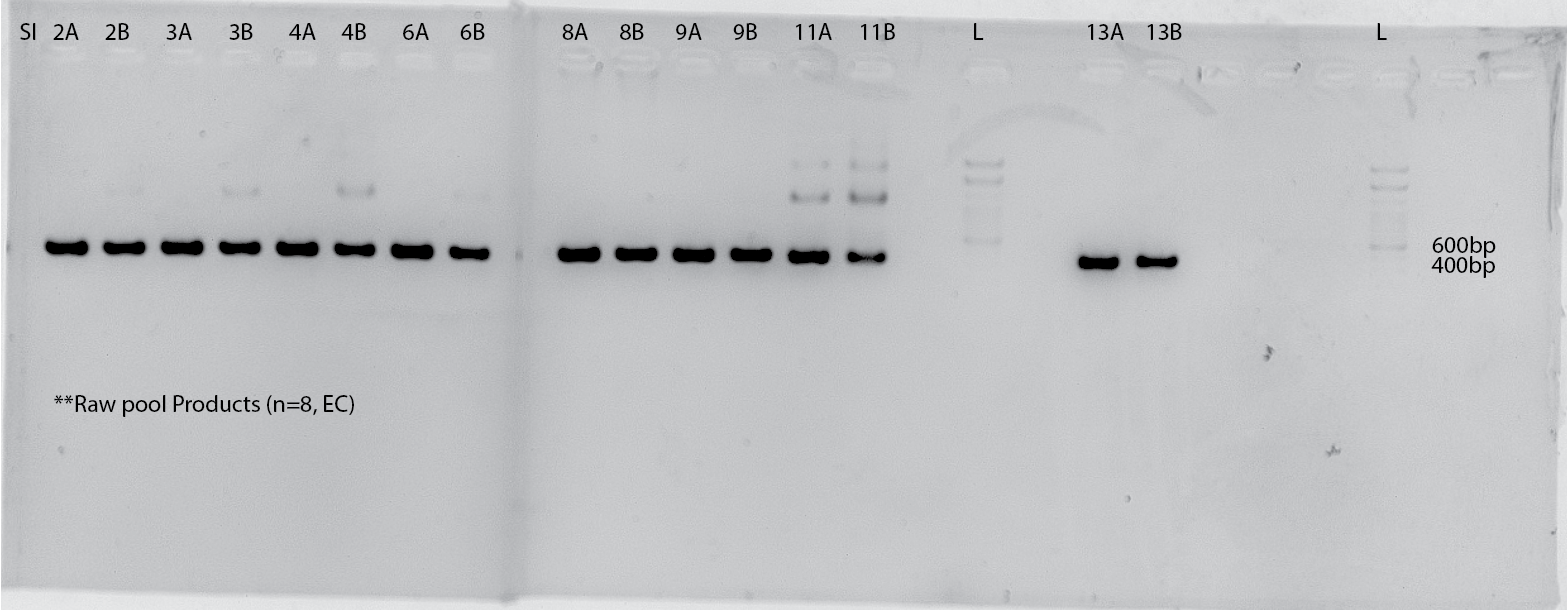
|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temp** | **Time** | **Cycles** |
| Heat Activation | 98 °C | 30s | 1 |
| Denaturation | 98 °C | 15s | 30 |
| Annealing | 64 °C | 05 min |
| Hold | 4 °C | indefinite | 1 |

**Note\*, products were stored at –80C.**

**Date-28.08.2022**

**Name of the experiment**:

1. Gel electrophoresis
2. PCR Clean-up of pooled products
3. Library prep
4. **Gel run:** We ran the raw pool products in 1.5% gel for 50 mins



After getting the gel image, we decided to select all for library prep.

* 1. **PCR Clean-up of pooled products**

Everything was done according to the protocol. We used (1X ratio) beads of total volume of amplified pooling products. Final Elution was 40uL.

* 1. **Illumina Library Preparation - DNA Libraries**

1. **Concentration measurement:**

We measured the concentration of extracted DNA

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen ID ID** | **Raw** | **DNA (100-fold) quantification with Qubit** | **DNA (10-fold) quantification with Qubit** | **Calculated DNA conc (neat) ul** | **Need for normalization (8ng/ul; 13 ul; total 104ng DNA)\_10F** | **Water (to make 13 ul)** |
| 2 | 11905022241 | 0.382 | 1.15 | 11.5 | 115 | 9.04 | 3.96 |
| 3 | 11906003412 | 0.176 | 1.23 | 12.3 | 123 | 8.46 | 4.54 |
| 4 | 11804021521 | 0.262 | 1.12 | 11.2 | 112 | 9.29 | 3.71 |
| 6 | 11806007711 | 162 | 1.27 | 12.7 | 127 | 8.19 | 4.81 |
| 8 | 12009027051 | 118 | 1.14 | 11.4 | 114 | 9.12 | 3.88 |
| 9 | 12102005521 | 0.432 | 0.886 | 8.86 | 88.6 | 11.74 | 1.26 |
| 11 | 12104023562 | 11.3 | 1.71 | 17.1 | 171 | 6.08 | 6.92 |
| 13 | 12203004511 | 0.58 | 1.07 | 10.7 | 107 | 9.72 | 3.28 |
| EC | water | 0 | 0 | 0 | 13 | 0.00 |  |

1. **Fragmentation**

|  |  |  |
| --- | --- | --- |
| Fragmentation | | |
| Reagent | 0.5x rxn | 10 |
| Normalized DNA (10-100ng) | 13 |  |
| (Yellow) NEBNext Ultra II FS Reaction Buffer | 3.5 | 35 |
| (Yellow) NEBNext Ultra II FS Enzyme mix | 1 | 10 |
| Total volume | 17.5 |  |
| Aliquote per tube | 4.5uL |  |

In a Thermocycler, with the heated lid set to 75°C, run the following program:

**5 min @ 37°C**

**30 min @ 65°C**

**Hold @ 4°C**

1. **Adapter Ligation**

|  |  |  |  |
| --- | --- | --- | --- |
| Adaptor Ligation: | | |  |
| Reagent | 0.5x rxn | 10 | Remark |
| FS Reaction Mixture | 17.5 |  |  |
| (Red) NEBNext Ultra II Ligation Master Mix\* | 15 | 150 |  |
| (Red) NEBNext Ligation Enhancer\* | 0.5 | 5 |  |
| (Red) NEBNext Adaptor for Illumina (1:100 dilution) | 1.25 |  | Add seperately |
| Total volume | 34.25 |  |  |
| Aliquote per tube | 15.5 |  |  |

Incubate at 20°C for 15 minutes in a thermocycler with the **heated lid off.**

1. SPRI Bead Clean-up and size selection:

\*allow beads to sit in RT for 30 mins prior

1. Use *SPRI bead* 0.9x ratio of beads-to-total volume of sample. Prep 80% EtOH.
2. Add 61uL (half reaction: 30.8uL) of room temperature beads (0.9x) to Adaptor Ligation reaction.  Mix well.
3. Pulse spin the tubes, but be sure not to spin down beads. Incubate for 5 mins at room temperature.
4. Place samples on magnetic rack, and incubate for 5 mins on the rack.
5. Remove supernatant.
6. Add 200uL of 80% EtOH to samples while on the magnetic rack. Incubate at room temperature for 30s then remove the supernatant.
7. Repeat EtOH wash step for a total of 2 times.
8. Air dry the beads for 5 mins while on the magnetic rack.
9. Remove tube from magnetic rack. Elute DNA from beads into 17uL (half reaction: **9uL)** of 0.1x TE Buffer, 10mM Tris-HCl, or Nuclease free water.
10. Vortex to mix. Spin tubes and incubate for 2 mins at room temperature off the magnetic rack.
11. Place on magnetic rack until solution is clear ~ 5 mins.
12. Remove 7uL of the supernatant and transfer to a clean nuclease free PCR tube.

**Date-29.08.2022**

**Name of the experiment**:

1. **Barcode PCR and PCR clean Up**
2. **Barcode PCR:**

|  |  |  |
| --- | --- | --- |
| **Reagent** | **0.5x rxn** | 10 |
| Purified, adaptor-ligated cDNA | 6 |  |
| (white) USER Enzyme (Cat no. M5505L, 250uL) | 1.5 | 15 |
| (blue) NEBNext Ultra II Q5 master mix | 12.5 | 125 |
| 5uM i7 barcoded primer (NEB index primer/TruSeq/or similar) | 5 |  |
| 5uM i5 barcoded primer (NEB Universal primer/TruSeq/or similar) |
| Total volume | **25** |  |

**Barcode Layout**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen\_ID** | **CSF\_Tetra\_Seq ID** | **Barcode layout** | **Barcode plate** |
| 2 | 11905022241 | CSF\_0036\_TP4 | B03 | IDT-1446944+945 |
| 3 | 11906003412 | CSF\_0037\_TP4 | C03 | IDT-1446944+945 |
| 4 | 11804021521 | CSF\_0038\_TP4 | D03 | IDT-1446944+945 |
| 6 | 11806007711 | CSF\_0039\_TP4 | F03 | IDT-1446944+945 |
| 8 | 12009027051 | CSF\_0040\_TP4 | G03 | IDT-1446944+945 |
| 9 | 12102005521 | CSF\_0041\_TP4 | G01 | IDT-1446944+945 |
| 11 | 12104023562 | CSF\_0042\_TP4 | H01 | IDT-1446944+945 |
| 13 | 12203004511 | CSF\_0043\_TP4 | G02 | IDT-1446944+945 |
| EC | water | EC\_tetra\_NS2k\_B14 | H02 | IDT-1446944+945 |

1. **0.8X clean up and Size selection:**

Everything was done according to the protocol. We used (0.8X ratio) beads of the total volume of amplified pooling products. Final Elution was 25 uL.

1. **0.75x Clean up and size selection**

Everything was done according to the protocol. We used (0.75X ratio) beads of the total volume of amplified pooling products. Final Elution was 15 uL.

1. **0.75x Clean up and size selection**

Everything was done according to the protocol. We used (0.75X ratio) beads of the total volume of amplified pooling products. Final Elution was 15 uL.

**Date-31.08.2022**

**Name of the experiment**:

1. **Measurement of RSV Conc.**
2. **Loading of NS2K**
3. **Equi of tetra libraries:**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Batch Sl** | **Qubit (ng/uL) after 40-fold dilution** | **Qubit (ng/uL) after 10-fold dilution** | **Original Conc** | **Volume picked (Desired DNA input 7ng) from Raw\_Qubit** | **Volume picked (Desired DNA input 7ng) from 10 fold\_Qubit** | **Decision for Pick** |
| 2 | 11905022241 | 0.392 | 3.92 | 1.79 |  | Raw |
| 3 | 11906003412 | 0.966 | 9.66 | 0.72 | 7.25 | 10F |
| 4 | 11804021521 | 0.808 | 8.08 | 0.87 | 8.66 | 10F |
| 6 | 11806007711 | 0.568 | 5.68 | 1.23 |  | Raw |
| 8 | 12009027051 | 0.298 | 2.98 | 2.35 |  | Raw |
| 9 | 12102005521 | 0.888 | 8.88 | 0.79 | 7.88 | 10F |
| 11 | 12104023562 | 0.648 | 6.48 | 1.08 |  | Raw |
| 13 | 12203004511 | 0.33 | 3.3 | 2.12 |  | Raw |
| EC | water | too low | too low | #VALUE! | #VALUE! | Raw |

Note\*\* we pooled all tetra libraries in a single tube.

1. **5% 650pM Phix preparation**

Working phix (650pM) was prepared with 1X con.

1. **Loading on NextSeq2000**

20uL of the diluted and pooled library was loaded with 5% PhiX (1.5ul for 650pM) at. 7.10 pm, 31.8.2022. Estimating time to finish the run is approximately.