**CSF metagenomics \_DNA libraries working outline**

**Date-10.04.2022**

**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.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Extraction sl** | **ScreeningID** | **StudyID** | **AdmDate** | **TLC** | **RemVolume** | **CtValue** |
| 1 | 1250004007 | 12204004541 |  | 60 | 830 | 20.57 |
| 2 |  | 12203026991 |  | 40 | 850 | 13.84 |
| 3 |  | 12203024741 |  | 20 | 2000 | 13.29 |
| 4 | 1200003877 | 12201034831 | 17-Jan-22 | 350 | 500 | 17.5 |
| 5 | 1010002333 | 12105004921 | 18-Apr-21 | 10 | 2000 | 19.62 |
| 6 | 1010002244 | 12103015842 | 10-Mar-21 | 180 | 350 | 18.11 |
| 7 | 1150002921 | 12101025462 | 23-Jan-21 | 20 | 800 | 17.58 |
| 8 | 1210001282 | 12010030701 | 22-Oct-20 | 150 | 570 | 20.42 |
| 9 | 1160001574 | 12010015311 | 24-Sep-20 | 60 | 800 | 19.28 |
| 10 | 1220000170 | 12003023362 | 22-Mar-20 | 10 | 600 | 20.43 |
| 11 | 1200000873 | 12003016171 | 23-Feb-20 | 300 | 2000 | 14.23 |
| 12 | 1260000168 | 12002019032 | 16-Feb-20 | 10 | 750 | 18.52 |
| 13 | 1030002744 | 12002002564 | 4-Feb-20 | 80 | 1200 | 14.62 |
| 14 | 1250000359 | 12001029023 | 28-Jan-20 | 30 | 500 | 17.25 |
| 15 | 1070001774 | 12001027713 | 27-Jan-20 | 20 | 400 | 19.72 |
| Water\_closed |  |  |  |  |  |  |
| Water01\_Opened |  |  |  |  |  |  |
| Water02\_opened |  |  |  |  |  |  |

We followed the protocol for extraction:

[COVID\_RNA\_Extraction\_Protocol\_Meta.docx](https://chrfbd-my.sharepoint.com/:w:/g/personal/genomics_chrfbd_org/EbvRF8JxKL5GhtsJJLTepDkBSw3H-idvjLoQcCllsF2W9A?e=BqJGVX) (Zymo Mini-Prep kit)

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

Due to mishandling of the centrifuge, the lids of samples 1,3,4,10, and 11 broke. These samples were excluded from the further procedure and stored in 200ul of 2x RNA shield at -80C for them to be extracted later. We continued with sample numbers- 2,5,6,7,8,9,12,13,14,15,16,17,18 and EC.

**Date-12.04.2022**

**Name of the experiment**:

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

We extracted DNA and RNA from sl 1,3,4,10,11 from protinease K steps.

We followed the protocol for extraction:

[COVID\_RNA\_Extraction\_Protocol\_Meta.docx](https://chrfbd-my.sharepoint.com/:w:/g/personal/genomics_chrfbd_org/EbvRF8JxKL5GhtsJJLTepDkBSw3H-idvjLoQcCllsF2W9A?e=BqJGVX) (Zymo Mini-Prep kit)

**Date-13.04.2022**

**Name of the experiment**:

1. DNA library preparation for 5 samples (Up-to 0.8X wash for Barcoded Library)
2. **Illumina Library Preparation - DNA Libraries**
3. **Concentration measurement:**

We measured the concentration of extracted DNA

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen ID ID** | **Calculated DNA conc (neat) ul** | **Need for normalization (5ng/ul; 13 ul; total 65ng DNA)** | **Water (to make 13 ul)** | **DNA input (ng)** |
| 1 | 12204004541 | 0.23 | 13.00 | 0.00 | 2.99ng input |
| 3 | 12203024741 | 6.08 | 10.69 | 2.31 |  |
| 4 | 12201034831 | 4.9 | 13.00 | 0.00 | 63.7ng |
| 9 | 12010015311 | 1.79 | 13.00 | 0.00 | 23.27ng |
| 11 | 12003016171 | 30.8 | 2.11 | 10.89 |  |
| EC | Hyclone water | too low | #VALUE! | 0.00 |  |

1. Fragmentation:

|  |  |  |
| --- | --- | --- |
| Fragmentation | | |
| Reagent | 0.5x rxn | 6 |
| Normalized DNA (10-100ng) | 13 |  |
| (Yellow) NEBNext Ultra II FS Reaction Buffer | 3.5 | 21 |
| (Yellow) NEBNext Ultra II FS Enzyme mix | 1 | 6 |
| Total volume | 17.5 |  |
| Aliquote per tube | 4.5uL |  |

In a Thermocycler, with the heated lid set to 105°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 | 6 | Remark |
| FS Reaction Mixture | 17.5 |  |  |
| (Red) NEBNext Ultra II Ligation Master Mix\* | 15 | 90 |  |
| (Red) NEBNext Ligation Enhancer\* | 0.5 | 3 |  |
| (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 15uL (half reaction: 7.5uL) of the supernatant and transfer to a clean nuclease free PCR tube.
13. **Barcode PCR:**

|  |  |  |
| --- | --- | --- |
| **Reagent** | **0.5x rxn** | 6 |
| Purified, adaptor-ligated cDNA | 7.5 |  |
| (white) USER Enzyme (Cat no. M5505L, 250uL) | 1.5 | 9 |
| (blue) NEBNext Ultra II Q5 master mix | 12.5 | 75 |
| 5uM i7 barcoded primer (NEB index primer/TruSeq/or similar) | 5 |  |
| 5uM i5 barcoded primer (NEB Universal primer/TruSeq/or similar) |
| Total volume | **26.5** |  |
|  | 21.2 |  |

**Barcode Layout**

**We used IDT plate for this batch**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen\_ID** | **CSF\_Tetra\_Seq ID** | **Barcode layout** | **Barcode plate** |
| 1 | 12204004541 | CSF\_0015\_TP4 | A12 | IDT\_15156265 |
| 3 | 12203024741 | CSF\_0016\_TP4 | B12 | IDT\_15156265 |
| 4 | 12201034831 | CSF\_0017\_TP4 | C12 | IDT\_15156265 |
| 9 | 12010015311 | CSF\_0018\_TP4 | D12 | IDT\_15156265 |
| 11 | 12003016171 | CSF\_0019\_TP4 | E12 | IDT\_15156265 |
| EC | Hyclone water | CSF\_NC\_NS2K\_Batch 03 | F12 | IDT\_15156265 |

***Note\**** *we directly washed the libraries by 0.75X and did it two times. We skipped 0.8X wash of libraries because of losing products.*

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

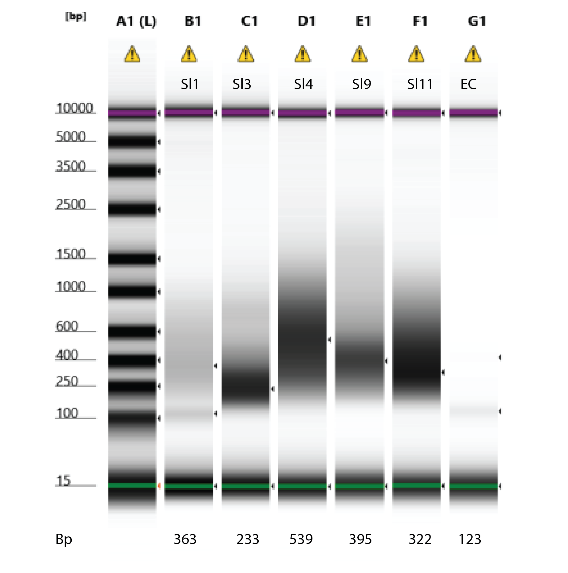
Everything was done according to the protocol. We used (0.75X ratio) beads of the total volume of amplified pooling products. Final Elution was 15 uL.

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.

1. Tapestation Run:

The libraries were run on Tapestation and the results are attached below



**Date-16.04.2022**

**Name of the experiment**:

1. RNA library preparation for 10 samples and 3 water controls (Up-to 0.8X wash for Barcoded Library)
2. **Illumina Library Preparation - DNA Libraries**
3. **Concentration measurement:**

We measured the concentration of extracted DNA

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen ID ID** | **Calculated DNA conc (neat) ul** | **Need for normalization (5ng/ul; 13 ul; total 65ng DNA)** | **Water (to make 13 ul)** | **DNA input (ng)** |
| 2 | 12203026991 | 1.51 | 13.00 | 0.00 | 19.63 |
| 5 | 12105004921 | 0.374 | 13.00 | 0.00 | 4.862 |
| 6 | 12103015842 | 0.164 | 13.00 | 0.00 | 2.132 |
| 7 | 12101025462 | too low | 13.00 | 0.00 |  |
| 8 | 12010030701 | 0.342 | 13.00 | 0.00 | 4.446 |
| 10 | 12003023362 | too low | 13.00 | 0.00 |  |
| 12 | 12003016171 | too low | 13.00 | 0.00 |  |
| 13 | 12002002564 | 0.718 | 13.00 | 0.00 | 9.334 |
| 14 | 12001029023 | 1.08 | 13.00 | 0.00 | 14.04 |
| **15** | 12001027713 | **too low** | 13.00 | 0.00 |  |
| 16 | water-closed | too low | 13.00 | 0.00 |  |
| 17 | w01-opened | too low | 13.00 | 0.00 |  |
| 18 | w02-opened | too low | 13.00 | 0.00 |  |

1. Fragmentation:

|  |  |  |
| --- | --- | --- |
| Fragmentation | | |
| Reagent | 0.5x rxn | 15 |
| Normalized DNA (10-100ng) | 13 |  |
| (Yellow) NEBNext Ultra II FS Reaction Buffer | 3.5 | 52.5 |
| (Yellow) NEBNext Ultra II FS Enzyme mix | 1 | 15 |
| Total volume | 17.5 |  |
| Aliquote per tube | 4.5uL |  |

In a Thermocycler, with the heated lid set to 105°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 | 15 | Remark |
| FS Reaction Mixture | 17.5 |  |  |
| (Red) NEBNext Ultra II Ligation Master Mix\* | 15 | 225 |  |
| (Red) NEBNext Ligation Enhancer\* | 0.5 | 7.5 |  |
| (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 15uL (half reaction: 7.5uL) of the supernatant and transfer to a clean nuclease free PCR tube.
13. **Barcode PCR:**

|  |  |  |
| --- | --- | --- |
| **Reagent** | **0.5x rxn** | 15 |
| Purified, adaptor-ligated cDNA | 7.5 |  |
| (white) USER Enzyme (Cat no. M5505L, 250uL) | 1.5 | 22.5 |
| (blue) NEBNext Ultra II Q5 master mix | 12.5 | 187.5 |
| 5uM i7 barcoded primer (NEB index primer/TruSeq/or similar) | 5 |  |
| 5uM i5 barcoded primer (NEB Universal primer/TruSeq/or similar) |
| Total volume | **26.5** |  |
|  | 21.2 |  |

**Barcode Layout**

We used IDT plate for this batch

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Batch Sl** | **Specimen\_ID** | **CSF\_Tetra\_Seq ID** | **Barcode layout** | **Barcode plate** |
| 2 | 12203026991 | CSF\_0020\_TP4 | A11 | IDT\_15156265 |
| 5 | 12105004921 | CSF\_0021\_TP4 | B11 | IDT\_15156265 |
| 6 | 12103015842 | CSF\_0022\_TP4 | C11 | IDT\_15156265 |
| 7 | 12101025462 | CSF\_0023\_TP4 | D11 | IDT\_15156265 |
| 8 | 12010030701 | CSF\_0024\_TP4 | E11 | IDT\_15156265 |
| 10 | 12003023362 | CSF\_0025\_TP4 | F11 | IDT\_15156265 |
| 12 | 12003016171 | CSF\_0026\_TP4 | G11 | IDT\_15156265 |
| 13 | 12002002564 | CSF\_0027\_TP4 | H11 | IDT\_15156265 |
| 14 | 12001029023 | CSF\_0028\_TP4 | A10 | IDT\_15156265 |
| **15** | 12001027713 | CSF\_0029\_TP4 | B10 | IDT\_15156265 |
| 16 | water-closed | CSF\_WC\_NS2K\_Batch 03 | C10 | IDT\_15156265 |
| 17 | w01-opened | CSF\_W01\_NS2K\_Batch 03 | D10 | IDT\_15156265 |
| 18 | w02-opened | CSF\_W02\_NS2K\_Batch 03 | E10 | IDT\_15156265 |

***Note\**** *we directly washed the libraries by 0.75X and did it two times. We skipped 0.8X wash of libraries because of losing products.*

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.

1. **Tapestation Run:**

The libraries were run on Tapestation and the results are attached below

Chart

Description automatically generated

**Date-17.04.2022**

**Name of the experiment**:

1. Dilution and Qubit
2. Equi-conc. and 0.75x wash
3. Dilution and Qubit
4. nM calculation
5. Loading in NextSeq 2000

1. **Equi-conc. calculation:**

After library preparation, equiconc was measured and all libraries were pooled in 2 tubes; tubes were distributed depending on library volume. Please check the calculation given below.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **SL** | **Neat\_17Apr** | **Conc from 10F** | **Volume picked (Desired DNA input 6ng) from Neat\_Qubit** | **Volume picked (Desired DNA input 6ng) from 10 fold\_Qubit** | Remark | Eq |
| 1 | 0.308 | 0.0308 | 19.48 | 194.8051948 | Full vol | 1 |
| 2 | 8.3 | 0.83 | 0.72 | 7.23 | 10F | 2 |
| 3 | 3.13 | 0.313 | 1.92 | 19.17 | Raw | 2 |
| 4 | 9.08 | 0.908 | 0.66 | 6.61 | 10F | 2 |
| 5 | 1.93 | 0.193 | 3.11 | 31.09 | Raw | 1 |
| 6 | 7.78 | 0.778 | 0.77 | 7.71 | 10F | 2 |
| 7 | 0.651 | 0.0651 | 9.22 | 92.17 | Raw | 1 |
| 8 | 8.8 | 0.88 | 0.68 | 6.82 | 10F | 2 |
| 9 | 8.31 | 0.831 | 0.72 | 7.22 | 10F | 2 |
| 10 | too low |  | #VALUE! | #DIV/0! | Full vol | 1 |
| 11 | 11.5 | 1.15 | 0.52 | 5.22 | Raw | 2 |
| 12 | 0.513 |  | 11.70 | #DIV/0! | Raw | 1 |
| 13 | 20.5 | 2.05 | 0.29 | 2.93 | 10F | 2 |
| 14 | 7.88 | 0.788 | 0.76 | 7.61 | 10F | 2 |
| **15** | too low |  | #VALUE! | #DIV/0! | Full vol | 1 |
| 16 | too low |  | #VALUE! | #DIV/0! | Full vol | 1 |
| 17 | 0.061 |  | 98.36 | #DIV/0! | Full vol | 1 |
| 18 | 0.055 |  | 109.09 | #DIV/0! | Full vol | 1 |
| nc | too low |  | #VALUE! | #DIV/0! | 0.4 | 1 |
| Ec | too low |  | #VALUE! | #DIV/0! | 0.4 | 1 |

**3. 0.75x Clean-up for equi tubes**

Everything was done according to the protocol. We used (0.75X ratio) beads of total volume of equitube. Final Elution was 15 uL for equitube-1, 2; 20uL for equi-tube 3.

4. **5% 650pM Phix preparation**

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

**5. Qubit of final library**

Final libraries were diluted and pooled according to the table below. We wanted to keep the loading conc. 650pM and Library size 420bp.

**6. Loading on NextSeq2000**

20uL of the diluted and pooled library was loaded with 5% PhiX (1.5ul for 650pM) at.6.30pm, 17.04.2022. Estimating time to finish the run is approximately 12.30am, 19.04.2022