



The logo for 'compare' features a central globe with a blue and white pattern. Surrounding the globe are several nodes connected by lines, forming a network. The nodes are colored in shades of blue and red. The word 'compare' is written in a blue, sans-serif font to the right of the globe. Below the logo, the word 'Deliverable' is written in a larger, blue, sans-serif font.

compare

Deliverable

2.3 Report on sequencing workflow

Version: 1

Due: Month 36

Completed: Month 36



This project has received funding from the *European Union's Horizon 2020 research and innovation programme* under grant agreement No 643476.



Contents

Deliverable Description	2
Workflow steps and tests	2
Overview statements	3
Actual Workflows	10
Summary and Conclusions	35

Deliverable Description

The overall aim of Deliverable D2.3 (*Report on sequencing workflow*) is ...

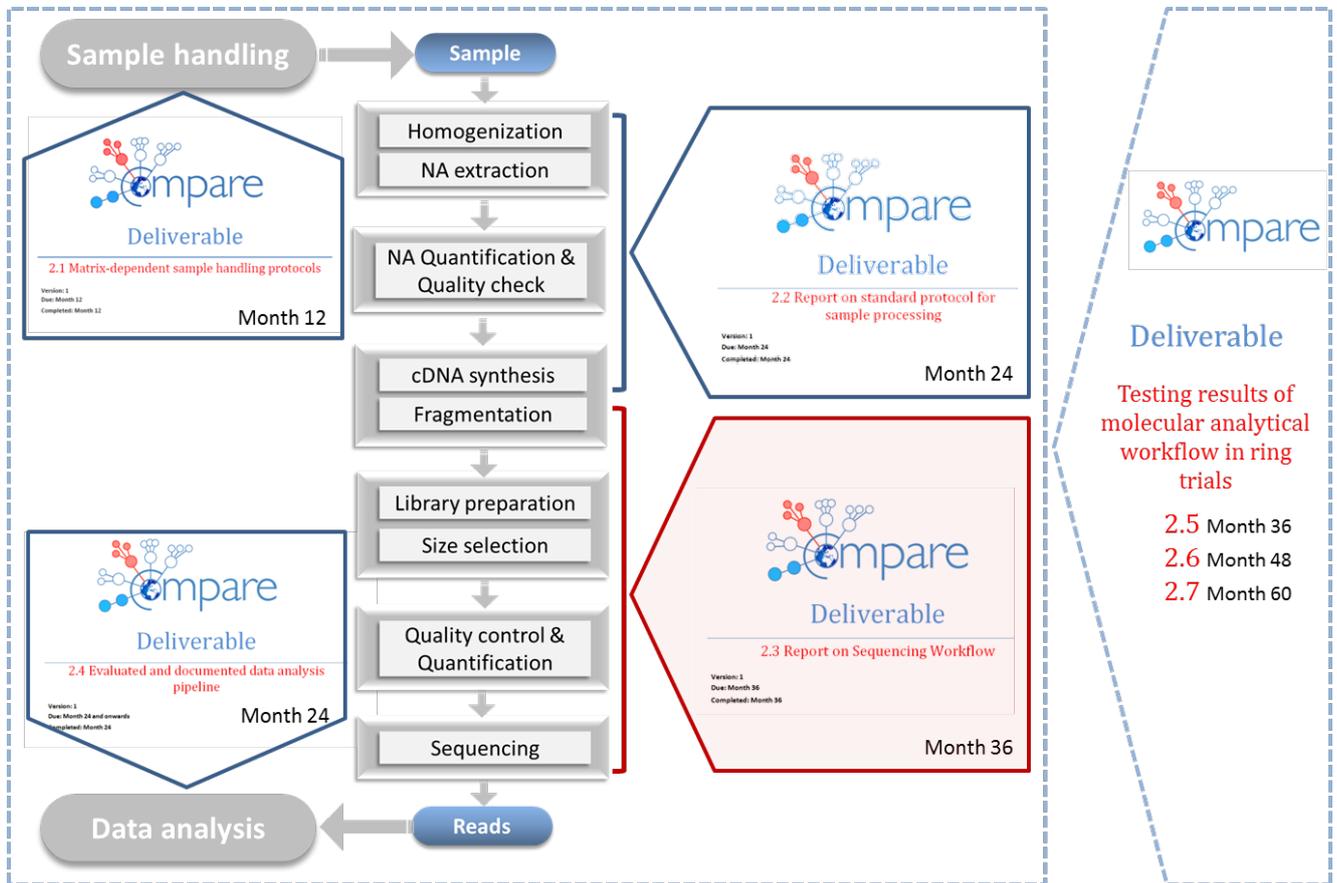


FIGURE 1: OVERVIEW OF DELIVERABLES (2.1 – 2.7) RELATED TO COMPARE WORK PACKAGE 2 WITH THEIR DUE-DATES AND SCOPES AS OUTLINED IN THE GRANT AGREEMENT. THE PRESENT DOCUMENT DEALS WITH D2.3 (RED MARKED).

Metagenomic Workflow – from cDNA to sequence reads

This Deliverable report is the continuation of Deliverable D2.2 (metagenomics workflow – from sample to cDNA) and will handle the protocols for fragmentation of DNA/cDNA, library preparation, size selection, quality and quantity checks, pooling and sequencing.

Tracking of the sequencing workflow:

While this report focuses on laboratory procedures that make up the COMPARE sequencing workflows, it is important to note for those following the workflows, that key to the ultimate utility

of the sequencing output is the tracking of relevant information as to those choices made during sequencing. Tracking this information will maximise the usability of sequence data shared through the COMPARE Data Hubs and Platform. Ultimately, those following sequencing workflows will report their data and will need to provide:

- a brief **text description** of the sequencing workflow, a reference to a published procedure or procedures or a reference to a laboratory workflow presented in an experimental repository, such as <https://www.protocols.io/>,
- the **library source** (such as "genomic", "metagenomic", "viral RNA", etc.),
- the **library strategy** (such as "whole genome shotgun", "amplicon", etc.),
- any **library selection or enrichment** that has been applied (such as "RT-PCR", "16S")
- information on **insert sizes** for paired libraries,
- sequencing vendor **platform** and instrument **model name** (such as 'Illumina HiSeq 2000' or 'AB SOLiD 5500') and
- (optionally) any further details and parameters relating to the sequencing workflow.

Workflow steps and tests

The sequencing process was divided into several steps and protocols were gathered from all COMPARE members for the following methods in use in their laboratories

1. DNA fragmentation.
2. Library preparation and size selection.
3. Quality control and quantification (of libraries)
4. Sequencing.

Requests for protocols were sent to all COMPARE members. Eight partners provided the requested overview and/or sequencing protocols, including ANSES (5), APHA (10), AUTH (20), DTU (1), EMC (2), FLI (4), IFREMER (21) and TIHO (17).

Overview statements

We felt it important to gather tested protocols so an attempt was made to document the successful use of the protocols. These are summarized by contributing group.

ANSES (5) Overview

Contact person: BLANCHARD Yannick <Yannick.BLANCHARD@anses.fr>

List of publication linked to this method:

1. Baillon L, Mérour E, Cabon J, Louboutin L, Quenault H, Touzain F, Morin T, Blanchard Y, Biacchesi S, Brémont M. A single amino acid change in the non-structural NV protein impacts the virulence phenotype of Viral hemorrhagic septicemia virus in trout. *J Gen Virol.* 2017 Jun;98(6):1181-1184. doi: 10.1099/jgv.0.000830. Epub 2017 Jun 22. PubMed PMID: 28640747.
2. Renson P, Touzain F, Leuret A, Le Dimna M, Quenault H, Normand V, Claude JB, Pez F, Rose N, Blanchard Y, Bourry O. Complete Genome Sequence of a Recombinant Porcine Reproductive and Respiratory Syndrome Virus Strain from Two Genotype 1 Modified Live Virus Vaccine Strains. *Genome Announc.* 2017 Jun 1;5(22). pii: e00454-17. doi: 10.1128/genomeA.00454-17. PubMed PMID: 28572326; PubMed Central PMCID: PMC5454209.
3. Sailleau C, Breard E, Viarouge C, Gorlier A, Quenault H, Hirchaud E, Touzain F, Blanchard Y, Vitour D, Zientara S. Complete genome sequence of bluetongue virus serotype 4 that emerged on the French island of Corsica in December 2016. *Transbound Emerg Dis.* 2017 May 11. doi: 10.1111/tbed.12660. [Epub ahead of print] PubMed PMID: 28497659.
4. Briand FX, Schmitz A, Ogor K, Le Prioux A, Guillou-Cloarec C, Guillemoto C, Allée C, Le Bras MO, Hirchaud E, Quenault H, Touzain F, Cherbonnel-Pansart M, Lemaitre E, Courtillon C, Gares H, Daniel P, Fediaevsky A, Massin P, Blanchard Y, Eterradossi N, van der Werf S, Jestin V, Niqueux E. Emerging highly pathogenic H5 avian influenza viruses in France during winter 2015/16: phylogenetic analyses and markers for zoonotic potential. *Euro Surveill.* 2017 Mar 2;22(9). pii: 30473. doi: 10.2807/1560-7917.ES.2017.22.9.30473. PubMed PMID: 28277218; PubMed Central PMCID: PMC5356430.
5. Lecollinet S, Blanchard Y, Manson C, Lowenski S, Laloy E, Quenault H, Touzain F, Lucas P, Eraud C, Bahuon C, Zientara S, Beck C, Decors A. Dual Emergence of Usutu Virus in Common Blackbirds, Eastern France, 2015. *Emerg Infect Dis.* 2016 Dec;22(12):2225. doi: 10.3201/eid2212.161272. PubMed PMID: 27869608; PubMed Central PMCID: PMC5189168.

6. Bréard E, Sailleau C, Quenault H, Lucas P, Viarouge C, Touzain F, Fablet A, Vitour D, Attoui H, Zientara S, Blanchard Y. Complete Genome Sequence of Bluetongue Virus Serotype 8, Which Reemerged in France in August 2015. *Genome Announc.* 2016 Apr 14;4(2). pii: e00163-16. doi: 10.1128/genomeA.00163-16. PubMed PMID: 27081120; PubMed Central PMCID: PMC4832148.

7. Grasland B, Bigault L, Bernard C, Quenault H, Toulouse O, Fablet C, Rose N, Touzain F, Blanchard Y. Complete genome sequence of a porcine epidemic diarrhea s gene indel strain isolated in france in december 2014. *Genome Announc.* 2015 Jun 4;3(3). pii: e00535-15. doi: 10.1128/genomeA.00535-15. PubMed PMID: 26044419; PubMed Central PMCID: PMC4457056.

APHA (10) Overview

Contact person: Ellis, Richard (APHA) <Richard.Ellis@apha.gsi.gov.uk>

The protocols described here have allowed APHA to achieve a rapid turnaround of multiple sample types and to make efficient use of the sequencing capacity available at APHA. This approach has been used to successfully generate sequence data from over 10,000 samples in the past 3 years. This has included a wide variety of bacteria genera (e.g. *Campylobacter* (1), *Chrysobacterium* (2), *Coxiella* (3), *Escherichia* (4), *Mycobacterium* (5), *Salmonella* (6), and *Staphylococcus* (7) and virus families (e.g. *Coronaviridae* (8), *Nairoviridae* (9), *Orthomyxoviridae* (10), *Phenuiviridae* (11), and *Rhabdoviridae* (12, 13)). APHA are currently seeking accreditation to ISO 17025 for the use of these methods for the characterization of bacteria and viruses.

References

1. Petrovska L, Tang Y, Jansen van Rensburg MJ, Cawthraw S, Nunez J, Sheppard SK, et al. Genome Reduction for Niche Association in *Campylobacter Hepaticus*, A Cause of Spotty Liver Disease in Poultry. *Frontiers in Cellular and Infection Microbiology.* 2017;7(354).
2. Verner-Jeffreys DW, Brazier T, Perez RY, Ryder D, Card RM, Welch TJ, et al. Detection of the florfenicol resistance gene *floR* in *Chryseobacterium* isolates from rainbow trout. Exception to the general rule? *FEMS Microbiol Ecol.* 2017;93(4).

3. Sidi-Boumedine K, Ellis RJ, Adam G, Prigent M, Angen Ø, Aspán A, et al. Draft Genome Sequences of Six Ruminant *Coxiella burnetii* Isolates of European Origin. *Genome Announcements*. 2014;2(3).
4. Duggett NA, Sayers E, AbuOun M, Ellis RJ, Nunez-Garcia J, Randall L, et al. Occurrence and characterization of *mcr-1*-harbouring *Escherichia coli* isolated from pigs in Great Britain from 2013 to 2015. *Journal of Antimicrobial Chemotherapy*. 2017;72(3):691-5.
5. Roberts T, O'Connor C, Nuñez-Garcia J, de la Rua-Domenech R, Smith NH. Unusual cluster of *Mycobacterium bovis* infection in cats. *Veterinary Record*. 2014;174(13):326.
6. Anjum MF, Duggett NA, AbuOun M, Randall L, Nunez-Garcia J, Ellis RJ, et al. Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. *Journal of Antimicrobial Chemotherapy*. 2016;71(8):2306-13.
7. Sharma M, Nunez-Garcia J, Kearns AM, Doumith M, Butaye PR, Argudín MA, et al. Livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) clonal complex (CC) 398 isolated from UK animals belong to European lineages. *Frontiers in microbiology*. 2016;7.
8. Dastjerdi A, Carr J, Ellis RJ, Steinbach F, Williamson S. Porcine epidemic diarrhea virus among farmed pigs, Ukraine. *Emerging infectious diseases*. 2015;21(12):2235.
9. Atkinson B, Marston DA, Ellis RJ, Fooks AR, Hewson R. Complete genomic sequence of Issyk-Kul virus. *Genome announcements*. 2015;3(4):e00662-15.
10. Hanna A, Banks J, Marston DA, Ellis RJ, Brookes SM, Brown IH. Genetic Characterization of Highly Pathogenic Avian Influenza (H5N8) Virus from Domestic Ducks, England, November 2014. *Emerging Infectious Disease journal*. 2015;21(5):879.
11. Lumley S, Horton DL, Marston DA, Johnson N, Ellis RJ, Fooks AR, et al. Complete genome sequence of Rift Valley fever virus strain Lunyo. *Genome announcements*. 2016;4(2):e00170-16.
12. Filippone C, Castel G, Murri S, Beaulieux F, Ermonval M, Jallet C, et al. Discovery of hantavirus circulating among *Rattus rattus* in French Mayotte island, Indian Ocean. *Journal of General Virology*. 2016;97(5):1060-5.
13. Gunawardena PS, Marston DA, Ellis RJ, Wise EL, Karawita AC, Breed AC, et al. Lyssavirus in Indian Flying Foxes, Sri Lanka. *Emerging Infectious Disease journal*. 2016;22(8):1456.

AUTH (20) Overview

Contact person: Anna Papa <annap@med.auth.gr>

Number of samples sequenced

11 viral isolates: (5 Crimean-Congo hemorrhagic fever viruses, 5 phleboviruses, 1 Greek goat encephalitis virus) 2 Crimean -Congo hemorrhagic fever viruses from ticks 6 Noroviruses, 1 astrovirus and 1 rotavirus from faeces from children with gastroenteritis. Total: 21 viral strains
One article is ready for submission. Title: Isolation and whole-genome sequencing of a CCHFV strain, Greece. The study was presented at the 2nd International Conference on CCHF. Three other studies (one on phylogeny of CCHFV lineage 2, one on noroviruses and one on a novel phlebovirus) were presented in conferences; the related articles are in preparation.

Comments. Whole genome was obtained from most samples. For few samples new primers were designed to fill the gaps by Sanger sequencing. We met difficulties for novel viruses for which there were no reference sequences available. A modification which we found useful is that we doubled the quantity of the initial cDNA due to the low viral load. We also realized that the initial centrifugation of the samples is very useful.

DTU (1) Overview

Contact person: Rene S. Hendriksen <rshe@food.dtu.dk>

Evaluation of Methods for the Concentration and Extraction of Viruses from Sewage in the Context of Metagenomic Sequencing. Hjelmsø MH, Hellmér M, Fernandez-Cassi X, Timoneda N, Lukjancenko O, Seidel M, Elsässer D, Aarestrup FM, Löfström C, Bofill-Mas S, Abril JF, Girones R, Schultz AC. PLoS One. 2017 Jan 18;12(1):e0170199. doi: 10.1371/journal.pone.0170199. eCollection 2017.

PMID: 28099518 Free PMC Article

A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. Munk P, Andersen VD, de Knecht L, Jensen MS, Knudsen BE, Lukjancenko O, Mordhorst H, Clasen J, Agersø Y, Folkesson A, Pamp SJ, Vigre H, Aarestrup FM. J Antimicrob Chemother. 2017 Feb;72(2):385-392. doi: 10.1093/jac/dkw415. Epub 2016 Nov 8.

PMID: 28115502

The association between measurements of antimicrobial use and resistance in the faeces microbiota of finisher batches. Andersen VD, DE Knecht LV, Munk P, Jensen MS, Agersø Y, Aarestrup FM, Vigre H. Epidemiol Infect. 2017 Oct;145(13):2827-2837. doi: 10.1017/S0950268817001285. Epub 2017 Jun 27. PMID: 28651652



Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. Nordahl Petersen T, Rasmussen S, Hasman H, Carøe C, Bælum J, Schultz AC, Bergmark L, Svendsen CA, Lund O, Sicheritz-Pontén T, Aarestrup FM. Sci Rep. 2015 Jul 10;5:11444. doi: 10.1038/srep11444. PMID: 26161690 Free PMC Article

COMPARE Global Sewage project: <http://www.compare-europe.eu/news/2016/12/global-sewage-surveillance-project-update-december-2016?id=a5f0798c-b8f2-4547-8037-04da04c0ece3>

EMC (2) Overview (Ion Torrent)

Contact person: B.B. Oude Munnink <b.oudemunnink@erasmusmc.nl>, My Phan <v.t.m.phan@erasmusmc.nl>

The Ion Torrent process was established in March 2016 and since then we have processed a variety of sample types including setup and testing runs (21), virus discovery samples (using random non-ribosomal hexamer primer (34 samples), specific virus sequencing (random or amplicon, 68 samples) and the Global Sewage samples (31 samples as of 22 Nov 17).

EMC (2) Overview (Roche 454)

Contact person: M. de Graaf <m.degraaf@erasmusmc.nl> , C.M.E. Schapendonk <c.schapendonk@erasmusmc.nl>

An estimated 350 samples have been successfully sequenced using these protocols. These protocols were used to sequence samples published in the following papers:

[Detection of Circovirus in Foxes with Meningoencephalitis, United Kingdom, 2009-2013.](#)

Bexton S, Wiersma LC, Getu S, van Run PR, Verjans GM, Schipper D, **Schapendonk** CM, Bodewes R, Oldroyd L, Haagmans BL, Koopmans MM, Smits SL.

FLI (4) Overview

Contact person: Wylezich, Claudia <Claudia.Wylezich@fli.de>, Beer, Martin <Martin.Beer@fli.de>

These workflows are for sequencing with Illumina MiSeq (automated protocol) and Ion Torrent platforms (manual protocol) from fragmentation to quantification of the library but excluding the sequencing procedure itself for that we have no LOP and use the manufacturer's protocol.

These protocols (either MiSeq or IonTorrent PGM) were used to sequence samples published in the following papers:

Hoffmann et al. 2012 Emerg Infect Dis 18:469

Jenckel et al. 2014 J Virol 88:6983

Hoffmann et al. 2015 N Engl J Med. 373:154

Hanke et al. 2015 Emerg Infect Dis 21:493

Akimkin et al. 2016 Emerg Infect Dis 22:1314

Hanke et al. 2016 PLoS Negl Trop Dis 10:e0004779

Schlottau et al. 2016 Transbound Emerg Dis 63:253

Schulz et al. 2016 J Gen Virol 97:2073

Abendroth et al. 2017 Arch Virol DOI 10.1007/s00705-017-3463-5

Hanke et al. 2017 Viruses 9:177

Pfaff et al. 2017 J Gen Virol 98:1

Pfaff et al. 2017 Trans Emerg Dis 64:677

Pfaff et al. in revision, Vaccine

Wylezich et al. in revision

Wylezich et al. in prep

We sequenced several hundred samples with each method. The Ion Torrent protocol was also successful used with Ion S5XL for a few samples.

IFREMER (21) Overview

Contact person: Julien SCHAEFFER <Julien.Schaeffer@ifremer.fr>, Soizick Le Guyader <Soizick.le.guyader@ifremer.fr>

The supplied workflow is in development and has not yet been tested on a large number of samples.

TIHO (17) Overview

Contact person: Hansmann, Florian <Florian.Hansmann@tiho-hannover.de>

This workflow was used to sequence samples published in the following publication:

- Jo WK, Grilo ML, Wohlsein P, Andersen-Ranberg EU, Hansen MS, Kinze CC, Hjulsgaard CK, Olsen MT, Lehnert K, Prenger-Berninghoff E, Siebert U, Osterhaus A, Baumgartner W, Jensen LF, van der Vries E. (2017). Dolphin Morbillivirus in a Fin Whale (*Balaenoptera physalus*) in Denmark, 2016. *J Wildl Dis*, 53(4):921-924.
- Jo WK, Pfankuche VM, Petersen H, Frei S, Kummrow M, Lorenzen S, Ludlow M, Metzger J, Baumgärtner W, Osterhaus A, van der Vries E. (2017). New Avian Hepadnavirus in Palaeognathous Bird, Germany. *Emerg Infect Dis*, 23(12):2089-2091.
- Chutchai P, Jo WK, Puff C, Ludlow M, van der Vries E, Banlunara W, Rungsipipat A, Kruppa J, Jung K, Techangamsuwan S, Baumgärtner W, Osterhaus ADME (2017). A novel strain of canine bocavirus type-2 infection associated with intestinal lesions. (Submitted)
- Pfankuche VM, Jo WK, van der Vries E, Jungwirth N, Lorenzen S, Osterhaus ADME, Baumgärtner W, Puff C (2017). Neuronal vacuolization in feline panleukopenia virus infection. (Accepted in Journal of Veterinary Pathology)
- Jo WK, Pfankuche VM, Lehmecker A, Martina B, Rubio-Garcia A, Becker S, Kruppa J, Jung K, Klotz D, Metzger J, Ludlow M, Baumgärtner W, van der Vries W, Osterhaus ADME (2017). Batai virus infection associated with encephalitis in a harbor seal (*Phoca vitulina*), Germany, 2016. (Submitted)

We sequenced around 60 samples including the samples from COMPARE GMI Virus Proficiency Test Part 2 (wet lab).

Actual Workflows

ANSES (5) Workflow Fragmentation to Sequencing

Contact person: BLANCHARD Yannick <Yannick.BLANCHARD@anses.fr>

The workflow followed is essentially the protocol from Life Total RNA-Seq Kit V2 (**Life, Ref 4475936**).

https://www.google.fr/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&cad=rja&uact=8&ved=0ahUKEwjF3_bZ0szXAhVhF8AKHWI2Cz0QFghDMAE&url=https%3A%2F%2Ftools.thermofisher.com%2Fcontent%2Ffs%2Fmanuals%2FMAN00010654_IonTotalRNASeqKit_v2_UG.pdf&usq=AOvVaw2FebtQxkWNDHW3JmikkME7

-The sample is assayed with 260/280 nm for quality and fluorimetry for quantity. If there is enough material 500 ng are used otherwise all the sample is used



- The RNA samples are Dnases treated (**Turbo DNA-free Kit**)
 - We use rRNA depleted samples (**Low Input RiboMinus™ Eukaryote System v2**)
 - RNAse III fragmentation (life protocol)
 - Size selection with (**NucleoMag NGS Clean-up and Size Select** (Macherey Nagel, Ref 744970.50)
 - Fragmentation evaluated by capillary electrophoresis (fragment analyser)
 - Library qualification: <50% of fragment below 160 nt are expected.
-
- Sequencing on Ion Proton according to protocol
 - Multiplexing is highly variable according to the expected complexity of the samples. max is 48 libraries (for purified virus samples)
 - For metagenomics (ex virus discovery) analysis 10 Millions reads are expected in first instance.

APHA (10) Workflow Fragmentation to Sequencing

Contact person: Ellis, Richard (APHA) <Richard.Ellis@apha.gsi.gov.uk>

APHA routinely utilize Illumina kits and instruments for high-throughput sequencing of a wide variety of samples. One of the key drivers of our workflow is that it is applicable for many different sample types and is suitable where minimal input material is available. Therefore, all sequencing libraries are generated with Illumina's Nextera XT kit, as it only requires 1 ng DNA as input and can be used for purified bacterial genomic DNA, crude heat-kill lysates of bacteria, PCR amplicons, and double-stranded DNA (generated from RNA samples). Our protocol is slightly modified from the manufacturers' recommendations, primarily by omitting the complicated and inefficient bead-normalization step, and is available on the COMPARE sharepoint site: [NexteraXT library preparation](#). The method incorporates both the fragmentation and size selection processes and can be completed in under 4 hours for up to 96 samples. Quantification of libraries is achieved using a simple fluorometric protocol ([Library Quantification](#)) and these are then sequenced either on an Illumina MiSeq ([MiSeq Operation](#)) or an Illumina NextSeq both with 2x150 paired end reads. This approach allows us to achieve a rapid turnaround of multiple sample types and to make efficient use of the sequencing capacity available at APHA.

AUTH (20) Workflow Fragmentation to Sequencing

Contact person: Anna Papa <annap@med.auth.gr>

Bacterial Metagenomics protocol - 16S rRNA next generation sequencing

Background

In order to find out the bacteria included in clinical samples and in vectors (e.g. ticks), the Ion 16S™ Metagenomics assay can be applied. The kit is designed for rapid analyses of polybacterial samples using Ion Torrent™ sequencing technology. The kit includes two primer sets that selectively amplify the corresponding hypervariable regions of the 16S region in bacteria. Sequencing of the amplified fragments is performed on the Ion PGM™ System and the analysis of the results is done with the Ion Reporter™ software Ion 16S™ Metagenomics Kit analyses module. The combination of the two primer pools allows for sequence-based identification of a broad range of bacteria within a mixed population.

The sequences are analysed using the metagenomics workflow in the Ion Reporter™ Software that enables the identification of bacteria present, at the genus or species level. Raw reads are quality filtered and aligned against a reference database of 16S rRNA gene sequences.

Equipments

- Personal Genome Machine, Ion Torrent (Life Technologies).
- Ion OneTouch 2 instrument
- Ion OneTouch ES instrument
- Qubit fluorometer (Life Technologies)
- Centrifuge, waterbath, vortex
- ABI7500 real time PCR system

1. DNA extraction

- Extract total genomic DNA from 200µl of sample using the Qiagen DNA mini kit (51304 Qiagen, Lubeck, Germany) according to the manufacturer's instructions
<https://www.qiagen.com/us/resources/resourcedetail?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en> (Blood or body fluid spin protocol)
- Final elution at 50 µl
- Store DNA at -20°C until use.
- Measure the total DNA concentration of the extracted DNA using the Qubit fluorometer (Life Technologies) with the Qubit dsDNA HS assay (Life Technologies).
https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

2. 16S rRNA amplicon and library preparation (A26216, Life Technologies)

Construction of DNA libraries for next-generation sequencing is performed using two primer sets that selectively amplify the corresponding hypervariable regions of the 16s region in bacteria (V2, 3, 4, 6, 7, 8, 9). These primers contain adaptors for Ion Torrent sequencing and Ion Xpress barcodes so that the PCR products could be pooled and sequenced directly.

- Amplify the 16S hypervariable regions
- Purify the amplification products
- Calculate DNA input for library preparation.
- End repair and purify pooled amplicons with AMPure beads
- Ligate the Ion Xpress barcode adapters and nick-repair using Ion Plus Fragment Library kit (Life Technologies).
- Purify the adapter-ligated and nick-repaired DNA using Agencourt AMPure beads (Leriva), eluted in low Tris-EDTA (TE) buffer, and quantified by using a Qubit dsDNA HS (high sensitivity) kit (Life Technologies).
- Determine library concentration using Ion Universal Library Quantification kit (Life Technologies Corporation, Carlsbad, CA, USA) in ABI7500 real time PCR system.
- Adjust library concentration to 26 pM.
- Pool the resulting PCR libraries equimolarly.

Detailed instructions are given in:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010799_Ion_16S_Metagenomics_UG.pdf

3. Template preparation (kit A27739, Life Technologies).

- Prepare the template in Ion Hi-Q Template One-Touch 2
- Enrich the amplified Ion Sphere Particles using Dynalbeads MyOne Streptavidin C1 beads (Life Technologies) using the Ion One-Touch ES system according to the manufacturer's protocol.
- Perform the **Emulsion PCR** of DNA linked ISPs in Ion OneTouch 2 instrument according to the manufacturer's instructions, using Ion PGM Hi-Q Template kit.
- **Enrich** the template positive ISPs with Ion OneTouch ES instrument according to the manufacturer's instructions using Ion PGM Hi-Q Template kit.

Detailed instructions are given in:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010902_PGM_HiQ_OT2_Kit_UG.pdf

or the quick guide:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010903_PGM_HiQ_OT2_Kit_QR.pdf

4. Sequencing

- Sequence the Ion Xpress barcoded library in the Personal Genome Machine, Ion Torrent (Life Technologies).

* A maximum of 16 barcoded samples can be pooled and loaded in a 316 chip (Life Technologies, MA, USA) using the Ion PGM Hi-Q (400) Sequencing Kit (Life Technologies Corporation, Carlsbad, CA, USA), according to the following recommended protocol:

https://tools.thermofisher.com/content/sfs/manuals/MAN0009816_Ion_PGM_HiQ_Sequencing_Kit_UG.pdf

or the quick guide:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010863_Ion_PGM_HiQ_Sequencing_Quick_Guide.pdf

Viral Whole Genome Sequencing

Equipments

Personal Genome Machine, Ion Torrent (Life Technologies).

Ion OneTouch 2 instrument

Ion OneTouch ES instrument

Qubit fluorometer (Life Technologies)

Centrifuge, water-bath, vortex

ABI7500 real time PCR system

1. RNA extraction

RNA extraction with QIAamp® Viral RNA mini kit (52904, QIAGEN) according to manufacturer's instructions:

<https://www.qiagen.com/mx/resources/resourcedetail?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en> (follow the spin protocol)

- Centrifuge 140 µl cell culture supernatant (or any other sample type) at 6.000g for 10 minutes at 4°C and use the supernatant
- Digest with RNase A (20mg/ml) and 20µl Proteinase K (20mg/ml)
- Incubate for 1 hour at RT, each step

- Digest on-column with DNase I (10µl DNase stock+70µl RDD buffer) for 15 min at RT
after the first wash with Wash Buffer 1, followed by a second wash with Wash Buffer 1.
- Final elution at 50 µl
- Store RNA at -80°C

2. cDNA first strand synthesis

Kit: Superscript III reverse transcriptase (18080051, Life technologies).

- 2µl random hexamers (50µM)
 - 1µl dNTPs (10mM)
 - 10µl RNA
 - Incubate for 5 min at 65°C and put immediately on ice for 1 min.
 - Add 4µl 5X First-Strand buffer, 1µl of 0,1M DTT, 1µl of RNase OUT, 1µl of SuperScript III
 - Final cDNA volume: 20µl/reaction
-
- Thermocycling conditions: 25°C for 5 min, 55°C for 60 min and 70°C for 15 min.

*sometimes two cDNA reactions per sample are applied

3. Second strand (dsDNA) synthesis

Kit: Klenow fragment (M0210S, New England Biolabs)

In the tube with the 1st strand cDNA add:

- 2,5µl of NE buffer 2
- 1µl of random hexamers (50µM)
- 1µl dNTP's (10mM)
- Heat at 95°C for 2 min.
- Add 1µl of DNA Polymerase I, Large (Klenow) Fragment

Thermocycling conditions: 37 °C for 60min and 75 °C for 15min.

Final volume after synthesis is 50µl

4. Qubit quantitation

Concentration of ds cDNA is measured with Qubit with Qubit dsDNA HS assay kit (Q32851, Life Technologies) and Qubit assay tubes (Q32856, Life Technologies) according to the manufacturer's instructions. 2µl of ds cDNA are used for the measurement. Detailed instructions are given at:

https://tools.thermofisher.com/content/sfs/manuals/Qubit_dsDNA_HS_Assay_UG.pdf

5. DNA fragmentation

Shear DNA with shearing enzymes included in the Ion Xpress™ Plus Fragment Library Kit (4471269, including Ion Shear Plus reagent kit, Life Technologies) according to the manufacturer's instructions.

For 200 bp fragment size, Ion Shear reaction time is 15min.

Purify with Agencourt AMPure XP reagent (Leriva, A63881) using x1.8 reagent per sample volume.

Use 1.5ml PCR clean DNA LoBind tubes (0030108051, Eppendorf).

6. Library preparation and size selection

Ligate Ion Xpress adapters with the Ion Xpress™ Plus Fragment Library Kit (4471269, Life Technologies) according to the manufacturer's instructions

Purify with Agencourt AMPure XP reagent (Leriva, A63881) using x1.2 reagent volume per sample volume.

Barcode with the Ion Xpress™ Plus Fragment Library Kit (4471269, Life Technologies) and Ion Xpress™barcode adapters 1-16 (4471250) according to the manufacturer's instructions

Select 200bp fragments with E-gel electrophoresis using E Gel size select 2% (G661002, Life technologies) and a 50bp DNA ladder (50µg, 10416014, Life Technologies).

Amplify the Library with Platinum PCR Supermix Hi-Fi (Life Technologies) from Ion Xpress™ Plus Fragment Library Kit (4471269, Life Technologies) according to the manufacturer's instructions for 8 cycles.

Purify with Agencourt AMPure XP reagent (Leriva, A63881) using x1.2 reagent volume per sample volume.

7. Library quantitation

Quantify the Library with real time PCR with Library TaqMan® Quantitation Kit (4468802, Life Technologies) according to the manufacturer's instructions.

Dilute the library at 26pM and mix the barcoded samples in equal concentrations.

For steps 5-7 follow instructions:

https://tools.thermofisher.com/content/sfs/manuals/MAN0009847_IonXpressPlus_gDNA_FragLibraryPrep_UG.pdf

8. Template preparation (A27739, Life Technologies).

Proceed in **Emulsion PCR** of DNA linked ISPs in the Ion OneTouch 2 instrument according to the manufacturer's instructions, using Ion PGM Hi-Q Template kit (A27739, Life Technologies).

Enrich the template positive ISPs with Ion OneTouch ES instrument according to the manufacturer's instructions using Ion PGM Hi-Q Template kit (A27739, Life Technologies).

For step 8 follow the instructions:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010902_PGM_HiQ_OT2_Kit_UG.pdf

or the quick guide:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010903_PGM_HiQ_OT2_Kit_QR.pdf

9. Sequencing

Use 316 chips (4483188, Life Technologies) with Ion PGM Hi-Q (200) chemistry (Ion PGM Hi-Q Sequencing kit, A25592) at the PGM genetic analyser (Ion Torrent, Life Technologies).

Detailed instructions are given at:

https://tools.thermofisher.com/content/sfs/manuals/MAN0009816_Ion_PGM_HiQ_Sequencing_Kit_UG.pdf

or in the quick guide:

https://tools.thermofisher.com/content/sfs/manuals/MAN0010863_Ion_PGM_HiQ_Sequencing_Quick_R.pdf

DTU (1) Workflow Fragmentation to Sequencing

Contact person: Rene S. Hendriksen <rshe@food.dtu.dk>

1. A summary paragraph of the method including the references and links and why this is important.

See item 4

3. The actual method as a lab protocol. If you have used manufacturer's protocols, simply send a link to the manufacturer's documentation. Same for a published method.

1. DNA fragmentation.

2. Library preparation.

https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-02.pdf

3. Quantification.

<https://tools.thermofisher.com/content/sfs/manuals/mp32866.pdf>

4. Sequencing.

https://support.illumina.com/content/dam/illumina-support/documents/documentation/system_documentation/miseq/miseq-system-guide-15027617-01.pdf

5. Modifications:

Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. Knudsen BE, Bergmark L, Munk P, Lukjancenko O, Priemé A, Aarestrup FM, Pamp SJ.

mSystems. 2016 Oct 18;1(5). pii: e00095-16. eCollection 2016 Sep-Oct. PMID: 27822556 Free PMC Article

4. If you made modifications in the original method, add a short description of what you modified, why, and how you checked that the modification was an improvement over the manufacturer's method.

Impact of Sample Type and DNA Isolation Procedure on Genomic Inference of Microbiome Composition. Knudsen BE, Bergmark L, Munk P, Lukjancenko O, Priemé A, Aarestrup FM, Pamp SJ.

mSystems. 2016 Oct 18;1(5). pii: e00095-16. eCollection 2016 Sep-Oct. PMID: 27822556 Free PMC Article

EMC (2) Workflow Fragmentation to Sequencing Ion Torrent

Contact person: B.B. Oude Munnink <b.oudemunnink@erasmusmc.nl>, My Phan <v.t.m.phan@erasmusmc.nl>

For library preparation (from fragmentation to final libraries for deep sequencing), we used the Thermo Fisher's protocol (available at

https://tools.thermofisher.com/content/sfs/manuals/MAN0007044_PrepAmpliconLibrFragment_using_IonXpressPlusFragKit_UB.pdf).

For quantification of each library, we used the Thermo Fisher’s protocol

(https://tools.thermofisher.com/content/sfs/manuals/MAN0015802_IonLibrary_Taqman_Quantitation_Kit_UG.pdf).

All libraries were normalised at 40 pM and pooled for Ion CHEF run, and then sequenced on an S5XL sequencer.

EMC (2) Workflow Fragmentation to Sequencing Roche 454

Contact person: M. de Graaf <m.degraaf@erasmusmc.nl> , C.M.E. Schapendonk <c.schapendonk@erasmusmc.nl>

* **A random PCR was done with the products of the klenow reaction of the samples:**

	One reaction (µl)	Lot no.
Buffer (10x)	5	N13191
MgCl2 (25 mM)	5	N13201
dNTP's (10mM)	1	11524000
FR20RV primer (10pmol/µl)	4	4081856
Amplitaq gold (5U/µl)	0,5	N11382
H2O	29,5	-
cDNA	5	-

PCR program:

10 min. 94°C

1 min. 94°C		→ 40 x
1 min. 65°C		
2 min. 72°C		

10 min. 72°C

PCR products are stored at -20°C

* **A part of the undiluted PCR products of the random PCR done with the samples was run on gel (454 seq):**

Note: - 5 µl PCR products + 1 µl loadingbuffer

- 5 µl smartladder was used; 30 minutes at 100V (100 ml gel).

* **PCR products of the random PCR done with the samples were pooled and purified (454 seq):**

- 25 µl of the PCR products of the RNA random protocol were pooled with 25 µl of the PCR products of the DNA random protocol for each sample and used for purification.

The PCR products were purified using the MinElute PCR Purification kit (Qiagen) using the following protocol:

*MinElute PCR Purification Kit Protocol
(using a microcentrifuge)*

Fragments ranging from 70 bp to 4 kb are purified from primers, nucleotides, polymerases, and salts using MinElute spin columns in a microcentrifuge.

Important points before starting:

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow

color of Buffer PB with pH indicator I indicates a pH of ≈ 7.5 .

- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR reaction and mix. It is not necessary to remove mineral oil or kerosene.

For example, add 250 μl of Buffer PB to 50 μl PCR reaction (not including oil).

50ul PCR mix + 250ul Buffer PB

2. If pH indicator I has been added to Buffer PB, check that the color of the mixture is yellow. If the color of the mixture is orange or violet, add 10 μl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.
4. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 min.
For maximum recovery, transfer all traces of sample to the column.
5. Discard flow-through. Place the MinElute column back into the same tube.
6. To wash, add 750 μl Buffer PE to the MinElute column and centrifuge for 1 min.
7. Discard flow-through and place the MinElute column back in the same tube.
Centrifuge the column for an additional 1 min at maximum speed.
IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place the MinElute column in a clean 1.5 ml microcentrifuge tube.

PCR Purification

9. To elute DNA, add 17 μ l **TE buffer** to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.
IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA.

* **DNA concentrations of the purified PCR products of the random PCR done with the samples were measured (454 seq):**

- DNA concentration was measured with the Nanodrop (VC-0366)
- Measure 1 μ l of the purified PCR products with the Nanodrop.

* **Dilutions were made of the purified PCR products of the random PCR done with samples E1000225-53 (454 seq):**

- Take 1500ng PCR products and resuspend them in a total volume of 16ul TE buffer

* **Fragment end repair was performed with diluted products of the random PCR done with the samples (454 seq):**

Fragment end repair was performed following the “Rapid Library Preparation Method Manual” section 3.2.

(*GS Junior Titanium Serie*, May 2010 (Rev. June 2010), Roche)

* **Thaw the RL 10x Buffer, dNTP Mix, ATP, Adaptor and Standard (-20°C, Rapid Library Reagents)**

* **Thaw the MID adaptors (-20°C, GS FLX Rapid Library MID Adaptors Kit)**

- **PCR machine**

1. In a 1.7 ml microcentrifuge tube, prepare the End Repair mix, as follows.

	For one reaction (µl)	Pre mix (µl) (14x)
RL 10x Buffer	2.5	35
RL ATP	2.5	35
RL dNTP	1	14
RL T4 polymerase	1	14
RL PNK	1	14
RL Taq polymerase	1	14

2. Pipet up and down to mix, and add the **9 µl** of End Repair mix to the DNA sample:
3. Vortex for 5 seconds, then spin for 2 seconds in a mini centrifuge.
4. Run the End Repair program on a thermocycler, with the heated lid turned on:

25°C for 20 min

72°C for 20 min

4°C on hold

* **An adaptor was ligated to the end repaired products of the random PCR done with the samples (454 seq):**

Adaptor ligation was performed following the “Rapid Library Preparation method manual” section 3.4.

(*GS Junior Titanium Serie*, May 2010 (Rev. June 2010), Roche)

1. Once the End Repair program has completed from Section 3.2, add **1 µl** of RL Adaptor or RL MID Adaptor to the reaction tube.
2. Add 1 µl of RL Ligase to the reaction tube.
3. Vortex 5 seconds, then centrifuge for 2 seconds in a mini centrifuge.
4. Incubate at **25°C** for **10 minutes** on the thermocycler.

* **The adaptor ligated products of the random PCR done with the samples were purified (454 seq):**

The PCR products were purified using the “Agencourt AMPure XP” (Agencourt, Beckman Coulter) using the following protocol:

Agencourt AMPure XP Protocol (using a Magnetic Particle Concentrator (MPC)).

Fragments > 100 bp are purified from primers, nucleotides, polymerases, and salts.

- Prepare 70% ethanol in the amount needed (2 ml per sample). For 10 ml, add 7 ml of 100% ethanol to 3 ml Molecular Biology Grade Water, and vortex.

- 1) Vortex the AMPure bead bottle for 20 seconds or until the beads are completely resuspended.
- 2) Add 125ul Agencourt AMPure XP to the sample (25ul).
- 3) Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
- 4) Incubate for 5 minutes at room temperature.
- 5) Place the tube on the MPC.
- 6) When the beads have fully pelleted on the wall of the tube, carefully remove and discard the supernatant.
- 7) Keeping the tube on the MPC, wash the beads **three times**, as follows:
 - a. Add 1 ml of 70% ethanol.
 - b. Completely remove and discard the ethanol.
- 8) Keeping the tube on the MPC, uncap the tube and air dry the pellet at room temperature for 2 minutes.
- 9) Remove the tube from the MPC.
- 10) Add 55 μ l of TE Buffer to the tube. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
- 11) Place the tube on the MPC, wait for the beads to pellet on the wall of the tube and transfer ~55 μ l of the SUPERNATANT containing the library into a new 1.7 ml tube. Make sure not to carry-over any beads in this process as they will cause incorrect readings during library quantitation.

(A clean up step must be done to remove the left over adaptor!!!)

* **Prepared library products of the random PCR done with the samples**

were quantified and working stocks were made (454 seq):

Library quantification was performed following the “Rapid Library Preparation method manual” section 3.7.1, 3.7.3, 3.7.4.1 and the “Template 454 library dilution” file.
(*GS Junior Titanium Serie*, May 2010 (Rev. June 2010), Roche)

To generate the standard curve, begin by labeling 8 tubes, 1 to 8.

1. In tube 1, prepare a 2.5×10^9 molecule/ μl solution of the RL Standard by mixing **90 μl** of the RL Standard (orange cap) with **90 μl** of TE Buffer.
2. Fill the remaining 7 tubes (tubes 2 to 8) with **60 μl** of TE Buffer.
3. Transfer **120 μl** from tube 1 into tube 2.
4. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
5. Change pipette tip and transfer **120 μl** of tube 2 into tube 3.
6. Vortex for 5 seconds and spin for 2 seconds in a mini centrifuge.
7. Proceed with the same serial dilution (transferring **120 μl** of one tube into the next, vortexing for 5 seconds, and changing pipette tip between each dilution) for the remaining 5 tubes.

3.7.3 Using a 96-well Plate Fluorometer

Excitation 485nm, Emission 535nm

1. Dilute the sample library 20 times in TE (3 μl + 57 μl TE)
2. Distribute on the black 96-well plate **50 μl** of each standard.
3. Transfer **50 μl** of the 20 times diluted sample library into an individual well.
4. Read and record the relative fluorescence units (RFU) for the standards and sample library.

Plate reader:

- Insert the filter with 485nm and 535nm filters:
 - Instrument > Movements > Filter out
 - Insert the 480/532nm filter

- Plate:
 - Select: Corning 96 flat black
 - Select the wells of the plate that must be measured

- Select: Fluorescence intensity (measurements)
 - Wavelength:
 - Excitation: 485nm
 - Emission: 535nm
 - Mode:
 - ✓ Top
 - Integration:
 - Lag time: 0 us
 - Integration time: 20 us
 - Multiple reads per well:
 - ✓ Multiple reads per well:
 - Type: Circle (filled)
 - Size: 4x4
 - Border: 500uM

- Read:
 - Number of Flashes: 25
 - Settle time: 0 ms
 - Gain:
 - ✓ Optimal
 - Label:
 - Name: Label 1

If the relative fluorescence units (RFU) for the sample library falls above the Standard curve, make a dilution of the sample and measure both the standards and the diluted sample again.

DO NOT DISCARD THE SAMPLE.

5. Transfer the sample library back to its tube.

3.7.4.2 Quantification by using Excel software

Calculation of the amount of molecules/ μl of the samples and how to dilute the samples to get $1 \cdot 10^7$ molecules/ μl :

- Fill in the RFU values of the measured standard solutions
- Change the values of the formula for calculation of the amount of molecules/ μl in the sample, to the values corresponding with those of the formula of the standard curve
- Make a copy of the excel file to your lab journal

3.8 Preparing Working Aliquots

Dilute an aliquot of the DNA library to a working stock of **1×10^7 molecules/ μl** , in TE Buffer. See results of 3.7.4.1.

FLI (4) Workflow Fragmentation to Sequencing

Contact person: Wylezich, Claudia <Claudia.Wylezich@fli.de>, Beer, Martin <Martin.Beer@fli.de>

These protocols are for sequencing with Illumina MiSeq (automated protocol) and Ion Torrent platforms (manual protocol) from fragmentation to quantification of the library but excluding the sequencing procedure itself for that we have no LOP and use the manufacturer's protocol (see https://tools.thermofisher.com/content/sfs/manuals/MAN0014580_IonPGM_HiQ_View_OT2_QR.pdf, https://tools.qa2.thermofisher.com/content/sfs/manuals/MAN0010849_Ion_520_530_OT2_QR.pdf for Ion Torrent protocols).

From fragmentation to quantification of the library

Library preparation: fragmentation

CAUTION Never run a method without AFA-grade water in the acoustic assembly; this could damage the transducer. Do not leave water in the water bath for an extended time. Empty the water bath and wipe it dry

after use with a lint-free cloth. Do not employ isopropyl alcohol, ammonia-based or abrasive cleaners on the acoustic assembly. Store the tube holder in a dry place.

- 1| Pipette 130 μ l sample in the sample tube (if the sample volume is less than 130 μ l fill up to 130 μ l with water). Place the tube in the microTube holder and fix it with the sample weight.
- 2| Start protocol via the start button, repeat fragmentation once by pressing the repeat button. Use the pre-installed DNA shearing protocol DNA_0500_bp_130_μl_Snap_Cap_microTUBE.
- 3| Pipette the fragmented sample into a clean 1.5 ml tube. If the sample volume is greater than 130 μ l, repeat steps 1-2 using the same microTube.

Purification and concentration of fragmented cDNA synthesis products

CAUTION When opening a new lot of AMPure XP beads, perform a bead calibration before first use. Aliquot the AMPure XP beads in order to prevent repeated warming and cooling as this will degrade the buffer and render the beads non-functional. Make sure the Agencourt AMPure XP beads are at room temperature and are thoroughly mixed. The reagent (magnetic particle solution) should appear homogenous and consistent in colour. Do not freeze.

- 1| Add 1.8 volumes of well-mixed Agencourt AMPure XP beads to each cDNA sample and mix the DNA-bead-solution thoroughly by pipette mixing (15x) and incubate 10 min at room temperature on a shaker at 300 r.p.m.
- 2| Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall).
- 3| Slowly aspirate the cleared solution from the reaction tube without disturbing the beads and discard the solution; keep the reaction tube in the MPC while aspirating the solution.
- 4| Dispense 1 ml 75% ethanol to the reaction tube and incubate 30 s at room temperature; slowly aspirate the ethanol from the reaction tube and discard.
- 5| Repeat step 4 while the reaction tube keeps on MPC.
- 6| Air-dry the pellet for 10 min with the cap open (keeping on MPC); do not over-dry!
- 7| Take reaction tube from MPC, elute DNA with 27 μ l Nuclease-free water by careful pipette mixing and incubate for 2 min.
- 8| Place the reaction tube onto the MPC, incubate for 2 min. Keeping the tube on the MPC, pipette 25 μ l of the concentrated DNA solution into a clean reaction tube.

Library preparation

There are 2 possibilities for library preparation, one manual preparation to be sequenced with the Ion Torrent PGM and one automated preparation to be sequenced with the Illumina MiSeq.

CAUTION To avoid contaminations: Tubes containing fragmented and concentrated DNA must exclusively be opened and handled in a template box. Buffers and reagents for preparing mastermixes must exclusively be opened and handled in a clean mastermix box. **Do not use the same barcode as in the sequencing run before.** Change gloves after handling template DNA before working in the mastermix box.

Library preparation for sequencing with Ion Torrent PGM: end-repair

- 1| Thaw, mix, and shortly centrifuge both end repair and ligation buffer, adapters, and dNTPs and keep them on ice.
- 2| Mastermix box: Pipette 2.5 µl of End-Repair Buffer in one 0.2 ml PCR tube per DNA sample. Add 2 µl End-Repair Enzyme Mix and keep the PCR tube on ice
- 3| Template box: Keep DNA samples on MPC some minutes before pipetting to avoid transfer of residual AMPure beads. Add 20.5 µl DNA sample to buffer and enzyme mix for a final total volume of 25 µl. Briefly vortex and centrifuge. *Optionally, save 2 µl of fragmented DNA for troubleshooting.*
- 4| Incubate in the thermocycler with following temperature profile:

Temperature	Time
25°C	20 min
70°C	10 min
4°C	∞

- 5| Briefly before the incubation is finished, start preparing the mastermix for the adapter ligation (see step 6). Before proceeding to the mastermix box, change gloves.

Library preparation for sequencing with Ion Torrent PGM: adapter ligation

- 6| Mastermix box: Prepare mastermix for the adapter ligation:

Components	1 x	x
RNase-free water	8.0 µl	µl
Ligation Buffer (2x)	40.0 µl	µl
dNTP Mix (10 mM)	1.0 µl	µl
Universal adapter P1 (to 0.5 µM final conc.)	1.0 µl	µl
Ligation and Nick Repair Mix	4.0 µl	µl
Total volume	54.0 µl	µl

- 7| Briefly vortex and centrifuge the mastermix.
 - 8| Mastermix box: Pipette 54 µl of mastermix in one 0.2 ml PCR tube per DNA sample.
 - 9| Mastermix box: Add 1 µl of the chosen barcode adapter to each mastermix aliquot.
- CAUTION** To avoid contaminations, open only one barcode adapter tube at a time and change gloves between pipetting the different adapters to avoid cross-contamination.
- 10| Template box: Add 25 µl end-repaired DNA (from part End-repair, above), briefly mix and centrifuge. Incubate in the thermocycler with pre-set temperature profile:

Temperature	Time
25°C	10 min
72°C	5 min
4°C	∞

Purification and concentration of IonTorrent Library

CAUTION The end-repaired and adapter-ligated library has to be purified to remove excess of polyethylene glycol (PEG), salts, and enzymes. Otherwise, the high concentration of PEG in the ligation mix will interfere with proper size selection.

- 11| Template box: (steps 11-19): Pipette 144 µl (1.8 volumes) of well-mixed Agencourt AMPure XP beads to 80 µl of end-repaired and adapter-ligated DNA library and mix the DNA-bead-solution thoroughly by pipette mixing (15x). Incubate 10 min at room temperature on a shaker at 300 r.p.m.
- 12| Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall).
- 13| Leaving the tube in the MPC, slowly aspirate the supernatant from the tube without disturbing the beads and discard the solution.
- 14| Dispense 1 ml 75% ethanol to reaction tube and incubate 30 sec at room temperature and slowly aspirate the ethanol from the reaction tube and discard.
- 15| Repeat step 14 while the reaction tube keeps on MPC.
- 16| Air-dry the pellet for 10 min with the cap open (keeping on MPC); do not over-dry!
- 17| Take reaction tube from MPC, elute DNA with 27 µl Nuclease-free water by careful pipette mixing and incubate for 2 min.
- 18| Place the reaction tube onto the MPC, incubate for 2 min, and pipette 25 µl of the concentrated DNA solution into a clean reaction tube.
- 19| Repeat steps 17-18 to increase the yield of library, pool both elutions. *Optionally, save 2 µl of the library for troubleshooting.*

Automated library preparation with the SPRIworks Fragment Library System II for sequencing with Illumina MiSeq:

CAUTION Blunt end adapters are not compatible with SPRIworks Fragment Library System II. Do not change the method card while instrument is powered on. To avoid contaminations, do not use the same barcode as in the sequencing run before.

- 1| Completely thaw, mix, and briefly centrifuge all components.
- 2| Mastermix box: Prepare 3 labelled screw cap tubes provided with the kit for each library: sample tube, adapter tube, library tube. Take off all caps of the tubes; keep the caps of the library tubes.

3| Pipet 2,5 µl of one of the different barcode adapters to the adapter tube and dilute with 2,5 µl Nuclease-free water.

CAUTION To avoid contaminations, **open only one barcode adapter tube at a time and change gloves between pipetting the different adapters to avoid cross-contamination.**

4| Template box: Transfer 500 ng fragmented DNA in a maximum of 400 µl to the **sample** tube, fill up to 400 µl with Nuclease-free water.

5| SPRI-TE Nucleic Acid Extractor (Steps 5-15): Set up the instrument: Turn off the instrument - Press the EJECT button, remove any method card if necessary and carefully insert the SPRIworks method card for Fragment Library System II (REF A85410) - Close the instrument door - Turn the power on.

6| Open the door only when the screen displays the TOP MENU.

7| When cartridges have completely thawed, ensure that all solutions including magnetic beads are at the bottom of the wells by gently tapping on the bench top.

8| Remove tip/tube rack and reagent rack (in this order) from the instrument.

9| Insert one cartridge per library into the grooved channel of the reagent rack by grasping the labelled lip and sliding it to the back of the channel.

10| Arrange the tubes and tips provided with the kit as displayed in the Table below:

Position in the tip tube rack	Consumables
1	Sheath with Piercing Tip
2	Sheath with 1 ml Tip
3	Sheath with 200 µl Tip
4	Uncapped 2 ml Adapter Tube with Adapters
5	Uncapped 2 ml Library Tube

11| Load reagent rack and tip/tube rack (in this order) into the instrument; the back edge of the tip/tube rack overlaps the reagent rack when loaded correctly.

12| Load the uncapped sample tube containing the fragmented DNA into the circular hole in position 13 of the reagent rack.

13| Start the run: Close the instrument door - Select the green START button - Specify 1 = no size selection and select ENTER to confirm - After a heat block test, the run begins automatically.

14| The run is finished when the instrument displays “Run Complete” - Open the door - Retrieve and cap the library tubes - Discard used sheaths, tips and cartridges.

15| To clean the instrument, select UV-Clean from the TOP MENU.

Size selection of library

1| Template box: (steps 1-16): Resuspend an aliquot of the magnetic particle solution via vortexing. For the upper size selection, dilute Agencourt AMPure XP beads per the table:

	1 x	x
AMPure XP beads	104.0 μ l	μ l
Water (PCR grade)	80.0 μ l	μ l
Sum	184.0 μl	μl

2| Fill the generated library (from steps above depending on sequencing platform) up to 100 μ l with Nuclease-free water.

3| Add 160 μ l **diluted** Agencourt AMPure XP beads per DNA library to remove large fragments and mix the DNA-bead-solution thoroughly by pipette mixing (15x). Incubate 5 min at room temperature on a shaker at 300 r.p.m.

4| Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall).

5| Slowly aspirate the cleared solution from the reaction tube without disturbing the beads and pipette the solution into a clean 1.5 ml tube in two steps á 125 μ l (exact final volume 250 μ l).

6| Add 30 μ l **undiluted** Agencourt AMPure XP beads per 250 μ l supernatant from step 5 to remove small fragments and mix the DNA-bead-solution thoroughly by pipette mixing (15x). Incubate 5 min at room temperature on a shaker at 300 r.p.m.

7| Place the reaction tube onto the MPC for 5 min to separate the beads from supernatant (until the beads have concentrated on the tube wall).

8| Leaving the tube in the MPC, slowly aspirate the supernatant in two steps á 138 μ l from the tube without disturbing the beads and discard the solution.

9| Leaving the tube in the MPC, dispense 200 μ l 75% ethanol to reaction tube and incubate 30 s at room temperature.

10| Slowly aspirate the ethanol from the reaction tube and discard.

11| Repeat step 9-10 while the reaction tube keeps on MPC.

12| Air-dry the pellet for 10 min with the cap open (keeping on MPC); do not over-dry!

13| Take reaction tube from MPC, add 18 μ l Nuclease-free water by careful pipette mixing and incubate for 2 min.

14| Place the reaction tube onto the MPC, incubate for 2 min and pipette 15 μ l of the DNA solution into a clean reaction tube.

15| Take reaction tube from MPC, add 15 μ l Nuclease-free water by careful pipette mixing and incubate for 2 min.

16| Place the reaction tube onto the MPC, incubate for 2 min and pipette 15 µl of the DNA solution into a clean reaction tube.

> **PAUSE POINT** Store the completed library in a safe-lock tube at 4°C or continue directly with quality check.

Quality check and quantification of library

1| Ensure the absence of too short or too long fragments, and of primer dimers using an Agilent Bioanalyzer High Sensitivity DNA assay (with the High Sensitivity DNA.xsy assay).

2| Continue directly with quantification of the library using the KAPA Library Quantification Kit for Illumina or Ion Torrent sequencing.

CAUTION Make sure to select the appropriate kit for the used sequencing platform and the real-time PCR cycler you use!

3| Completely thaw, mix, and briefly centrifuge all kit components.

4| Mastermix box: Before first use, prepare buffer qPCR/Primer mix from the Kit: Add 1 ml of Primer Premix (10×) to 5 ml bottle of KAPA SYBR FAST qPCR Master Mix (2×) and mix thoroughly. This mix is stable for 1 year when stored closed at -20°C.

5| Mastermix box: Prepare a mastermix using the following Table; calculate 6 reactions per DNA library plus 12 reactions for standards plus additional positive controls, if used, plus 2 no-template-controls (NTC) plus 0.5 reactions in excess.

Component	1 x	x
Nuclease-free water	4.0 µl	µl
KAPA SYBR FAST qPCR Mastermix (including Primer mix)	12.0 µl	µl
Total volume	16.0 µl	µl

6| Vortex and briefly centrifuge.

7| Mastermix box: Pipette 16 µl mastermix in each used well of a 96-well microtiter plate.

8| Template box: Dilute sample DNA only with dilution buffer: 3 dilutions with duplicates are needed. Keep sample DNA on MPC during pipetting to avoid transferring AMPure XP beads. Based on the Bioanalyzer data (see step 1), select adequate library dilutions (each 3 dilutions); use the following values as guidance:

- No detection with Bioanalyzer DNA HS Chip → use dilutions 1:20; 1:40; 1:80
- Weak signal with Bioanalyzer DNA HS Chip → use dilutions 1:200; 1:400; 1:800
- High signal with Bioanalyzer DNA HS Chip → use dilutions 1:2,000; 1:4,000; 1:8,000
- Amplified Libraries → use dilutions 1:20,000; 1:40,000; 1:80,000

9| Template box: Add 4 µl of diluted DNA library or standard to the respective wells, final reaction volume 20 µl.

10| Seal the plate with sealing film, press well on. Briefly centrifuge the plate using a microtiter plates centrifuge.

11| Insert the plate into the cycler and wipe the seal clean before closing the lid. Enter parameter, sample names and dilutions in the cycler software and start the KAPA PCR program.

	Step	Temperature	Time
1x	1	95°C	5 min
35x	2	95°C	30 sec
	3	60°C	45 sec
1x	4	4°C	∞
1x	Melting curve analysis		

12| After completion of the run, check the integrity and plausibility of the PCR results, e.g. fluorescence intensity, amplification curves, melting curves, R^2 of the standard curve ≥ 0.95 , PCR efficiency ≥ 0.95 , efficiencies between 0.90 and 0.95 are acceptable if R^2 is ≥ 0.99 , Cq values of different sample dilutions and the positive control (Illumina).

Calculate DNA library molarities from the qPCR results and optionally pool several libraries with different barcode adapters for one sequencing run.

For the reagents and equipment needed as well as the reagent setup, please see the single LOPs provided in the appendix of this Deliverable.

FLI LOPs (in WP2_D2.3_Appendix)

LOP_DNA_Frag_Covaris_FLI_v3.pdf

LOP_LibQual-check_HS-chip_FLI_v1.pdf

LOP_LibQuant_Kapa_FLI_v1.pdf

LOP_Library-prep_IonTorrent_FLI_v1.pdf

LOP_Library-prep_automated_IlluminaMiSeq_FLI_v1.pdf

LOP_Purification_IonTorrent-Library_FLI_v1.pdf

LOP_Purification_fragmented-cDNA_FLI_v1.pdf

LOP_SizeSelection_Library_FLI_v1.pdf

IFREMER (21) Workflow Fragmentation to Sequencing

The following LOP for RNA fragmentation, cDNA synthesis and library preparation were used.

For library quantification Qubit ssDNA kit (life technology Cat Q32850) and Kapa Library



Quantification (cat KK4873, real time sybr green PCR) were used. For the sequencing a MiSeq instrument was used with the kit Miseq Reagent kit v2-300cycles multiplexing no more than 7 samples per run.

[See LOP_RNA to library_IFREMER.pdf \(in WP2_D2.3_Appendix\)](#)

TIHO (17) Workflow Fragmentation to Sequencing

Contact person: Hansmann, Florian <Florian.Hansmann@tiho-hannover.de>

DNA fragmentation, Library preparation and size selection (0.7X; fragments should be larger than 200bp) are performed using the “Nextera XT DNA Library Prep Kit” and handling of the samples is done according to the kit’s reference guide:

https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera-xt/nextera-xt-library-prep-reference-guide-15031942-02.pdf.

Regarding quality control and quantification we use:

Quantification: Qubit (Qubit® dsDNA HS Assay Kit) and the procedure is performed according to the manufacturer’s instructions (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_dsDNA_HS_Assay_UG.pdf).

Quality control: Quality is measured using the agilent Chip (Agilent High Sensitivity DNA Kit) and measurement is done according to the manufacturer’s instructions:

http://www.chem.agilent.com/library/usermanuals/Public/G2938-90321_SensitivityDNA_KG_EN.pdf

For sequencing we are using an Illumina MiSeq according to the manufacturer’s protocol.

Summary and Conclusions

We provide here protocols for next generation sequencing used by members of COMPARE. The protocols cover the sequencing steps from fragmentation of DNA or cDNA, to library preparation, quantitation and finally the actual sequencing process. Protocols for the Illumina, Ion Torrent and 454 platform are included. Summaries of each group's use of the methods were provided (pages 4-11) to give an indication of their successful application.