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Birchler De Allende - Using The Oxford Nanopore Ligation Sequencing Kit to Sequence Zebrafish Larvae

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We use this protocol and it's working

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Abstract

Using The Oxford Nanopore Ligation Sequencing Kit to Sequence Zebrafish Larvae

Introduction

1 The protocols described outline methods for extracting high-quality genomic DNA from zebrafish larvae for sequencing applications. The process typically begins with collecting a small tissue sample, such as a fin clip, from 3-day post-fertilization larvae. The QIAGEN DNeasy Blood & Tissue Kit is commonly used for DNA extraction. The procedure involves tissue lysis with proteinase K, followed by binding of DNA to the silica membrane, washing steps to remove contaminants, and elution of pure DNA. This method yields PCR-ready genomic DNA suitable for various downstream applications, including genotyping and next-generation sequencing.

Following DNA extraction, library preparation for sequencing may be performed using kits like the Oxford Nanopore Ligation Sequencing Kit, which involves DNA repair, end-preparation, and adapter ligation steps. These methods enable researchers to efficiently genotype and sequence zebrafish larvae, facilitating early identification of desired genotypes for disease modeling and other genetic studies.

Extraction of DNA from Zebrafish Larvae - The QIAGEN DNeasy Blood & Tissue Kit

2

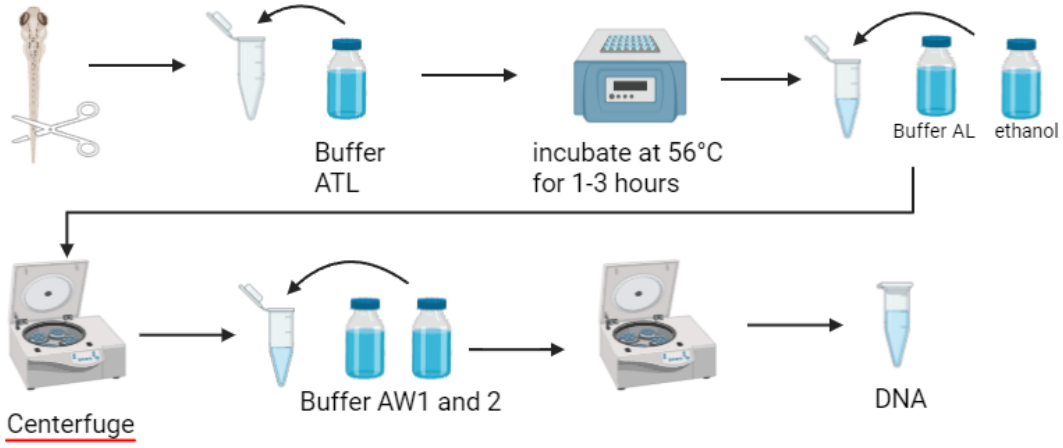

Figure 1. Basic steps for QIAGEN DNeasy Blood & Tissue Kit zebrafish larvae tail clip DNA extraction. Biorender Image made by Ian Birchler De Allende.

3 Materials For **Qiagen DNeasy Blood and Tissue Kit**:
Zebrafish larvae (2-5 days post-fertilization recommended)
Proteinase K
Lysis buffer (e.g., ATL buffer for Qiagen kits)
Ethanol (96-100%)

Microcentrifuge tubes (1.5 mL)
Vortex mixer
Water bath or incubator (set to 56°C)
Nuclease-free water or TE buffer

4 Transfer the sample (e.g., animal tissue, cells, or larvae) to a microcentrifuge tube.

5 Add 180 µL Buffer ATL and 20 µL proteinase K to the sample.

6 Vortex thoroughly and incubate at 56°C for 1-3 hours , depending on the sample type.

For tough tissues, mechanical disruption using a bead mill or rotor-stator homogenizer can improve lysis efficiency

7 After lysis, vortex the sample and add 200 µL Buffer AL. Mix thoroughly.

8 Incubate at 70°C for 10 minutes to enhance DNA yield.

9 Add 200 µL ethanol (96-100%) and mix well.

10 Transfer the entire mixture to a DNeasy Mini spin column placed in a collection tube.

11 Centrifuge at 8000 rpm (6000 x g) for 1 minute. Discard the flow-through

12 Add 500 µL Buffer AW1 to the spin column and centrifuge for 1 minute at 8000 rpm

13 Add 500 µL Buffer AW2 and centrifuge for 3 minutes at 13,000 rpm (20,000 x g) to dry the membrane

14 Place the spin column in a clean 1.5 mL microcentrifuge tube

15 Add 200 µL Buffer AE directly onto the membrane.

16 Incubate at room temperature for 1 minute, then centrifuge for 1 minute at 8000 rpm to elute the DNA

Library Preparation Using the Oxford Nanopore Ligation Sequencing Kit

17 Start with 1 µg of high molecular weight genomic DNA.

18 Adjust the volume to 47 µL using nuclease-free water.

19 Prepare the End-Repair and dA-Tailing reaction:
47 µL DNA sample
3 µL NEBNext FFPE DNA Repair Buffer
2 µL NEBNext FFPE DNA Repair Mix
3.5 µL NEBNext Ultra II End Prep Reaction Buffer
Mix thoroughly by flicking the tube or gentle pipetting.

20 Incubate using a thermal cycler:
20°C for 5 minutes
65°C for 5 minutes

21 Add 60 µL of AMPure XP beads to the reaction.

22 Incubate at room temperature for 5 minutes.

23 Place on a magnetic rack for 5 minutes and remove the supernatant.

24 Wash twice with 200 µL of 70% ethanol without disturbing the beads.

25 Air-dry the beads for 5 minutes.

26 Elute the DNA in 60 µL of nuclease-free water.

27 Prepare the Adapter Ligation Mix:

60 µL end-prepped DNA
25 µL Blunt/TA Ligase Master Mix
10 µL Adapter Mix (AMX)

28 Mix gently by pipetting.

29 Incubate at room temperature for 10 minutes.

30 Add 40 µL of AMPure XP beads to the ligation reaction.

31 Incubate and wash as in the previous clean-up step.

32 Elute in 15 µL of Elution Buffer.

Priming and Loading the Flow Cell

33 Priming:
Prepare the flow cell by attaching it to the MinION device.
Gently flush the flow cell with 800 µL of priming mix (provided in the sequencing kit) using the flow cell priming port.

34 Sample Loading:
Mix the eluted DNA with 37.5 µL of Running Buffer with Fuel Mix (RBF), and 25.5 µL of Loading Beads (LB).
Gently pipette the mixture into the flow cell via the sample loading port.
Start Sequencing:
Close the lid of the flow cell and initiate sequencing using the MinKNOW software.
Sequencing and Data Analysis

35 Run MinION:
Monitor sequencing through the MinKNOW software, ensuring that the flow cell performs optimally and tracks data output.
Sequencing runs can range from a few hours to days, depending on the required depth and application.

Bioinformatics:

36 **Key file types produced by Nanopore sequencing:**

1. POD5 files: These contain the raw signal data from the nanopore sequencing process. POD5 is the newer format that has replaced the legacy FAST5 format. It's more efficient for storage and data access.
2. FASTQ files: These contain the base-called sequence data, which is the raw signal converted into nucleotide sequences. FASTQ is a standard format for storing sequence data along with quality scores.
3. BAM files: These are used for storing alignment information when reads are mapped to a reference genome. BAM files are also used for storing information about modified bases when performing modified base calling.
4. Sequencing summary file: This contains metadata and summary statistics for the sequencing run, including information about each read such as read length, quality scores, and other metrics.

37 **The bioinformatics workflow for nanopore data typically involves several key steps:**

1. Quality control using tools like NanoPlot to assess read quality, length distribution, and other metrics.
2. Optional read filtering and trimming to remove low-quality sequences or adapter contamination.
3. Genome assembly using long-read assemblers such as Flye for de novo projects, or alignment to a reference genome using tools like Minimap2.
4. Variant calling with specialized tools like Medaka, which can also detect structural variants and methylation patterns.

1. Quality Control using NanoPlot

38 Install NanoPlot:

```
pip install NanoPlot
```

1. Run NanoPlot on your FASTQ files:

```
NanoPlot --fastq sample.fastq -o nanoplot_output
```

Examine the output in the nanoplot_output directory, including:

- NanoPlot-report.html: Interactive report with plots and statistics
- Summary statistics in .txt files

- Various plots as .png files showing read length distribution, quality scores, etc.

Key metrics to assess:

- N50 read length
- Mean read quality
- Total bases sequenced
- Read length distribution

2. Optional read filtering and trimming to remove low-quality sequences or adapter contamination.

39 Adapter Trimming:
Use Porechop to remove adapter sequences from both ends of reads.

```
porechop -i raw_reads.fastq -o trimmed_reads.fastq
```

Use NanoFilt to filter reads based on quality and length.

```
cat trimmed_reads.fastq | NanoFilt -q 10 -l 1000 >
filtered_reads.fastq
```

3. Genome Assembly using Flye

40 Install Flye:

```
conda install -c bioconda flye
```

Run Flye assembly:

```
flye --nano-raw reads.fastq --out-dir flye_assembly --threads 16 -
-genome-size 5m
```

Adjust threads and estimated genome size as needed.

Examine the output:

- assembly.fasta: Final assembled contigs
- assembly_graph.gfa: Assembly graph
- assembly_info.txt: Statistics on contigs

Assess assembly quality:

- Check N50 contig length
- Examine total assembly size vs expected genome size
- Look for potential misassemblies or contamination

4. Variant Calling using Medaka:

41 Install Medaka:

```
pip install medaka
```

42 Run Medaka for variant calling:

```
medaka_variant -i alignment.sorted.bam -f reference.fasta -o
medaka_variants
```