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Nematostella vectensis genomic DNA preparation and Telomeric Restriction Fragment Analysis (TRF)

Rationale and background: To generate high-quality genomic DNA from Nematostella vectensis and use it for Telomeric Restriction Fragment Analysis in order to describe the telomere structure in this model system

DNA preparation

It is important that everything is absolutely free of traces of plasmids or probes. Use clean buffers, take a new box of pipette tips, etc. Clean your pipettes thoroughly before you start. Do not prepare probes or plasmids on the same day on the same bench!

And never use a VORTEX, avoid shearing of the DNA, so treat it gently throughout the procedure!

- For genomic DNA extraction 20 juveniles were collected and washed three times with diluted sea water (1:3 diluted with distilled water). The pelleted juveniles can be used right away, but can also be frozen in liquid nitrogen and stored at -80°C before continuing to DNA preparation
- Thaw out pellet quickly at room temperature (RT)
- Resuspend animals in 1 ml of DNA Extraction buffer
- Incubate at 50°C for 2-3 h or overnight (ON) to get better lysis
- Transfer lysate to a Phase Lock Gel tube (Heavy Phase Lock Gel tubes from 5PRIME)
- Add 1 volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v, UltraPure, Invitrogen), mix GENTLY by inverting several times
- Spin 10 min., 3000 rpm, RT

- Transfer water-phase to a 15 ml tube containing 1 volume of Iso-propanol and 0.1 volume of 2M NaAc pH 5.5
- Mix by inverting several times
- Spin max. speed, 1 hour, 4°C
- Resuspend pellet in 0.3 ml TNE+100 μg/ml RNase A (DNase free)
- Incubate for 30 min at 37°C
- GENTLY resuspend using a 1 ml tip with its end cut off
- Incubate 1-2 h at 37°C
- Add 0.3 ml TENS/protK mix
- Incubate 1 hr at 37°C
- Transfer sample to a 2 ml phase lock tube
- Add 0.6 ml Phenol:Chloroform:Isoamyl Alcohol, mix well by inverting
- Spin max. speed, 10 min, RT
- Transfer upper phase to a new 1.5 ml tube with 600 μl Iso-propanol + 66 μl 2M NaAc
- Mix by inverting
- Spin max. speed, 1 hour, 4°C
- Wash with 70% EtOH
- Resuspend in 50-100 μl of 10 mM Tris (pH 7.4) (depending on the amount of DNA)
- Incubate 30 min at 37°C, resuspend with a 100 μl tip with its end cut off
- Incubate ON at 4°C until DNA is dissolved. It will be VERY viscous.

Restriction digest of DNA

Frequently cutting enzymes are used to digest the bulk of genomic DNA, but not the telomeric repeats. It is hard to measure the actual concentration of genomic DNA at this point, so the amount used depends on the pellet size obtained during DNA preparation.

DNA	25-50 μl
10x NEB buffer 4	10 μl
MboI	10 μl
AluI	5 μl
RNAse A	0.1 μl

Add H_2O to a total of 100 μl

- Digest ON at 37°C.

Measuring DNA concentration with Hoechst fluorimetry

- Dilute Hoechst 33256 to 1 μg/ml in filter-sterilized TNE
- Place 2 ml in cuvette and press <ZERO>
- Add 2 ml of reference DNA solution (e.g. 100 ng/ml)
- Press <CALIB> and enter concentration of reference DNA solution, press <ENTER>
- Remove sample with suction
- Replace with 2m fresh solution and zero again
- Add 2 μl of DNA and mix
- Measure DNA concentration for each sample in duplicate or triplicate

Gel-electrophoresis of digested DNA

- Prepare a large agarose gel (0.7%) in 0.5xTBE; add ethidium bromide (1 μl per 100 ml)
- Load 1-3 μg of digested DNA per lane with Orange G gel loading buffer

- Run gel in 0.5xTBE; run initially at 100 volts for a couple of minutes until all loading dye has entered the gel, then switch to 38 volts
- Run ON (~ 15 h)
- Take picture of gel under UV to check if all DNA has been properly digested
- Dry gel on gel dryer for 2.5 h; 51°C (use 2 sheets of 3M paper below gel and cover with cling film)

Southern blotting with telomeric probe

- After drying, carefully rinse gel in DI water to float it off the 3M paper
- Denature: gently shake for 30 min in Denaturing solution
- Neutralize: gently shake in Neutralizing Solution, 2 times 15 min
- Rinse gel with DI water
- Pre-hybridize in hybridization bag in Church buffer for at least 1 h at 50°C
- Remove Church buffer from pre-hybridization and add fresh Church buffer (I usually use a volume of 25 ml) with labeled telomeric probe (see below)
- Hybridize ON at 50°C
- Remove probe and add to liquid radioactive waste
- Wash gel 3 times for 30 min in 4xSSC at 50°C. Add the first wash to liquid radioactive waste
- Wash once in 4xSSC with 0.1% SDS at 50°C for 15 min
- Dry gel carefully with paper towels and seal in hybridization bag or wrap in cling film
- Expose ON on phosphoscreen
- Scan – if signal is too weak, re-expose for longer period

Preparation of γ32P-labeled telomeric probe for Southern blotting

The telomeric oligo probe $(TTAGGG)_4$ is end-labeled with γ32P-ATP using T4 Polynucleotide Kinase (PNK) from NEB.

- Set up labeling reaction: 1 μl $(TTAGGG)_4$ oligo (20 pmol total)
  1 μl 10x PNK buffer
  1 μl PNK
  2μl H_2O
  5 μl γ32P-ATP
- Incubate at 37°C for 1-2 h
- Cleanup of the probe is done using Micro Bio-Spin® Chromatography Columns from BIORAD according to the manufacturer's protocol

Buffers and solutions

- **DNA extraction buffer**

  100 mM Tris HCl (pH 8.5)

  100 mM NaCl

  50 mM EDTA

  1% SDS

  1% ß-mercaptoethanol

  100 µg/ml Proteinase K.

- **TNE**

  10 mM Tris pH 7.4

  100 mM NaCl

  10 mM EDTA

- **TENS/protK**

  10 mM Tris pH 7.4

  10 mM EDTA

  100 mM NaCl

  1% SDS

  100 µg/ml proteinase K

  (prepare fresh)

- **5xTBE (1 liter)**

  54.5 g Tris base

  4.65 g Na_2EDTA

  27.52 g Boric acid

- **Orange G gel loading buffer 6x**

  50% glycerol

  0.5% Orange G

- **Denaturing solution**

  1.5 M NaCl

  0.5 M NaOH

- **Neutralizing solution**

  3 M NaCl

  0.5 M Tris-HCl pH 7.0

- **Church buffer (1 liter)**

  500 ml 1 M NaPi pH 7.2 (see below)

  2 ml 0.5 M EDTA pH 8.0

  70 g SDS

  10 g BSA

  add H_2O to 1000 ml

- **1 M NaPi pH 7.2 (2.3 liter)**

  308 g $Na_2HPO_4 * 7H_2O$

  9.2 ml H_3PO_4

  Add H_2O close to final volume, adjust pH with H_3PO_4 and adjust volume to 2.3 liter

- **20xSSC**

  3 M NaCl

  0.3 M Sodium citrate