Protocols for Ragworms

Duy Nghia Pham, Inna M. Sokolova

2023-01-07

Contents

Chapter 1

Homogenization

1.1 Materials

- 0.1 M Tris:HCl pH 8.5 containing 153 M MgSO4, 0.2% w/v Triton X-100, 0.1 mM PMSF:
  - 2.5 M HCl: in 250 mL conical flask pre-filled with 100 mL water add 51.754 mL[1] 37% HCl (d = 1.19 g/mL) using glass pipet. Transfer to 250 mL volumetric flask and fill up with water. Transfer to 250 mL bottle. Store at 25°C.
  - In 1 L beaker add 12.114 g[2] Tris base (M = 121.14) and 0.0377 g MgSO4*7H2O (M = 246.48) then fill with 800 mL water. Add 21-23 mL 2.5 M HCl to reach pH 8.5. Transfer to 1 L volumetric flask and fill up with water. Transfer to 1 L bottle then dissolve 1869 L Triton X100 (d = 1.07 mg/ L). Store at 4°C.
  - 200 mM PMSF stock: in 1.5 mL tube add 0.035 g PMSF (M = 174.2) and 1 mL isopropanol, store at -20 °C.
- 1.5 mL flat[3] screw cap tubes pre-filled with 500 mg zirconium beads (250 mg 2 mm beads and 250 mg 1 mm beads).
- 2 mL socket screw cap tubes.
- Avery[4] freeze-resistant round labels, no. 1-15 for each sample.

[1] https://www.sigmaaldrich.com/DE/en/support/calculators-and-apps/molarity-calculator

[2] https://www.sigmaaldrich.com/DE/en/support/calculators-and-apps/mass-molarity-calculator

[3] compatible with FastPrep

[4] https://www.avery-zweckform.com/blanko-etiketten/rund-10-mm

1.2 Methods

- Quickly cut the head (50 mg) and wrap it in the aluminum foil maintained in liquid nitrogen.
- Record mass of the headless worm, transfer to 15 mL pre-cool glass homogenizer.
- Prepare 1:25 tissue homogenate:
  - in 50 mL tube add 2.5 L PMSF stock to every 5 mL cold buffer (2000-fold dilution) right before use. Invert briefly.
  - Add 2500 L buffer containing PMSF to every 100 mg wet tissue.
- Record mass of the head, transfer to the 1.5 mL tube pre-filled with beads (no. 1). Cool down in liquid nitrogen then temporarily store at -20 °C.
- Surround glass homogenizer by ice, rotate at 200 rpm for ~3 min.
- Aliquot 1.8 mL homogenate to 2 mL tubes (no. 2+) on ice then temporarily store at -20 °C.
- Store tubes (no.1 for gene expression and no. 2+ for other biomarkers in separated cryoboxes) at -80 °C for downstream applications.

Chapter 2

CEA

2.1 Organization

- Transfer one homogenate tube (e.g., no. 2) of all samples to new cryoboxes.
- Thaw out homogenate (max. 24 samples each run, 8 samples each subrun).
- Vortex and transfer 375 L homogenate to 2 mL tube, store at -80 °C for later analysis of lipids (read more in 6).
- Vortex and transfer 300 L homogenate to 1.5 mL tube, centrifuge 3,000 $\times g$ for 5 min at 4 °C.
- Transfer supernatant to 1.5 mL tube, use for ETS on the same day (read more in 3) and store the leftover at -80 °C for later analysis of carbohydrates and proteins (read more in 4 and 5).

Chapter 3

ETS

Modified after De Coen and Janssen (1997).

3.1 Materials

- Buffer solution: 0.13 M Tris:HCl pH 8.5 containing 0.3% w/v Triton X-100
  - Add 1.574 g Tris base (M = 121.14) to conical flask then fill with ~80 mL water. Add ~3.1 mL 2.5 M HCl to reach pH 8.5. Add 280.4 L Triton X100 (d = 1.07 mg/ L). Dissolve, adjust pH if needed and add water to a volume of 100 mL, aided by volumetric flask. Transfer to DURAN bottle, store cold.
- Substrate solution: 1.7 mM NADH and 0.25 mM NADPH Add 0.0241 g NADH (M = 709.4) and 0.0042 g NADPH (M = 833.35) to 20 mL buffer solution, store dark cold
- 8 mM INT: 0.2023 g INT (M = 505.7) in 50 mL water, store dark cold
- For each batch of 24 samples, take out 10 mL buffer in Falcon tube, 1.3 mL Substrate in 1.5 mL tube, 5.3 mL INT in 50 mL glass bottle
- 0.5 M KCN (M = 65.12): 32.6 mg KCN in 1mL water, freeze at – 20
- 1 mM rotenone (M= 394.42): 0.39 mg rotenone in 1ml ethanol absolute, freeze at -20

3.2 Methods

- Rxn in duplicate wells:
  - 25 L supernatant + 75 L buffer + 23 L KCN + 2 L rotenone + 50 L INT for self-control
  - 25 L supernatant + 75 L buffer + 25 L substrate + 50 L INT (Handystep) for rxn

- Measure kinetically at 490 nm in 10 min with 10s interval, at room temperature (25 °C), shake 5s before reading

Chapter 4

Proteins

Modified after Bradford (1976).

4.1 Materials

- 5 mg/mL BSA stock solution: e.g., 50 mg BSA in 10 mL homogenization buffer
- 2 mg/mL BSA stock solution: 0.5 mL 5 mg/mL BSA + 0.75 mL water
- Bradford reagent

4.2 Methods

- dilute supernatant 8 times in 1.5 mL tube: 10 L supernatant + 70 L water, on ice
- Prepare standard curve using 2 mg/mL BSA stock solution in 1.5 mL tubes on ice

mg/mL standard	uL stock	uL water
2	200	0
1	100	100
0.5	50	150
0.25	25	175
0.125	12.5	187.5
0.0625	6.25	193.75
0	0	200

- Add 200 L Bradford reagent using handystep to 10 L diluted supernatant or standards in wells
- Shaking 300s (5 min) and measure in triplicate at 595 nm

Chapter 5

Carbohydrates

Modified after Masuko et al. (2005).

5.1 Materials

- Concentrated H2SO4, store dark room
- 5% phenol: 2 mL 90% phenol + 34 mL water in Falcon tube, vortex then transfer to 50 mL glass bottle, store dark
- 100 mM glucose stock solution: e.g., 180.16 mg in 10 mL water, store cold
- 2 mM glucose stock solution: 200 L stock solution + 9800 L water, store cold

5.2 Methods

- dilute supernatant 8 times in 2 mL tube: 25 L supernatant + 175 L water = 200 L
- Prepare standard curve using 2 mM stock solution in 2 mL tubes: 2, 1, 0.5, 0.25, 0.125, 0.0625, 0 mM (200 L in final)

mM standard	uL stock	uL water
2	200	0
1	100	100
0.5	50	150
0.25	25	175
0.125	12.5	187.5
0.0625	6.25	193.75
0	0	200

- Medium vortex samples and standards
- Add 500 L concentrated H2SO4 and 100 L 5% phenol both using Handystep
- VORTEX FOR A WHILE and heat at 90 °C for 5 min, cool down for 5 min then vortex.
- measure 200 L in triplicate at 492 nm. Shake 5s before reading.

Chapter 6

Lipids

Modified after Inouye and Lotufo (2006).

6.1 Materials

- Chloroform: methanol 1:1 v/v: mix 150 mL chloroform and 150 mL methanol in a 500 mL bottle, store dark room, pour to 100 mL bottle to use
- Concentrated H2SO4, store dark room
- 5 mg oil/mL acetone stock solution: 5.7 L oil (d = 0.87 mg/ L) in 1 mL acetone[1]
- Vanillin reagent: dissolve 750 mg vanillin in 125 mL hot water then add 500 mL 85% H3PO4 in a 1 L bottle, store dark room, pour to 250 mL bottle to use

6.2 Methods

- Prepare standard curve using stock solution in 1.5 mL tubes: 5, 2.5, 1.25, 0.625, 0.3125, 0 mg/mL

mg/mL standard	uL stock	uL acetone
5	200	0
2.5	100	100
1.25	50	150
0.625	25	175
0.3125	12.5	187.5

[1] Pre-wetting 1 mL tip can prevent acetone dropping

mg/mL standard	uL stock	uL acetone
0	0	200

- Add 375 L homogenate to 2 mL tube[2]
- thawing homogenate, medium vortex, add 1.5 mL Chloroform: methanol, vortex and incubate 10 min
- Centrifuge 3000 g for 5 min at 4 °C, wait some time for methanol going up
- transfer 200 L of low chloroform phase [3] to 1.5 mL tube
- spin down standards and samples
- evaporate solvent in standards and samples by heating at 90 °C for 10 min (open cap)
- Add 100 L concentrated H2SO4 using Handystep, vortex twice and heat at 100 °C for 10 min (close cap)
- After cooling, transfer 50 L to 1.5 mL microtubes tube already filled with 1 mL vanillin reagent, vortex
- After 5 min, measure 200 L in triplicate at 490 nm

[2]already done when preparing for ETS assay
[3]wait for methanol getting out of the tip

Chapter 7

MGO

Modified after Mitchel and Birnboim (1977).

7.1 Materials

- 0.1 M borax: in 50 mL tube add 1.5255 g borax (M[1] = 381.37) and 40 mL water. Make another one. Rotate 20 min. Transfer to 100 mL bottle. Store in dark at 25°C[2].
- 0.2 M Girard's reagent T: in 50 mL tube add 1.3411 g Girard T (M = 167.64) and 40 mL water. Rotate 5 min. Transfer to 50 mL bottle. Store in dark at 4°C.
- 1232 uM methylglyoxal stock solution: in 50 mL tube add 10 uL 6.16 M methylglyoxal (M = 72.06, 40% w/w in water, d = 1.11) and 50 mL homogenization buffer[3] (5000-fold dilution). Rotate 5 min. Store at 4°C.

7.2 Methods

- Thaw out homogenate (max. 24 samples each run). **Vortex** and transfer 250 uL to 1.5 mL tube. Centrifuge at 10,000 $\times g$ for 15 min at 25°C. Transfer 200 uL supernatant to 1.5 mL tube.
- Prepare 200 uL methylglyoxal standards in 1.5 mL tube by stock dilution.

uM standard	uL stock	uL homogenization buffer
1232	200	0
616	100	100

[1] borax decahydrate

[2] avoid lower temperature to prevent precipitation

[3] 0.1 M Tris:HCl pH 8.5 containing 153 uM MgSO4, 0.2% w/v Triton X-100, no PMSF and EDTA

uM standard	uL stock	uL homogenization buffer
308	50	150
154	25	175
77	12.5	187.5
38.5	6.25	193.75
0	0	200

- Add 250 uL borax and 50 uL Girard T to samples and standards using repeating pipette.
- **Vortex** and heat at 60°C[4] for 10 min. Cool to 25°C for 5 min.
- Centrifuge at 10,000 $\times g$ for 5 min at 25°C[5].
- Measure[6] absorbance of 200 L in **duplicate** at 325 nm. Shake 5s before reading.

[4] trials at 30°C and 90°C showed lower absorbance

[5] may need another centrifuge for standards; small pellet could appear in samples but not in standards

[6] pre-wetting the tip helps minimize Triton bubbles and increase homogeneity in samples (since supernatant is taken directly for measurement without transferring to new tube and vortexing)

Chapter 8

MDA

Modified after Buege and Aust (1978).

8.1 Materials

- 15% w/v TCA - 0.375% w/v TBA - 0.25 M HCl reagent: Add 0.1875 g TBA in 50 mL tube. Add 34 mL 20% w/w TCA (density 1.1 g/mL, equivalent to 22% w/v). Add 5 mL 2.5 M HCl. Add 10 mL water to a volume of nearly 50 mL. Wrap by aluminum, rotate for 1h, store dark room. Rotate for 5 min before use each day.

8.2 Methods

- Thaw out homogenate (max. 22 samples each run). **Vortex** and transfer 200 uL to 1.5 mL tube **with snap cap with hole made by needle**. Use 200 uL homogenization buffer for control in **duplicate**.
- Add 500 uL reagent to samples and controls (24 tubes in total), **NOT vortex**.
- Heat at 90 °C for 20 min. Cool for 15 min.
- Centrifuge at 10,000 $\times g$ for 3 min at 25 °C. Rotate tubes 180° then centrifuge one more time with same settings.
- Allow air to escape (especially in control). Measure absorbance of 200 L in **triplicate** at 530 nm. Shake 5s before reading.

Chapter 9

Enzyme assays

9.1 Organization

- Thaw out homogenate (max. 22 samples each run, 11 samples each sub-run).
- Vortex and transfer 300 L homogenate to 1.5 mL tube, centrifuge 6,000 $\times g$ for 10 min at 4 °C.
- Transfer supernatant to 1.5 mL tube, use for GR on the same day (read more in 10) and store the leftover at -80 °C for later measurement of GST (read more in 11).

Chapter 10

GR

Modified after Mannervik (1999).

10.1 Materials

- 1 mM NADPH: in 5 mL tube add 4.2 mg NADPH (M = 833.4) and 5 mL homogenization buffer. Store at 4°C.
- 10 mM GSSG: in 5 mL tube add 30.6 mg GSSG (M = 612.6) and 5 mL water. Store at 4°C.
- 1% BSA: in 5 mL tube add 50 mg BSA and 5 mL homogenization buffer. Store at 4°C.

10.2 Methods

- Master mix for 24 rxn: in 5 mL tube add 3120 uL homogenization buffer, 520 uL NADPH, 520 uL GSSG, and 520 uL BSA (120:20:20:20). **Vortex**.
- Rxn in duplicate wells:
  - 180 uL master mix + 20 uL homogenization buffer for control
  - 180 uL master mix + 20 uL supernatant for sample
- Measure kinetically at 340 nm in 15 min with 30s interval, at room temperature (25 °C), shake 5s before reading. Use 21/31 points for slope.

Chapter 11

GST

Modified after Habig et al. (1974).

11.1 Materials

- 5 mM GSH: in 5 mL tube add 7.7 mg GSH (M = 307.33) and 5 mL homogenization buffer. Use fresh only.
- 5 mM CDNB:
  - Prepare 100 mM CNDB stock: e.g., in 15 mL tube add 286.9 mg CDNB (M = 202.55) and 14.16 mL absolute ethanol. Store at -20°C.
  - Dilute 20X: in 2mL tube add 50 uL stock and 950 uL absolute ethanol.

11.2 Methods

- Master mix for 24 rxn: in 5 mL tube add 4160 uL homogenization buffer, 260 uL GSH, and 260 uL CDNB (160:10:10). **Vortex**.
- Rxn in duplicate wells:
  - 180 uL master mix + 20 uL homogenization buffer for control
  - 180 uL master mix + 20 uL supernatant for sample
- Measure kinetically at 340 nm in 15 min with 30s interval, at room temperature (25 °C), shake 5s before reading. Use 21/31 points for slope.

Chapter 12

TAC

Modified after Re et al. (1999).

12.1 Materials

- 63.63 mM potassium persulfate: in 5 mL tube add 86 mg $K_2S_2O_8$ (M = 270.32) and 5 mL water. Vortex.
- 7.29 mM ABTS: in 15 mL tube add 40 mg ABTS salt (M = 548.68) and 10 mL water. Vortex.
- ABTS radical cation stock solution: add 0.4 mL $K_2S_2O_8$ to 10 mL ABTS (final conc. 2.45 mM $K_2S_2O_8$ + 7 mM ABTS). Vortex. Store in dark at 25°C for 24h before use.
- 10 mM Trolox: in 5 mL tube add 12.5 mg Trolox (M = 250.29) and 5 mL homogenization buffer. Rotate 5 min. Use fresh.

12.2 Methods

- Thaw out supernatant (max. 40 samples each run). **Vortex** before use.
- Prepare 200 uL Trolox standards in 1.5 mL tube by stock dilution. **Vortex** and keep on ice.

uM standard	uL stock	uL homogenization buffer
0	0	200
500	10	190
1000	20	180
1500	30	170
2000	40	160
2500	50	150

uM standard	uL stock	uL homogenization buffer
3000	60	140

- Dilute[1] $ABTS^{\bullet+}$ 10X: in 50 mL tube add, e.g., 2.5 mL $ABTS^{\bullet+}$ stock solution and 22.5 mL water. Vortex.
- Rxn in duplicate wells: 10 uL standards or samples + 200 uL diluted $ABTS^{\bullet+}$ using repeating pipette.
- Measure endpoint at 734 nm at room temperature (25 °C), shake 300s (5 min) before reading.

[1] check Abs beforehand with 3000 and 0 uM standards to fit the reader capacity. Adjust by adding stock solution or water

Chapter 13

Gene expression

updating

Bibliography

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248–254. https://doi.org/10.1016/0003-2697(76)90527-3
172736 citations (Crossref) [2022-08-26].

Buege, J. A., & Aust, S. D. (1978, January 1). [30] Microsomal lipid peroxidation. In S. Fleischer & L. Packer (Eds.), *Methods in Enzymology* (pp. 302–310, Vol. 52). Academic Press. https://doi.org/10.1016/S0076-6879(78)52032-6

De Coen, W., & Janssen, C. (1997). The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: A new methodology to assess the energy budget of toxicant-stressed Daphnia populations. *Journal of Aquatic Ecosystem Stress and Recovery*, *6*(1), 43–55. https://doi.org/10.1023/A:1008228517955
311 citations (Crossref) [2022-08-26].

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *The Journal of Biological Chemistry*, *249*(22), 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8

Inouye, L. S., & Lotufo, G. R. (2006). Comparison of macro-gravimetric and micro-colorimetric lipid determination methods. *Talanta*, *70*(3), 584–587. https://doi.org/10.1016/j.talanta.2006.01.024
40 citations (Crossref) [2022-08-26].

Mannervik, B. (1999). Measurement of Glutathione Reductase Activity. *Current Protocols in Toxicology*, *00*(1), 7.2.1–7.2.4. https://doi.org/10.1002/0471140856.tx0702s00
27 citations (Crossref) [2022-08-26].

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry*, *339*(1), 69–72. https://doi.org/10.1016/j.ab.2004.12.001
1273 citations (Crossref) [2022-08-26].

Mitchel, R. E. J., & Birnboim, H. C. (1977). The use of Girard-T reagent in a rapid and sensitive method for measuring glyoxal and certain other -dicarbonyl compounds. *Analytical Biochemistry*, *81*(1), 47–56. https://doi.org/10.1016/0003-2697(77)90597-8
51 citations (Crossref) [2022-08-26].

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9), 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3