# Measuring Genetic Variation in Zebra Mussels Using Protein Electrophoresis

***Corey A. Goldman***

Departments of Botany and Zoology
University of Toronto
Toronto, Ontario M5S 3G5
(416) 978-7163, corey.goldman@utoronto.ca

Reprinted from: Goldman, C. A. 1999. Measuring genetic variation in zebra mussels using protein electrophoresis. Pages 343-351, in Tested studies for laboratory teaching, Volume 20 (S. J. Karcher, Editor). Proceedings of the 20th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 399 pages.



At the 1997 ABLE meeting in Calgary Jim Bader (Case Western Reserve University) presented a major workshop on using cellulose acetate electrophoresis to measure genetic variability in natural populations (Bader, 1998). At the University of Toronto we have been using a very similar protocol, with fruit flies as the study organism. In the 1997-98 academic year I incorporated several of Jim's ideas into our existing protocol and changed the study organism to zebra mussels, with considerable success (1,500 first-year students completed this exercise over a two-week period).

In this article I share my experiences with you and provide the "details" so you can conduct the lab yourself, such as which enzyme system gives results that are easy for the students to interpret. An illustrated experimental protocol is given in Figure 1. Three appendices are also included: stain instructions for students (Appendix A); buffer, enzyme stain, and agar recipes (Appendix B); and analysis of sample data (Appendix C).

## Getting Started

**Selecting a study organism:** The study organism should have relevance, appeal, and familiarity to students (e.g., a pest species); be easy to collect; not require an animal-care protocol; students will not object to "killing" the organism; ease of sample preparation; small size (easy to store in a freezer); and published reports in the literature of the degree of genetic variation for many enzyme systems.

**Selecting an enzyme system:** The enzyme system should be highly active (stains quickly); stable (will not degrade quickly in the lab and stores well in a deep freezer); monomeric (two bands for heterozygote) or dimeric (three bands for heterozygote); shows good separation of bands using a least-harmful and inexpensive buffer; and have few allozymes (to simplify data analysis; allozymes are enzymes differing in electrophoretic mobility as a result of allelic differences at a *single gene locus*) and few isozymes (multiple isozymes may confound gel interpretation; isozymes are multiple forms of an enzyme from *multiple loci*). If you can find two closely related species where one species is fixed for a given allele for the enzyme system you chose, while the other shows allellic variation, then the first species provides a benchmark to greatly simplify the students' ability to interpret the banding patterns that they observe.

**Background reading:** When selecting an enzyme system the following contain recipes for stains and buffers, and important background information about the technique:

Richardson, B. J., and P. R. Baverstock, and M. Adams. 1986. *Allozyme electrophoresis: A handbook for animal systematics and population studies.* Academic Press, 410 pages. [Hardcover, ISBN 0-12-587840-0, $89 US from 1-800-782-4479, *definitely worth the money*]

Hebert, P. D. N., and M . J. Beaton. 1989. *Methodologies for allozyme analysis using cellulose acetate electrophoresis.* Helena Laboratories*, Beaumont, Texas, 32 pages. [Softcover, revised March 1993; free from publisher, who also sells acetate gels and applicator assembly system.]

* Helena Laboratories: 1-800-231-5663 in U.S., 1-800-668-6929/6944 in Canada.

If you choose to use zebra mussels the following articles are very helpful:

May, B., and J. E. Marsden. 1992. Genetic identification and implications of another invasive species of dreissenid mussel in the Great Lakes. *Canadian Journal of Fisheries and Aquatic Sciences*, 49:1501–1506. [In my course all students read this article, which was reprinted in their lab manual at no cost for copyright since the journal is published by the Government of Canada. Article reports on the discovery of the quagga mussel, gives allozyme variability for zebra and quagga mussels for 12 loci, provides background information, and is not overly technical (i.e., can be understood by a first-year student).]

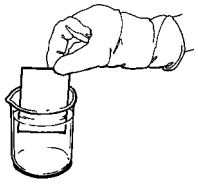
Marsden, J. E., A. P. Spidle, and B. May. 1995. Genetic similarity among zebra mussel populations within North America and Europe. *Canadian Journal of Fisheries and Aquatic Sciences*, 52:836–847.

———. 1996. Review of genetic studies of *Dreissena* spp. *American Zoologist,* 36:259–270.

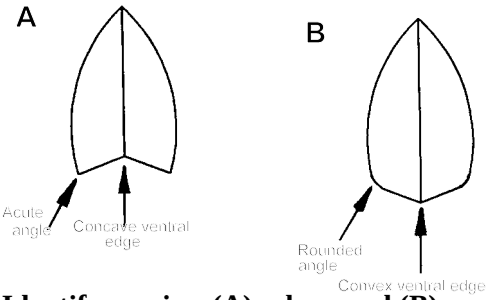
## Materials

C **Mussels** can be used fresh or frozen. We collect specimens ourselves and store in a &70°C freezer.

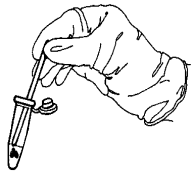
C A 3 $mm^3$ **tissue sample** is added to two drops of double-distilled water (dispensed with a disposable micropipette) in a 1.5-mL disposable microcentrifuge tube and homogenized with a small glass rod. Grinding on ice is not required for a robust enzyme (such as MDH).



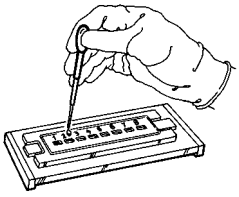
**1 - Soak gel in buffer solution**



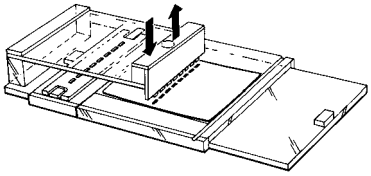

**2 - Identify species: (A) zebra, and (B) quagga**



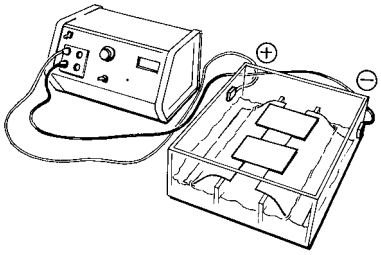
**3 - Prepare tissue samples**



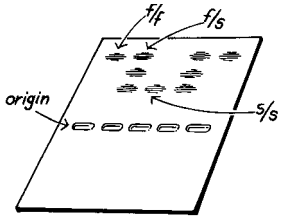
**4 - Load the sample plate**



**5 - Set up the applicator**
**6 - Transfer samples to gel**



**7 - Apply tracking dye**
**8 - Electrophorese samples**




**9 - Stain gel**
**10 - Incubate gel**
**11 - Interpret gel**

| Anode (+) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FF | FF | FF | SS | FF | SS | SS | SS | genotype |
| — | — | — | — | — | — | — | — | MDH-1 |
| | | | | | | | | origin |
| — | — | — | = | — | — | = | — | MDH-2 |
| FF | FF | FF | FS | FF | SS | FS | SS | genotype |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | well # |
| ♀ | ♀ | ♀ | ♂ | ♂ | ♂ | ♂ | ♂ | species id |

Notes: Run time = 25 MIN. Voltage = 200 VOLTS MY WELLS: #1 + #5

**12 - Record banding patterns observed by group**
**13 - Analyse class data**

**Figure 1.** Experimental protocol.

- A 60 × 76 mm **cellulose acetate gel** (Helena Titan III #3023, about $4 US each) will hold eight samples. A group of four uses one gel; each student prepares one zebra and one quagga sample.
- The **buffer solution** used to soak the gel and in the electrophoresis chamber depends on the enzyme system being studied. For the enzyme *malate dehydrogenase* (MDH), we use Tris-maleate (TM) buffer as per Richardson et al., which can be re-used each day. The gel is blotted surface-dry between blotting paper before being transferred to the alignment base.
- The **sample applicator assembly** consists of the sample well plate, sample alignment base, gel alignment base, and applicator. The system we use is home-made (see Figure 1). Helena sells the complete system (Super Z) for about $450 US. Ideally, each group of four students has their own applicator assembly. Samples are applied to the middle of the gel.
- We use a home-made **electrophoresis chamber** (see Figure 1). Wicks are cut from sheets of filter paper. For MDH, gels are run at 200 volts for 25 minutes. A very small amount of tracking dye is applied to the long edge of one gel to confirm that a current is present.
- A **histochemical stain** is prepared (by each group immediately before application) and applied as an agar overlay to the gel. We stain for the dimeric enzyme *malate dehydrogenase* (MDH). Stain consists of: Tris HCl buffer, NAD, malic substrate, MTT, PMS, and agar. All ingredients are dispensed with disposable pasteur pipets into a falcon tube. Stock stain ingredients can be kept in a refrigerator for several weeks. Recipes are given on Appendix B.
- **Safety precautions** include: wearing latex gloves when handling gel, buffer, and stain; wearing lab coats; only the instructor operates the electrophoresis chamber; and informing students about the hazardous effects and of the chemicals and the proper disposal of hazardous waste.

## Notes for the Instructor

**Timing:** A class of 24 students (six groups of four) can easily complete this lab in 3 hours, assuming each group has their own applicator assembly and the class has two electrophoresis chambers (three gels per chamber). A thorough analysis of the class data is carried over into the following lab period.

**Soaking the gel:** Students immerse the gels *slowly* into the buffer and soak for at least 20 minutes. If the gel is immersed quickly the plastic backing will peel away and the gel cannot be used.

**Setting up electrophoresis chamber:** Wicks are cut from filter paper, moistened in buffer, and placed in each chamber with the leading edge resting on each gel-support arm. Each gel is positioned (face down) so that the gel makes contact with the two moist wicks. Glass slides are placed over the contact of gel and wick to keep gel flat and ensure an even current through the gel (not shown in Figure 1).

**Mechanism of histochemical stain reaction:** MDH enzyme catalyses the conversion of malic substrate to oxaloacetate. This reaction requires NAD, which becomes reduced to NADH. NADH reacts with MTT (which is reduced) and PMS to yield formazon, which is purple in colour, therefore the site of the MDH enzyme on the gel can be identified by a purple band.

**Gel interpretation:**

C Stained gels are incubated in the dark for 10–15 minutes or until bands are clearly visible. To simplify scoring wash off the agar since the bands actually form in the gel itself.

C MDH has two isozymes (i.e., it is encoded by two different loci): MDH-1 is the cytoplasmic form which migrates to the anode (+), MDH-2 is the mitochondrial form migrating slowly to the cathode (&), hence 20–25 minutes run time. (The difference in migration positions is due to the differences in pH between the cytoplasm and mitochondria.)

C With Tris-maleate (TM) buffer, MDH-1 bands stain thick, while MDH-2 bands can be scored with ease (i.e., heterozygotes appear as three distinct thin bands). Best to score only MDH-2.
C MDH is a dimeric enzyme in which the two polypeptide chains can combine in any way to form one protein, and in this way three enzyme variants (bands) are formed; the FS association is twice as abundant as either the SS or FF associations, thus it stains twice as intensely.

C Two common alleles (fast and slow) are present in zebra mussels for both MDH-1 and MDH-2.

C Quagga mussels are fixed for the fast allele for both MDH-1 and MDH-2 in our sample, and thus provide a reference for scoring the zebra mussels, which have two alleles.

C In Lake Ontario populations of zebra mussels, heterozygotes (three bands) are common for MDH-2; for MDH-1 there is a high frequency of slow alleles, heterozygotes are not common. Homozygotes appear as single bands, either close to the origin (slow) or further from the origin (fast).

**Analysis of class data:** (1) Compute genotype frequencies for each species. (2) Allele frequencies are used to estimate expected genotype frequencies and to assess whether the populations (for each species) are at Hardy-Weinberg equilibrium. (3) How do the class results compare with the results reported in the literature (May and Marsden, 1992: Table 1)? Sample class data are provided and analysed in Appendix C.

## Literature Cited

Bader, J. M.. 1998. Measuring genetic variability in natural populations by allozyme electrophoresis. Pages 25-42, *in* Tested studies for laboratory teaching. Volume 19 (S. J. Karcher, Editor). Proceedings of the 19th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 365 pages.

APPENDIX A
*Stain Instructions (for students)*

| Step | Ingredient | Amount | Dispensed with... |
|---|---|---|---|
| 1 | Tris HCl buffer, pH = 8.0 | 1.0 mL | calibrated pasteur pipette labelled “Tris” |
| 2 | NAD | 1.5 mL | calibrated pasteur pipette labelled “NAD” |
| 3 | Malic substrate | 13 drops | pasteur pipette labelled “Malic” |
| 4 | MTT | 5 drops | pasteur pipette labelled “MTT” |
| 5 | PMS | 5 drops | pasteur pipette labelled “PMS” |
| 6 | Agar | 2 mL | calibrated pasteur pipette labelled “Agar” |

**Instructions:**

(A) After gel is put in chamber, add ingredients in **steps 1 to 4** to a 15 mL falcon tube. Cap tube and place in ice bucket in the dark. (Label the tube with your group’s bench #.)

(B) Immediately before gel is removed from the chamber, add PMS (**step 5**) and return tube to ice.

(C) After 25-minute run time, remove gel from rig and place in a clear plastic dish on a flat surface.

(D) Add molten agar (**step 6**), swirl quickly, and pour over center of gel (agar will expand out over entire gel). This must be done within 5 seconds of adding the agar to avoid clumps in the stain mixture (as the agar hardens quickly).

(E) Do not move stained gel for 1 minute (to allow agar to harden). Cover stained gel with green plastic tray.

(F) After 1 minute, move the dish to your bench and monitor the progress of the stain. Notify your instructor (who will remove the agar overlay) when bands can be scored.

APPENDIX B
*Buffer, Enzyme Stain, and Agar Recipes*

**Chemicals/materials:**

| Item | Used for | Sigma # | Comments |
|---|---|---|---|
| Agar | stain overlay | - | bacterial grade |
| HCl | stain Tris buffer | - | 1 M or 4 M, hydrochloric acid |
| Maleic acid | TM buffer reagent | M-9009 | obtain purest grade possible |
| L-Malic acid | MDH stain substrate | M-9138 | = L-malate |
| MTT | stain stock solution | M-2128 | methyl thiazolyl blue<br>protect from light, possible carcinogen |
| NAD | MDH stain stock soln. | N-7004 | nicotinamide adenine dinucleotide |
| NaOH | adjust pH malate substrate | | 4 M, sodium hydroxide |
| PMS | stain stock solution | P-9625 | phenazine methosulphate<br>protect from light, possible carcinogen |
| Sodium azide | for chemical storage | S-2002 | 250 mg/mL |
| Trizma base | Tris buffer | T-1503 | 99% purity |

**Tris-maleate (TM) buffer for gel soaking and tank:** (from Richardson et al.)
0.05 M Tris-maleate buffer, pH 7.8
Final molarity of buffer constituents: 50 mM Tris and 20 mM Maleic acid
Quantities for 1 liter of buffer: 6.06 g Tris and 2.32 g Maleic acid

**Agar overlay:** (from Hebert and Beaton)
4.0 g bacterial grade agar
250 mL water
Heat mixture until boils vigorously (heating 2-3 minutes in microwave). Store covered at 60°C between use.

**Enzyme stain** (for MDH - Malate Dehydrogenase, EC 1.1.1.37; from Hebert and Beaton)
1.0 mL 0.09M Tris HCl, pH=8.0 (optional)
(44.4 g Trizma Base, 248 mL 1M HCl, make up to 4 L, adjust pH as necessary)
1.5 mL NAD (2 mg/mL, fix with 1 mL sodium azide stock/mL for storage)
13 drops Malic substrate
5 drops MTT (6 mg/mL)
5 drops PMS (2 mg/mL)
2 mL agar
Malic substrate: 180 mL water; 20 mL 0.20 M Tris HCl, pH = 9.0, (98.6 g Trizma Base, 120 mL 1M HCl, make up to 4 L, adjust pH as necessary); 3.68 gm L-Malic acid; adjust to pH of 8.0

APPENDIX C
*Sample Class Data for Zebra Mussels*

**Calculation of observed genotype and allele frequencies**

| | Genotypes | | | |
|---|---|---|---|---|
| | fast-fast | fast-slow | slow-slow | Total |
| No. individuals | 12 | 6 | 18 | 36 |
| Genotype frequency | 0.33 | 0.167 | 0.5 | 1 |
| No. fast alleles | 24 | 6 | 0 | 30 |
| No. slow alleles | 0 | 6 | 36 | 42 |
| Frequency of fast allele = 30/72 = 0.417<br>Frequency of slow allele = 42/72 = 0.583 | | | | |
| Observed heterozygosity, $H$ = 0.167 | | | | |

***Is the population at Hardy-Weinberg equilibrium?*:**

**Calculation of expected genotypes from Hardy-Weinberg**

| | Genotype | | | |
|---|---|---|---|---|
| | fast-fast | fast-slow | slow-slow | Total |
| Hardy-Weinberg | $p^2$ | $2pq$ | $q^2$ | 1 |
| Expected frequency | $(0.417)^2$ = 0.174 | 2(0.417×0.583) = 0.486 | $(0.583)^2$ = 0.340 | 1 |
| Expected individuals | 0.174 × 36 = 6.3 | 0.486 × 36 = 17.5 | 0.34 × 36 = 12.2 | 36 |
| Expected heterozygosity, $H$ = 0.486 | | | | |

**Testing the hypothesis of Hardy-Weinberg equilibrium: Calculation of $\chi^2$**

| | Genotype | | | |
|---|---|---|---|---|
| | fast-fast | fast-slow | slow-slow | Total |
| Observed No. (O) | 12 | 6 | 18 | 36 |
| Expected No. (E) | 6.3 | 17.5 | 12.2 | 36 |
| O ! E | 5.7 | ! 11.5 | 5.8 | 0 |
| $(O ! E)^2$ | 32.5 | 132.25 | 33.65 | |
| $(O ! E)^2/E$ | 5.16 | 7.56 | 2.76 | $\chi^2 = 15.48$ |

We obtain an observed chi-square value of $\chi^2 = 15.48$. The number of degrees of freedom is 1 (not 2, since we obtain the allele frequency *p* from the data, once we know the number of individuals in one of the three genotype classes, we then know the numbers in the other two classes).

The chi-square value is significant at the 5% level of significance (critical $\chi^2 = 3.84$). For the sample data, we can conclude, therefore, that at least for the enzyme locus being studied, there is no evidence that this population is at Hardy-Weinberg equilibrium, suggesting that some evolutionary force is at work.