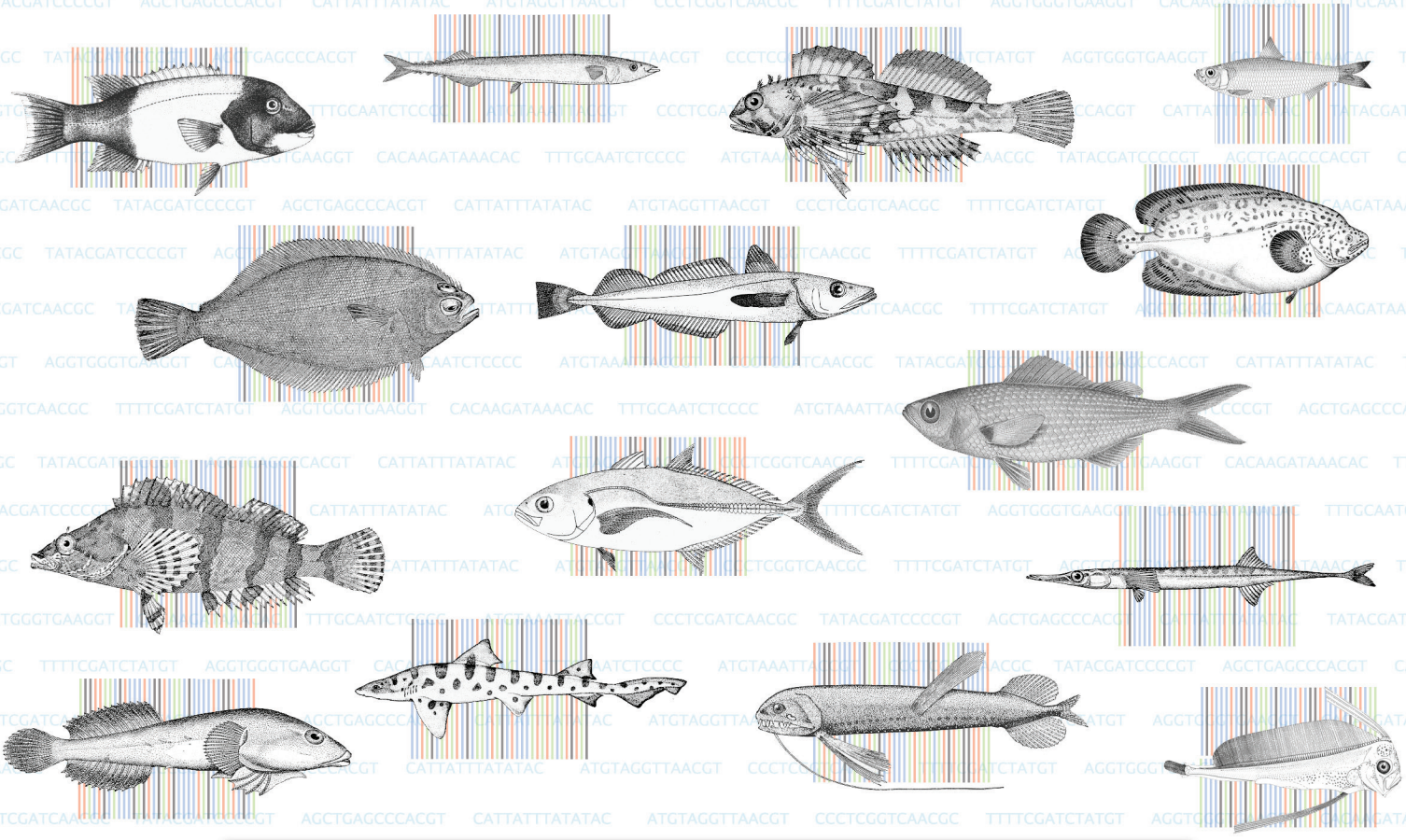
CCCTCGATCAACGC TATACGATCCCCGT AGCTGAGCCCACGT CATTATTTATATAC ATGTAGGTTAACGT CCCTCGGTCAACGC TTTTCGATCTATGT AGGTGGGTGAAGGT CACAAGATAAACAC

BARCODING LIFE'S MATRIX

Engaging students as citizen scientists in the International Barcode of Life project



LABORATORY BENCH MANUAL

v1.4

FISH DNA BARCODING

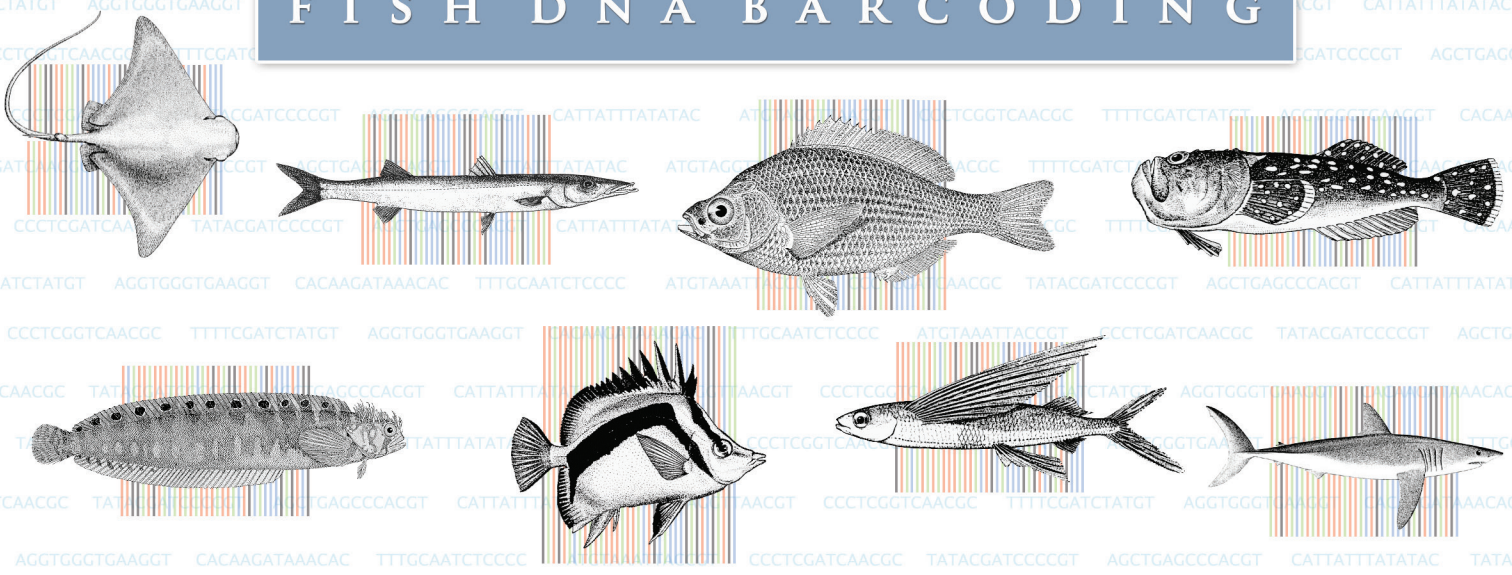
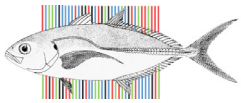


Table of Contents



Brief History of DNA Barcoding

Linnaean taxonomy – the hierarchical grouping of organisms according to common physical characteristics – has been the standard method of species identification and discovery for the last 250 years. In that span of time, fewer than 2 million of Earth's estimated 10 – 50 million plant and animal species have been formally described and cataloged. The current rate of species loss from human overpopulation, habitat destruction, pollution, overharvesting, and other anthropogenic impacts threatens to outpace the rate of species discovery using this traditional classification system, which is slow and reliant upon the expertise of a dwindling pool of experts[1]. In an effort to accelerate the discovery of new species and develop powerful new tools to monitor and preserve Earth's vanishing biodiversity, a multinational alliance of scientists is cataloging life using a DNA barcoding system[2]. The practical value of this new genetic identification system extends beyond biodiversity research and into a variety of other areas with enormous benefit to society (e.g. food authentication, the identification of agricultural pests, the detection of disease-carrying insects, etc.).

The system consists of two primary components: 1) a genetic tag or barcode that is unique to a particular species in much the same way that a Universal Product Code (UPC) barcode is unique to a particular product; and 2) an electronic database capable of providing the identity or name of the species by *reading* and matching its genetic barcode to a library of *reference* barcodes. A DNA barcode is a short, standardized genomic region represented by its constituent nucleotide sequence. As a general rule, DNA barcodes exhibit fewer nucleotide differences between members of the same species, and larger differences across members of different species groups. Discrete genomic loci were chosen as barcode regions for animals, plants, and fungi based on their ability to distinguish species groups within each kingdom. A 650 base pair (bp) segment of the mitochondrial *cytochrome c oxidase subunit 1 (COI)* gene is the standard barcode region for animals[2], whereas a segment of the nuclear ribosomal internal transcribed spacer region (ITS) is the accepted barcode region for fungi[3]. Nucleotide sequences from two chloroplast genes – the *ribulose-1,5-bisphosphate carboxylase (rbcL) and maturase K (matK)* genes – are used as standard barcode regions to identify land plants[4,5].

The central component of the barcode system is a searchable, enterprise-scale database that links DNA barcode sequences generated by researchers from known biological specimens to their formal names and other important information (including data that allows the origin and current location of the source specimen to be readily tracked and verified). This information is assembled within **reference** DNA barcode records and stored in the Barcode of Life Data Systems (BOLD) reference library[6]. As discussed in greater detail below, the scientific community established a set of data standards to ensure an extremely high level of concordance between a reference barcode sequence and the species name to which it is linked. Through the BOLD Identification System (BOLD-IDS), a **query** barcode sequence obtained from an unknown tissue sample or food product is compared against **reference** barcode sequences contained in the BOLD reference library to determine the identity of the source specimen.

Brief History of DNA Barcoding

DNA barcoding first came to the attention of the scientific community in 2003 when Paul Hebert's research group at the University of Guelph published a paper entitled: *Biological identifications through DNA barcodes*[1]. In this seminal publication, the authors proposed a genetics-based system of species identification and discovery to overcome profound and insurmountable limitations inherent to traditional taxonomy. In 2004, only a year after Hebert's publication appeared in print, the Consortium for the Barcode of Life (CBOL) was established to promote the use of DNA barcoding as a global standard for the identification of biological species. CBOL operates through the Smithsonian Institution's National Museum of Natural History (Washington, D.C) and encompasses over 200 member organizations from 50 countries.

The first international barcode conference was held in February 2005. At that time, 220 biodiversity researchers representing 44 nations convened at London's Natural History Museum to share the results of their early barcoding projects and to present their plans to conduct a number of ambitious new initiatives. In the same year, the first efforts to coordinate the assembly of standardized reference barcode libraries for Earth's fish and bird species were launched through two global campaigns: the *Fish Barcode of Life* (FISH-BOL) initiative and the *All Birds Barcoding Initiative* (ABBI), respectively. Two years later, delegates from 25 countries traveled to the University of Guelph in Ontario, Canada to discuss the formation of a *United Nations of DNA barcoding* – a global community of biodiversity scientists dedicated to the common goal of creating a DNA barcode registry for all multicellular life on the planet. Committees were soon established to coordinate and fund the activities of researchers from each participating country, and in July 2009 work began on finalizing the research plans of the International Barcode of Life (iBOL) project. Representing the largest biodiversity genomics initiative ever undertaken, iBOL was formally launched in October 2010.

Global Barcoding Initiatives

A particular specimen can only be identified in BOLD-IDS when reference sequences from its corresponding *parent* species are already represented in the BOLD reference library. The utility of DNA barcoding as a global species identification tool will therefore continue to expand as the number of reference barcodes in the BOLD database grows. At its formal launch ceremony, iBOL announced its goal to provide coverage for 500K species of plants and animals by 2015. To meet this landmark challenge, a global alliance of scientists has organized its barcoding activities into a number of large-scale campaigns. Each campaign coordinates the efforts of multinational teams to assemble reference DNA barcode libraries for targeted groups of eukaryotic organisms, with an initial focus on groups with the highest practical importance to humanity.

Global Barcoding Initiatives

The *All Birds Barcoding Initiative* (ABBI) is among the oldest barcoding campaigns. ABBI seeks to build a standardized reference barcode library from the estimated 10K bird species found in North America.
http://www.barcodingbirds.org/

The *Fish Barcode of Life Initiative* (FISH-BOL) is a global effort to construct a standardized reference barcode library for all marine and freshwater fish species.
http://www.fishbol.org/

The *Lepidoptera Barcode of Life* campaign aims to build a reference barcode library for the world's butterflies and moths. The campaign is subdivided into smaller continental initiatives that are coordinated in North America and Australia.
http://lepbarcoding.org/

The *Mammal Barcode of Life* campaign aims to create a reference library of DNA barcodes for the Earth's mammals.
http://www.mammaliabol.org/

The *Bee Barcode of Life Initiative* (Bee-BOL) is an international campaign to assemble a standardized reference sequence library for Earth's 20K known bee species.
http://www.bee-bol.org/

HealthBOL organizes the construction of reference barcode libraries from parasites, pathogens, and organisms that transmit them to humans.
http://www.healthbol.org/

The *Mosquito Barcoding Initiative* is aimed at developing a global mosquito species identification system (with a particular emphasis on disease-bearing species and their closest relatives).
http://mosquitobarcode.org/Index.htm

The *Quarantine Barcoding of Life* (QBOL) project is dedicated to the use of DNA barcoding as a diagnostic tool for the detection of plant pathogens.
http://www.qbol.org/en/qbol.htm

The *Trichoptera Barcoding of Life* campaign was launched to build a comprehensive CO1 barcode reference library for the world's caddisfly species (with the goal of developing new tools for freshwater biosurveillance).
http://trichopterabol.org/

Global Barcoding Initiatives

Other active campaigns focus their efforts on creating barcode libraries for species that inhabit entire ecosystems.

The *Moorea Biocode Project* represents the first complete inventory of all marine, terrestrial, and aquatic non-microbial life in a complex tropical ecosystem.
http://mooreabiocode.org/

The *Polar Barcode of Life* (PolarBOL) campaign conducts barcoding-based bioinventory projects in Earth's polar regions.
http://www.polarbarcoding.org/

Several countries – including Germany, Mexico, Norway, and a coalition of European nations – now sponsor initiatives aimed at assembling reference libraries for their respective flora and fauna. The growing list of DNA barcoding campaigns and national initiatives, and the sheer scale of their efforts, underscores the shared commitment of the international community to constructing the BOLD reference library. The continual growth of the BOLD reference database through this international commitment has led to a concomitant increase in the number of publications describing new applications and methods for DNA barcoding. Each month, research groups around the globe publish the results of nearly 40-60 studies that utilize DNA barcoding and related technologies to address a variety of important issues.

The *Education and Barcode of Life* (eBOL) initiative is a newly established campaign that aims to advance DNA barcoding as a discovery science-based strategy to innovate life science education.
http://www.educationandbarcoding.org/

Student Participation in iBOL

Operating under the auspices of eBOL, the Barcoding Life's Matrix program[7] provides exciting opportunities for students to join a global alliance of scientists in its efforts to create a digital genetic registry of Earth's multicellular life. Through their involvement in the Barcoding Life's Matrix program, high school and undergraduate students are equipped with the information and resources necessary to generate and integrate reference DNA barcode sequences into records that will ultimately benefit future studies aimed at preserving and protecting Earth's biodiversity.

Procedurally, the generation of reference barcode sequences follows a relatively straightforward and stepwise laboratory process. Genomic DNA is extracted from a tissue source and a ~ 650 base pair fragment of the CO1 gene is amplified using polymerase chain reaction (PCR). The size of the resulting CO1 amplicon is then verified by agarose gel electrophoresis before being purified and

Student Participation in iBOL

submitted to a commercial facility for bidirectional sequencing. The sequencing facility will return two trace files containing the nucleotide sequence for the corresponding strands of the submitted DNA fragment. An integrated suite of informatics tools available through the BOLD Student Data Portal (BOLD-SDP) is subsequently utilized by students to create contigs assembled from both trace files.

It bears noting here that although **query** barcode sequences and **reference** barcode sequences are generated and edited in the same manner, the latter are subject to a variety of data standards established by the scientific community[6]. For instance, a reference barcode sequence is generated from a specimen that must ultimately be deposited as a voucher in a curated collection maintained by a museum or other biorepository. If the voucher specimen represents a previously described species (as is often the case), then an expert taxonomist must verify its species name based on its morphology, or provide some other form of provisional designation. The sequence of a COI reference barcode must be at least 500 nucleotides in length, contain <1% ambiguous base calls, and be devoid of stop codons, contaminating sequences, or insertions or deletions.

Reference barcode sequences are integrated into comprehensive electronic data records that contain additional forms of mandatory information related to the source specimen and the collection event. This information minimally includes the original and unaltered trace files or electropherograms, the primer names and sequences used to generate the reference barcode, a unique identifier for the voucher specimen and the name of the institution where it is curated, and a collection record that identifies the specimen collector, the date and location of the collection, and GPS coordinates for the collection site[6]. These records form the basic data unit of the BOLD reference database that enables accurate species identifications to be made by its end-users through BOLD-IDS. Strict compliance with data standards therefore ensures the fidelity of BOLD as a reliable species identification tool.

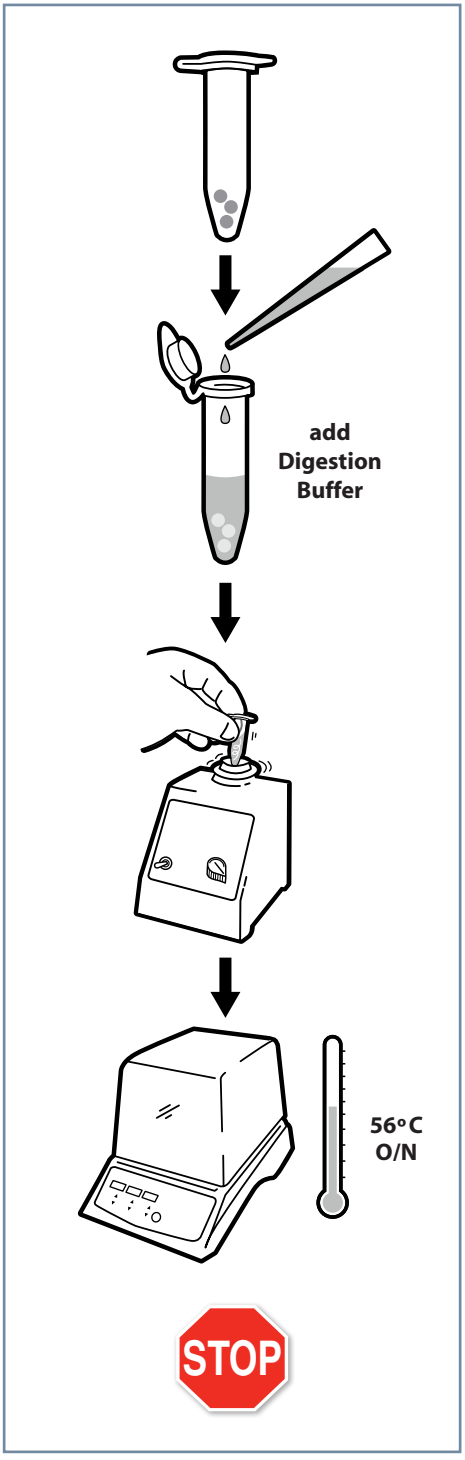
The Barcoding Life's Matrix program provides instructors with the training, instructional resources, and Web-based informatics tools needed to help their students meet these data standards and join iBOL scientists in their efforts to create a global reference DNA barcode library of Earth's multicellular life. This manual constitutes only a small proportion of the instructional resources developed to support student engagement in this landmark scientific initiative. It contains a series of optimized procedures and classroom management suggestions that enable instructors to successfully guide students through the laboratory segment of the DNA barcoding pipeline. Detailed information on the informatics segment of the pipeline, which includes the assembly and validation of reference barcode records, can be found in the Quick Start Guide of the BOLD SDP, which is accessible online at:

www.boldsystems.org/index.php/SDP_Home.

References

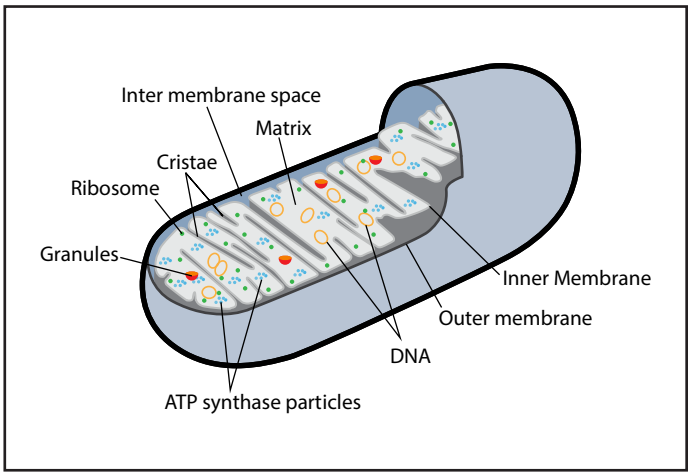
1. Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proceedings of the Royal Society of London 270: 313-321.
2. Stoeckle MY, Hebert PDN (2008) Bar Code of Life: DNA Tags Help Classify Animals. Scientific American 299(4): 66-71.
3. Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, et al. (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. Proceedings of the National Academy of Sciences 109(16): 6241-6246.
4. CBOL Plant Working Group (2009) A DNA barcode for land plants. Proceedings of the National Academy of Sciences 106(31): 12794-12797.
5. Vijayan, K, Tsou CH (2010) DNA barcoding in plants: taxonomy in a new perspective. Current Science 99(11): 1530-1541
6. Ratnasingham S, Hebert PDN (2007) The barcode of life data system (www.barcodinglife.org). Molecular Ecology Notes 7: 355-364.
7. Santschi L, Hanner RH, Ratnasingham, S, Riconscente M, Imondi R (2013) Barcoding Life's Matrix: Translating Biodiversity Genomics into High School Settings to Enhance Life Science Eduction. PLoS Biology (11)(1): e1001471.

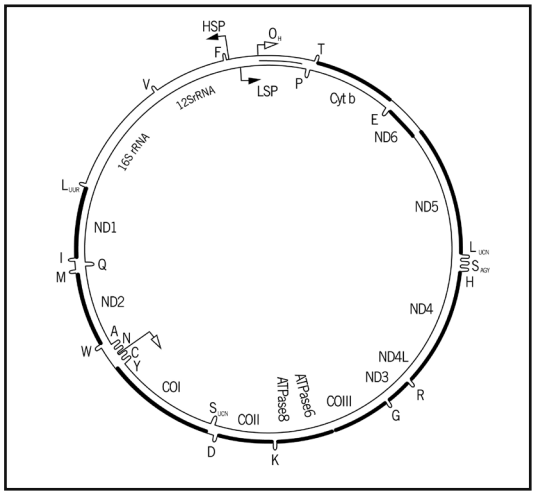
Procedural Overview



Background

Mitochondria are membrane-bound organelles that generate most of the cell's supply of ATP (adenosine triphosphate) through oxidative phosphorylation. The COI barcoding gene resides in the mitochondrial genome, a circular, double-stranded DNA molecule between 15,000 and 17,000 base pairs (bp) in length. In order to copy and amplify a fragment of the COI gene for the purposes of DNA barcoding, you must first isolate total DNA from fish tissue. Total DNA (sometimes called genomic DNA or gDNA) consists of both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). In general, each cell contains between 100 and 10,000 separate copies of mtDNA. In addition to containing the COI gene, the mitochondrial genome encodes 12 protein coding genes involved in oxidative phosphorylation and ATP production, 22 transfer RNA (tRNA) genes, and genes encoding the small and large subunits of ribosomal RNA (rRNA).





The first step in isolating gDNA from your specimens involves lysing cells in the presence of Proteinase K (an enzyme that digests nucleases and other proteins that hold cells together in tissue).

Equipment/Materials

SL-200 micropipettes and tips

shaking incubator

vortex mixer

disposable gloves

microcentrifuge tubes labeled with appropriate specimen ID containing fish tissue

Digestion Buffer

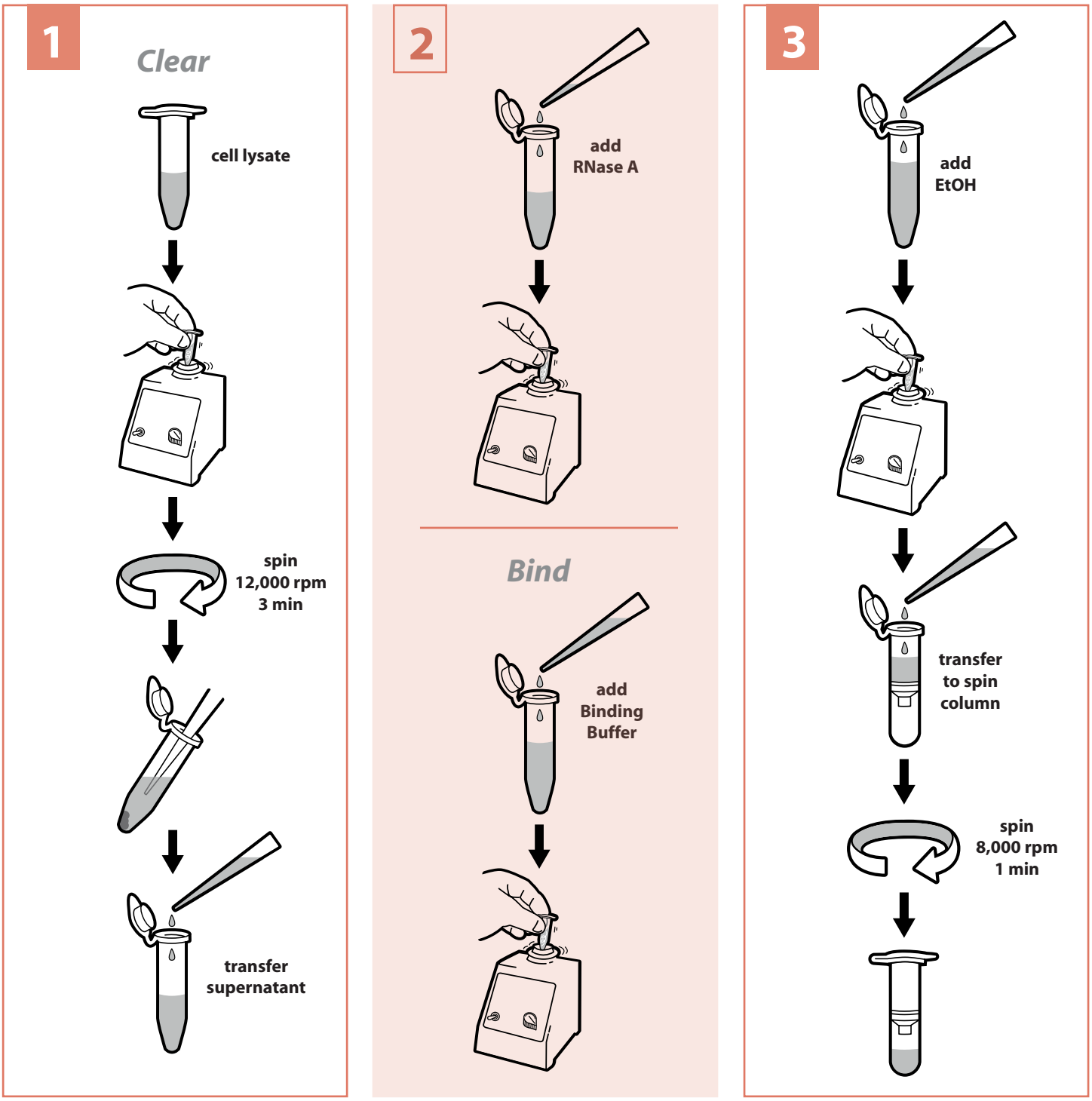
Proteinase K

Methods *(read through the entire protocol before beginning)*

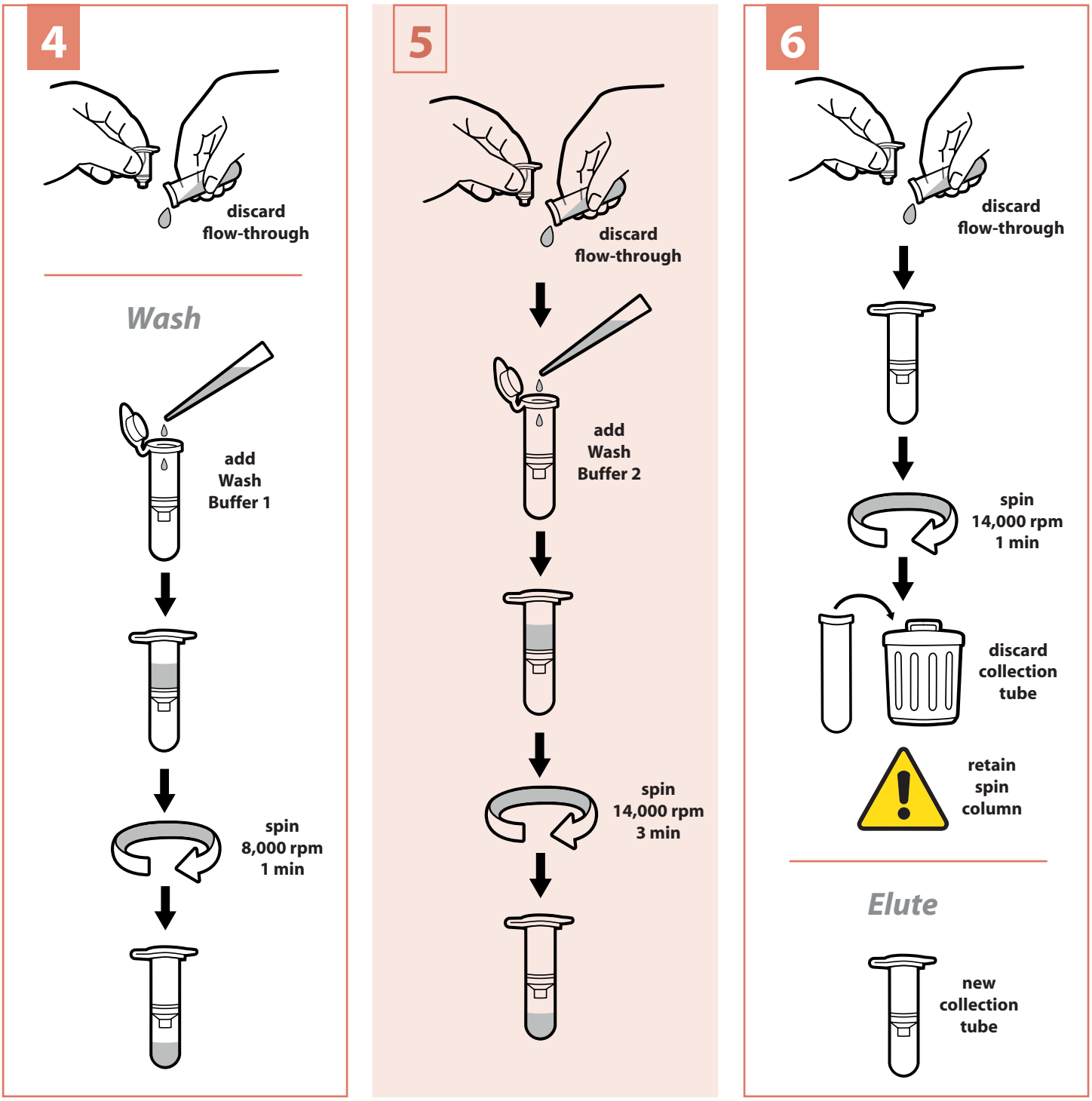
Important Note: Before getting started, set a shaking incubator (temperature, 56˚; speed, 500 rpm; and time, ≥2000 minutes).

- ☐ 1. Obtain pre-labeled 1.5 mL microcentrifuge tubes containing ~ 200 mg of tissue from the freezer. Be sure to use the specimen IDs that appear on these tubes for labeling tubes in the protocols outlined in this manual.
- ☐ 2. Set an SL-200 micropipette to 180 µL.
- ☐ 3. Using a fresh tip, add 180 µL **Digestion Buffer** to each tube containing tissue. If the salts in the Digestion Buffer are precipitated, incubate at 56˚C before using.
- ☐ 4. Set an SL-200 to 20 µL.
- ☐ 5. Using a fresh tip, add 20 µL **Proteinase K** to each tube.
- ☐ 6. Mix the contents of each tube thoroughly by vortexing for 15 seconds.
- ☐ 7. Incubate tubes overnight at 56˚ C in shaking incubator. For complete digestion, it is essential to vortex tubes every 15 minutes during the first 60 to 90 minutes of incubation. Be sure to vortex tubes for at least 15 seconds each time you remove the tubes from the incubator. After at least 12 hours of incubation, you may freeze samples at -20˚ C if you are not immediately proceeding to the next lab. The total incubation time should not exceed 24 hours.

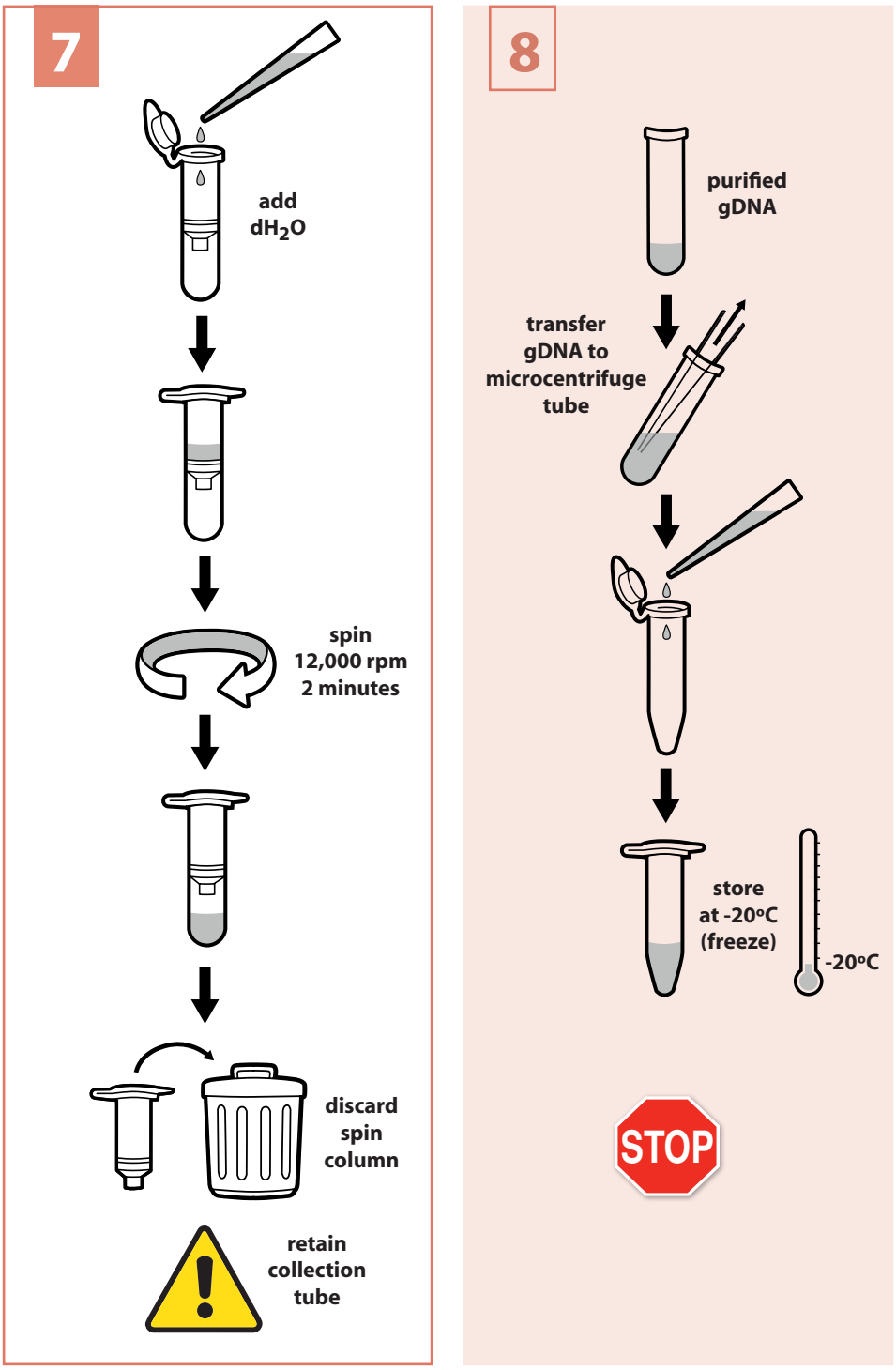
Procedural Overview



Procedural Overview



Procedural Overview



Laboratory 2: Purifying total DNA (gDNA) from tissue lysates

Background

In Laboratory 1, you incubated specimen tissue in lysis buffer containing Proteinase K. The tubes now contain a cell lysate consisting of digested proteins, carbohydrates, lipids, RNA, DNA, and other material. In order to isolate gDNA (nuclear and mitochondrial DNA) from these other macromolecules, you will add binding buffer to the cell lysates and transfer them into a spin column containing a silica matrix that selectively binds DNA. When the spin column is placed in a microcentrifuge, centripetal force pulls the cell lysate through the silica matrix; DNA binds and becomes trapped in the silica matrix as the other macromolecules freely pass through the matrix and into a collection tube. Once the DNA is bound to the matrix, two wash steps are performed to remove unbound contaminants from the matrix. In the last step of the protocol, a small volume of water is used to remove (elute) the DNA from the column matrix. During the final centrifugation step, unbound DNA will be pulled to the bottom of a clean collection tube. This purified DNA solution will contain a mixture of nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). mtDNA will be used in Laboratory 5 as a template to copy and amplify a COI gene fragment using Polymerase Chain Reaction (PCR).

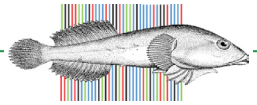
Equipment/Materials

- refrigerator/freezer
- SL-1000 micropipettes and tips
- SL-200 micropipettes and tips
- SL-20 micropipettes and tips
- microcentrifuge
- vortex mixer
- disposable gloves
- fine point permanent markers
- spin columns
- collection tubes
- 1.5 mL microcentrifuge tubes
- RNase A
- Binding Buffer
- 100% ethanol (EtOH)
- Wash Buffer 1
- Wash Buffer 2
- dH_2O

Laboratory 2: Purifying total DNA (gDNA) from tissue lysates

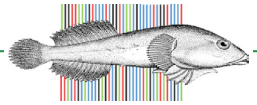
Methods *(read through the entire protocol before beginning)*

- ☐ 1. Remove cell lysates from the incubator (lysates will range in color from clear to amber, depending on the amount of blood present in the tissue obtained from each specimen).
- ☐ 2. Vortex cell lysates for 15 seconds.
- ☐ 3. Place the tubes containing the cell lysates in a microcentrifuge rotor and centrifuge for 3 minutes at 12,000 rpm. During this centrifugation step, any undigested particulate materials in the cell lysate will form a compact pellet at the bottom of the centrifuge tube. If the tissue was completely digested, then no pellet will form. Regardless of whether or not a pellet formed, you should proceed to the next step.
- ☐ 4. For each sample, label the lid of new 1.5 mL microcentrifuge tube with the appropriate specimen ID. This step should be performed while the tissue lysates are spinning in the microcentrifuge.
- ☐ 5. Set an SL-200 to 190 µL.
- ☐ 6. Remove tubes from the microcentrifuge. Place the tubes in a tube rack and carry to your workstation.
- ☐ 7. Using a fresh tip, transfer the supernatant of the cell lysates (190 uL) to the new 1.5 mL microcentrifuge tubes that you labeled with the appropriate specimen IDs (be careful not to disturb the pellet during this step).
- ☐ 8. Set an SL-200 to 20 µL.
- ☐ 9. Using a fresh tip, add 20 µL RNase A to the cleared lysates and vortex immediately.
- ☐ 10. Set an SL-1000 to 200 µL.
- ☐ 11. Using a fresh tip, add 200 µL Binding Buffer to each lysate and vortex immediately.
- ☐ 12. Using a fresh tip, add 200 µL 100% ethanol (EtOH) to each lysate and vortex immediately.
- ☐ 13. For each sample, label a spin column with the appropriate specimen ID.
- ☐ 14. Set an SL-1000 to 600 µL.
- ☐ 15. Spin columns have already been inserted into a collection tube. Using a fresh tip, transfer the contents of each lysate into a preassembled spin column that has been labeled with the appropriate specimen ID. Be sure not to touch the pipette tip to the silica matrix during the transfer. To avoid doing so, carefully eject the lysate down the wall of the spin column.



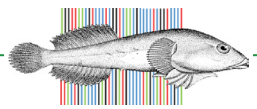
Methods *(read through the entire protocol before beginning)*

- ☐ 16. Place the spin columns containing the lysate in a microcentrifuge rotor and centrifuge for 1 minute at 8000 rpm. During this centrifugation step, DNA will bind the silica matrix as the lysate is pulled through the spin column. Before proceeding to the next step, be sure that all of the lysate was pulled through the spin column and into the collection tube. If any lysate remains in the spin column, repeat the centrifugation step for 2 minutes at 12,000 rpm.
- ☐ 17. Remove the spin columns from the microcentrifuge, making sure to pick up both the collection tube and the spin column. Place the spin columns in a tube rack and carry to your workstation. Discard the contents of the collection tubes into a waste container and place the spin columns back into the same collection tube.
- ☐ 18. Set an SL-1000 to 500 µL.
- ☐ 19. Using a fresh pipette tip, add 500 µL **Wash Buffer 1** into each column.
- ☐ 20. Place the spin columns containing **Wash Buffer 1** into a microcentrifuge rotor and centrifuge for 1 minute at 8000 rpm.
- ☐ 21. Remove spin columns from the microcentrifuge, discard the contents of the collection tubes into a waste container, and place the spin columns back into the same collection tubes.
- ☐ 22. Using a fresh pipette tip, add 500 µL **Wash Buffer 2** into each tube.
- ☐ 23. Place the spin columns containing **Wash Buffer 2** into a microcentrifuge rotor and centrifuge for 3 minutes at 14,000 rpm.
- ☐ 24. Remove spin columns from the microcentrifuge, discard the contents of the collection tubes into a waste container, and place the spin columns back into the same collection tubes.
- ☐ 25. Place the **empty** spin columns with collection tubes into a microcentrifuge rotor and centrifuge for 1 minute at 14,000 rpm. This step is necessary to remove any residual Wash Buffer from the spin columns.
- ☐ 26. Remove spin columns from the microcentrifuge, place the empty spin columns into **new** collection tubes, and discard the used collection tubes.
- ☐ 27. Set an SL-200 to 100 µL.

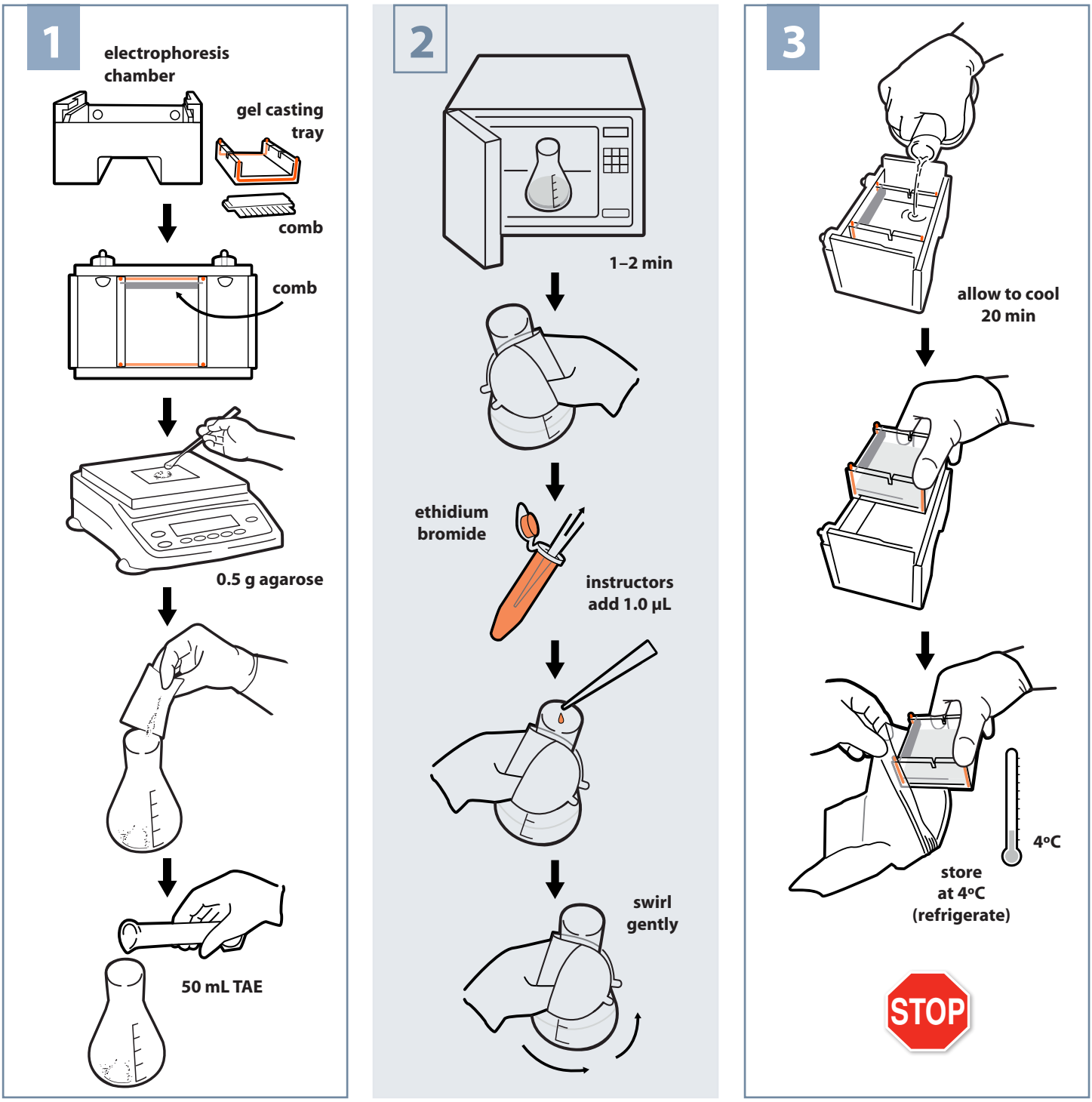


Methods *(read through the entire protocol before beginning)*

- ☐ 28. Using a fresh pipette tip, add 100 µL distilled water (dH_2O) to the center of each spin column and let stand for 1 minute. Be careful to avoid damaging the silica matrix with the pipette tip during this step.
- ☐ 29. Place the spin columns containing dH_2O into a microcentrifuge rotor and centrifuge for 2 minutes at 12,000 rpm. Your purified solution of gDNA will be in the bottom of the collection tube after this step, so **do not discard.**
- ☐ 30. While your columns are spinning, label the lid of new 1.5 mL microcentrifuge tubes with the appropriate specimen IDs followed by gDNA and the date.
- ☐ 31. Carefully remove the spin columns from the microcentrifuge.
- ☐ 32. Set an SL-200 to 100 µL.
- ☐ 33. Using a fresh pipette tip, transfer the gDNA solution (~ 100 µL) from the collection tubes to the new 1.5 mL microcentifuge tubes that you labeled with your specimen ID codes.
- ☐ 34. Store the tubes containing your gDNA stock solution in a freezer (-20° C) until ready to proceed to Laboratory 4.



Procedural Overview



Laboratory 3: Casting an agarose gel to examine gDNA by agarose gel electrophoresis

Background

Agarose gel electrophoresis is a laboratory procedure that is routinely used to separate and visualize DNA fragments by their size. For this procedure, a gel containing a porous matrix of agarose (a complex polysaccharide) is cast. The porous gel is then submerged in an electrically conductive buffer (TAE) and DNA is loaded into small depressions (or wells) that are molded into the gel. Once the DNA is loaded into the wells, a DC electrical current is passed through the agarose gel and conductive buffer. Because DNA carries a net negative charge (due to the negatively charged phosphate molecules that form its backbone), it will migrate through the agarose matrix toward the anode (or positive terminal) of a buffer-filled electrophoresis chamber. If DNA fragments of different sizes are loaded into a single well, they will migrate at different rates through the agarose matrix; smaller DNA fragments are able to move more quickly through the matrix than larger fragments.

DNA fragments are visualized in the gel with ethidium bromide (EtBr). EtBr is a fluorescent compound that will be added to your agarose gel when it is cast. The compound will bind to your gDNA as it migrates through the gel. DNA labeled with EtBr will fluoresce with an intense orange color when it is exposed to ultraviolet (UV) light. To visualize DNA labeled with EtBr, the agarose gel slab is removed from the electrophoresis chamber and placed on top of a UV light source (a transilluminator).

In Laboratory 2, you completed the gDNA isolation protocol. In Laboratory 4, you will use gel electrophoresis to evaluate the success of your efforts. In preparation for this lab, you will now cast a 1% agarose gel by following the steps outlined in this protocol.

Equipment/Materials

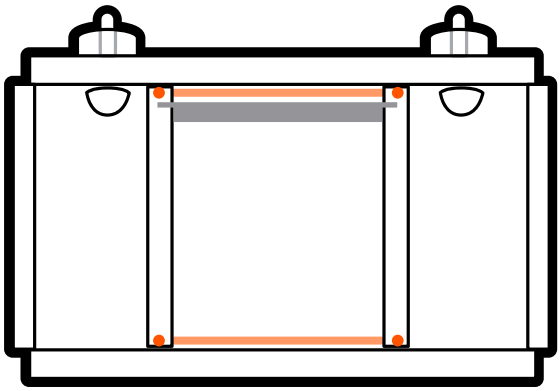
- digital balance
- microwave oven
- 4˚C refrigerator
- graduated cylinders
- electrophoresis chamber
- gel casting tray
- 10-well gel comb
- 250 mL or 500 mL flasks
- Kimwipes
- weighing paper
- rubber "hot hand" protector
- disposable gloves
- freezer bags
- fine point permanent markers
- 1X TAE buffer
- agarose powder
- ethidium bromide solution (10 mg/mL)

Methods *(read through the entire protocol before beginning)*

Important Note: Ethidium bromide is a mutagen, a suspected carcinogen, and is irritating to the eyes, skin, and mucous membranes in high concentrations. Wear a lab coat, safety goggles, and nitrile rubber gloves during this protocol.

Assembling the casting tray for OWL gel electrophoresis system:

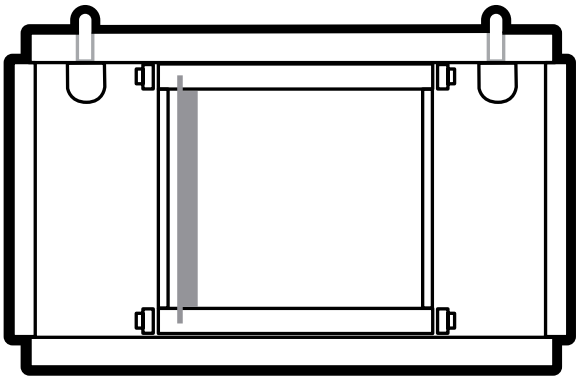
- [ ] 1. Position the gel casting tray so that its open ends are oriented perpendicular to the electrophoresis chamber.
- [ ] 2. Wet the gaskets along the edge of the open ends of the casting tray with a moist Kimwipe.
- [ ] 3. Gently press the casting tray into the electrophoresis chamber. To prevent leaks, be sure that the rubber gaskets along the open edges of the casting tray make a tight seal against the inside walls of the chamber.
- [ ] 4. Insert a 10-well comb into the grooves near the top edge of the casting tray. A figure of correctly positioned casting tray and comb is shown below:



Methods *(read through the entire protocol before beginning)*

Assembling the casting tray for the Fotodyne (screw gate) gel electrophoresis system:

- [ ] 1. Position the gel casting tray so that its gates are in the up position. Loosen screws to move gates and then tighten when gates are in the proper position. It may be necessary to tape the gates to prevent leaking.
- [ ] 2. Gently position the casting tray in the electrophoresis chamber.
- [ ] 3. Insert a 10-well comb into the grooves near the top edge of the casting tray. A figure of correctly positioned casting tray and comb is shown below:



Casting agarose gels

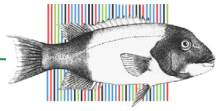
- [ ] 1. Use a balance to weigh 0.5 g of agarose on weighing paper.
- [ ] 2. Transfer the agarose from the weighing paper to an empty 250 mL or 500 mL flask. Avoid pouring the agarose powder along the sides of the flask when transferring.
- [ ] 3. Measure 50 mL of 1X TAE buffer with a graduated cylinder and add to flask containing agarose powder.
- [ ] 4. Plug the mouth of the flask with two or three dry Kimwipes.
- [ ] 5. Place flask in a microwave and heat for 1-2 minutes on the highest setting. If the agarose isn't completely melted, microwave for an additional 15 – 30 seconds or until melted.

Methods *(read through the entire protocol before beginning)*

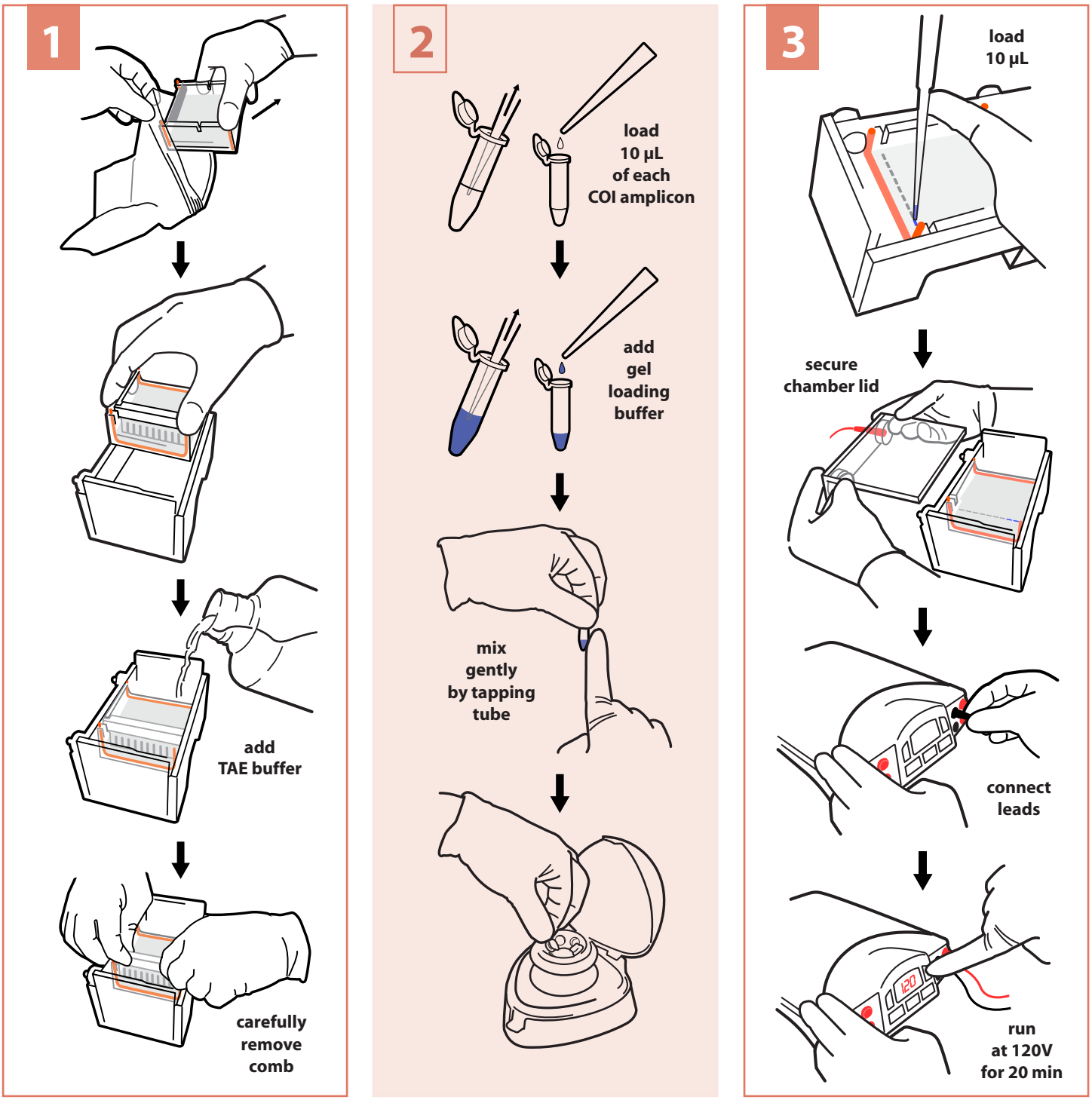
- ☐ 6. Remove the hot flask from microwave with a rubber hot hand protector or insulated gloves.
- ☐ 7. Instructors: add 1.0 µL of ethidium bromide stock solution (10 mg/mL) to each flask of melted agarose.
- ☐ 8. Gently swirl melted agarose to mix the ethidium bromide (swirl gently to avoid making bubbles).

Warning: Do not to pour hot agarose into Fotodyne electrophoresis chambers. If you are using the Fotodyne gel electrophoresis system, please allow the flask to cool for 5 minutes at room temperature approximately before proceeding to step 9.

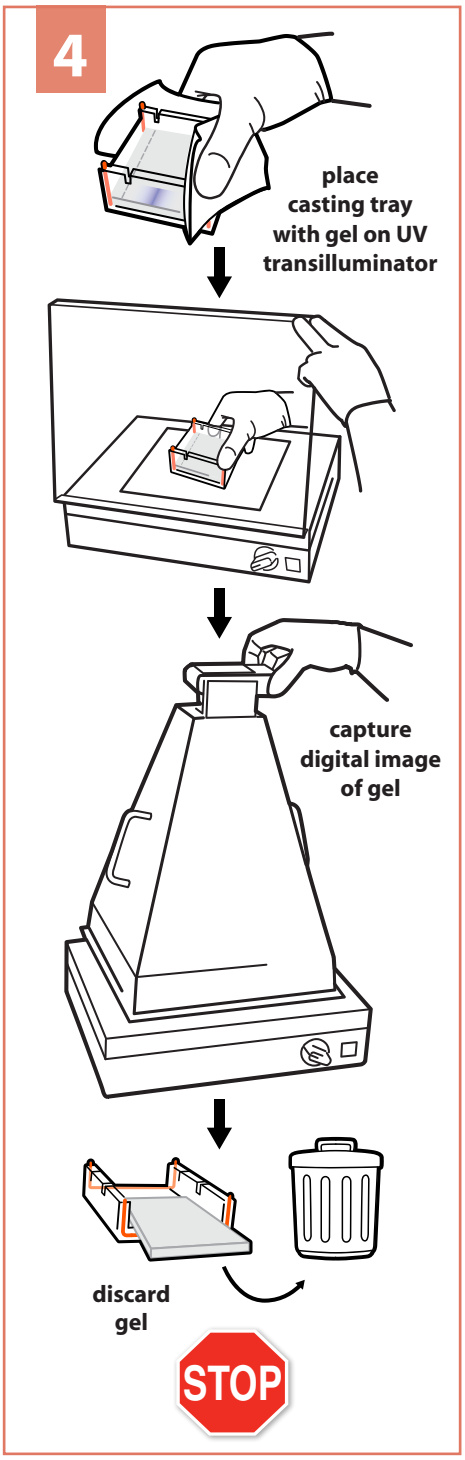
- ☐ 9. Pour the contents of the flask into the gel casting tray and allow to solidify. If any bubbles form when you pour the gel into the casting tray, simply move them to the lower part of the gel (away from the side containing comb) with a clean pipette tip.
- ☐ 10. Allow the gel to cool for 20 minutes. **Do not attempt to move the gel while it is cooling**.
- ☐ 11. When the gel is completely solidified, remove the casting tray containing the agarose gel from the electrophoresis chamber. Do not remove the comb from your gel as this may damage the wells where you will load your DNA in the next lab.
- ☐ 12. Place the entire casting tray containing your gel and comb in an air-tight freezer bag. Use a fine point permanent marker to label the bag with the date and group name. Store the bag containing your gel in a refrigerator (~ 4˚C) until you are ready to proceed to Laboratory 4.



Procedural Overview



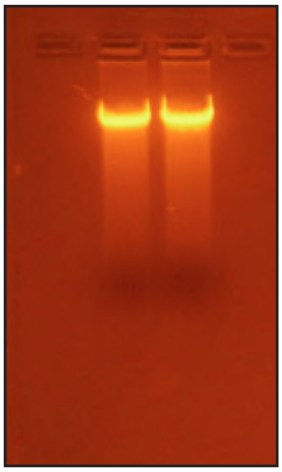
Procedural Overview



Laboratory 4: Examining purified gDNA with agarose gel electrophoresis

Background

In Laboratory 3, you cast an agarose gel and stored it in a refrigerator at 4˚C. In this protocol, you will use this gel to determine if you were successful in your efforts to purify gDNA from fish tissue. To accomplish this goal, you will submerge your agarose gel in an electrically conductive buffer, carefully remove the comb, and load a small volume (aliquot) of your purified gDNA samples from Laboratory 2 into the wells (or depressions) that the comb created in the gel when it solidified. To make it easier to see your DNA solution as you load it into a well of the agarose gel, you will add a small volume of loading buffer to your DNA. The loading buffer contains a blue indicator dye that makes it easier to see your DNA when you load it into a well. It also contains a carrier that will help your DNA sink to the bottom of each well.



When you apply an electrical current through the electrophoresis chamber containing your gel, the DNA loaded into the wells will migrate through the porous gel matrix toward the anode (the red/positive terminal) due to the negative charge of the phosphate backbone. The blue indicator dye that you added to your DNA samples is also negatively charged and will independently migrate through the gel toward the positive terminal at its own characteristic rate. It's important to realize, however, that this dye **does not label your DNA**. Recall from Laboratory 3 that your instructor added a small amount of EtBr to your gel. EtBr is a fluorescent compound that will bind to your DNA as it is pulled through the gel by an electrical current. By placing your gel on an ultraviolet light source (a UV transilluminator), you will be able to determine if your gDNA extraction was successful. The figure to the left shows a photograph of gDNA visualized in this way. gDNA isolated from fish gill tissue typically appears as a bright, high molecular weight band that is trailed by a very faint smear. This banding pattern can vary based on a variety of different factors. The DNA appears bright orange because it is bound to EtBr, which emits light of this wavelength when excited by UV light.

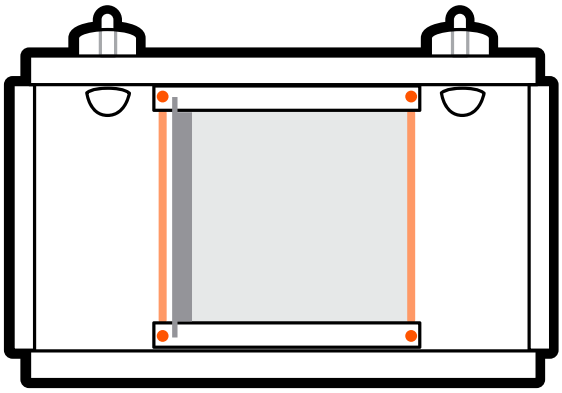
Equipment/Materials

- SL-20 micropipette and tips
- microcentrifuge
- gel electrophoresis chamber and lid
- DC power supply
- UV transilluminator
- digital camera with attached hood
- agarose gel from Laboratory 3
- 0.5 mL microcentrifuge tubes
- disposable gloves
- safety goggles
- gel loading buffer
- 1X TAE buffer

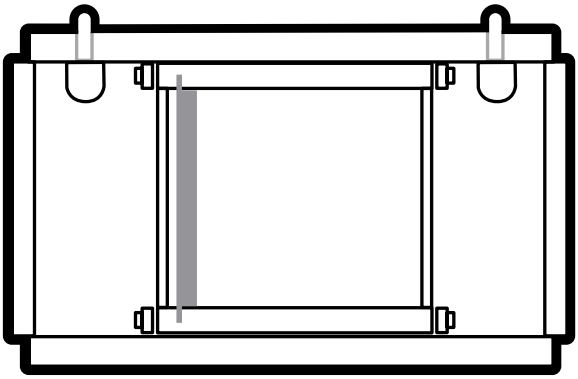
Methods *(read through the entire protocol before beginning)*

Important Note: Before getting started, thaw tubes containing purified gDNA (on ice), remove your agarose gel from the refrigerator, and allow to warm at room temperature on a lab bench. Students should wear lab coats and nitrile rubber gloves throughout the protocol below.

OWL system: Rest the casting tray containing the agarose gel and comb on the platform in the center of the electrophoresis chamber as shown in the figure below:



Fotodye (screw gate) system: Remove tape (if necessary), loosen thumb screws, lower gates, and position tray on the platform in the center of the electrophoresis chamber as shown in the figure below:Set an SL-20 to 10 µL.



Loading agarose gels

☐ 1. Pour 1X TAE buffer into each reservoir of the electrophoresis chamber and completely submerge the agarose gel. The amount of buffer above the gel should be roughly equivalent to the thickness of the gel itself.

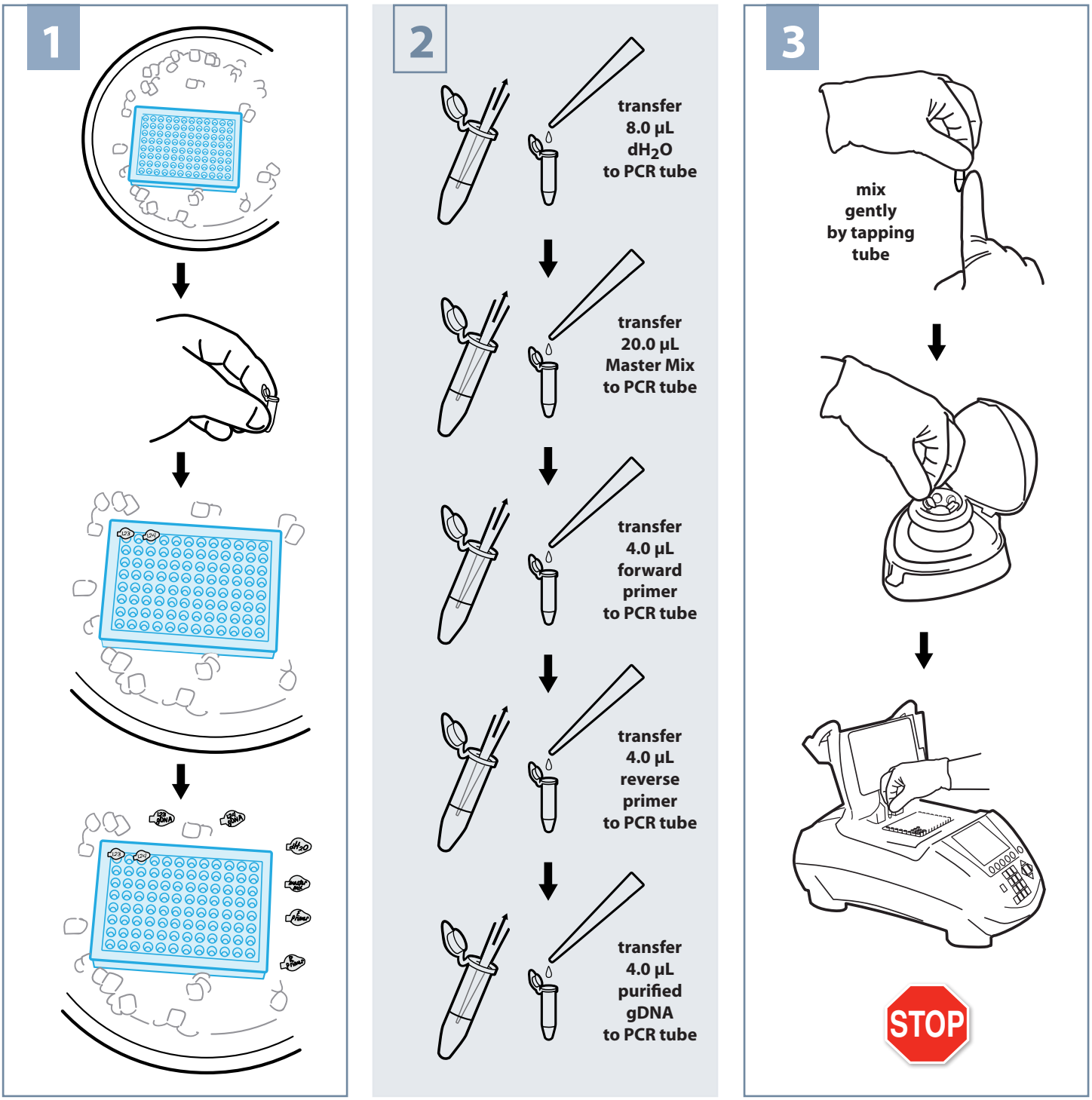
Methods *(read through the entire protocol before beginning)*

- ☐ 2. Carefully remove the comb by pulling it straight up from both sides.
- ☐ 3. Set an SL-20 to 10 µL.
- ☐ 4. Using a fresh pipette tip, transfer 10 µL of purified (and thawed) gDNA stock solution (from Laboratory 2) to a 0.5 mL microcentrifuge tube labeled with only the specimen ID code. Instructors: be sure to collect the tubes containing the remaining gDNA stock solution (90 µL) and store in a freezer at the end of this step.
- ☐ 5. Set an SL-20 to 2 µL.
- ☐ 6. Using a fresh tip, add 2 µL of gel loading buffer to each of the tubes containing 10 µL gDNA.
- ☐ 7. Mix by gently tapping each tube with your index finger.
- ☐ 8. Briefly spin each tube in a picofuge to bring the contents down to the bottom of the tubes.
- ☐ 9. Set an SL-20 to 10 µL.
- ☐ 10. Using a fresh tip, load 10 µL of each sample into a separate well of the agarose gel. In your notebook, be sure to indicate the well number that corresponds to each of your samples.
- ☐ 11. When samples are loaded, carefully secure the acrylic lid of the electrophoresis chamber by gently sliding it over the top of the chamber. Be sure that the connections are made between the outlets of the lid and the plugs of the chamber. It is very important at this stage of the protocol to **avoid moving or bumping** the electrophoresis chamber as this may result in gDNA spilling out of its designated well and into adjacent wells.
- ☐ 12. Ensure that the positive (red) lead is located at the bottom of the gel (the side opposite the comb). If this is not the case, consult your instructor for guidance on how to proceed.
- ☐ 13. Connect the electrical leads from the lid to the power supply according to the color coding scheme.
- ☐ 14. Set the voltage on the power supply to 120V, press <RUN>, and monitor the progress of the blue indicator dye in the gel. This dye will migrate through the gel at a rate comparable to a DNA fragment that is approximately 300 bp in length.
- ☐ 15. Your gel will be ready for inspection under UV light after approximately 20 minutes. Consult your instructor for guidance on how to visualize and take photographs of your gel.

Record Data

10-WELL GEL ***Label this figure to document where you loaded your samples*** *(if you skip a well, place an X in the corresponding box below)*								*Group #* *Picture #*	
well 1	***well 2***	***well 3***	***well 4***	***well 5***	***well 6***	***well 7***	***well 8***	***well 9***	***well 10***

Procedural Overview



Background

In Laboratory 2, you purified gDNA from fish tissue. Recall that gDNA contains both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). The COI barcoding gene resides on the mitochondrial genome. In order to generate DNA barcodes for your fish specimens, you must first copy and amplify a segment of this mitochondrial gene with a technique called Polymerase Chain Reaction (PCR). You will use short DNA fragments called primers to define the 650 bp region of the COI gene that will be copied and amplified by PCR.

To perform PCR, you will set up a reaction mixture containing primers, your gDNA **template, dNTPs** (dATP, dCTP, dGTP, and dTTP), a heat-stable **DNA polymerase**, and a buffer containing **magnesium chloride** ($MgCl_2$). The reaction mixture is immediately placed in a thermocycler that has been programmed to run through repeated cycles of heating and cooling. At the start of the first cycle, the temperature of the mixture is raised to near-boiling (94˚C). At this temperature, the hydrogen bonds that hold the two strands of template DNA together are temporarily broken (**denaturation step**). The temperature then lowered to 55˚C, which allows the primers to bind to the top and bottom DNA strands of the COI gene according to the base pairing rules (**annealing step**). Next, the temperature is raised to 72˚C. During this step, a heat stable DNA polymerase begins synthesizing new strands of the COI gene region bracketed by the primers (**elongation step**). Each cycle of denaturation, annealing, and elongation is repeated 35 times. After each cycle, the number of COI gene fragments doubles. When complete, the reaction will produce over a billion copies of the 650 bp COI gene fragment defined by your primers.

Equipment/Materials

SL-20 micropipettes and tips

picofuge

thermocycler

bins to hold ice (1 per group)

freezer

benchtop lab cooler

crushed ice

fine-tip Permanent Markers

racks for PCR tubes

PCR tubes

disposable gloves

fine point permanent marker

reagents (in table below)

Methods *(read through the entire protocol before beginning)*

Important Note: Accurate pipetting is essential to the success of this protocol. Be sure to carry out the complete protocol on ice.

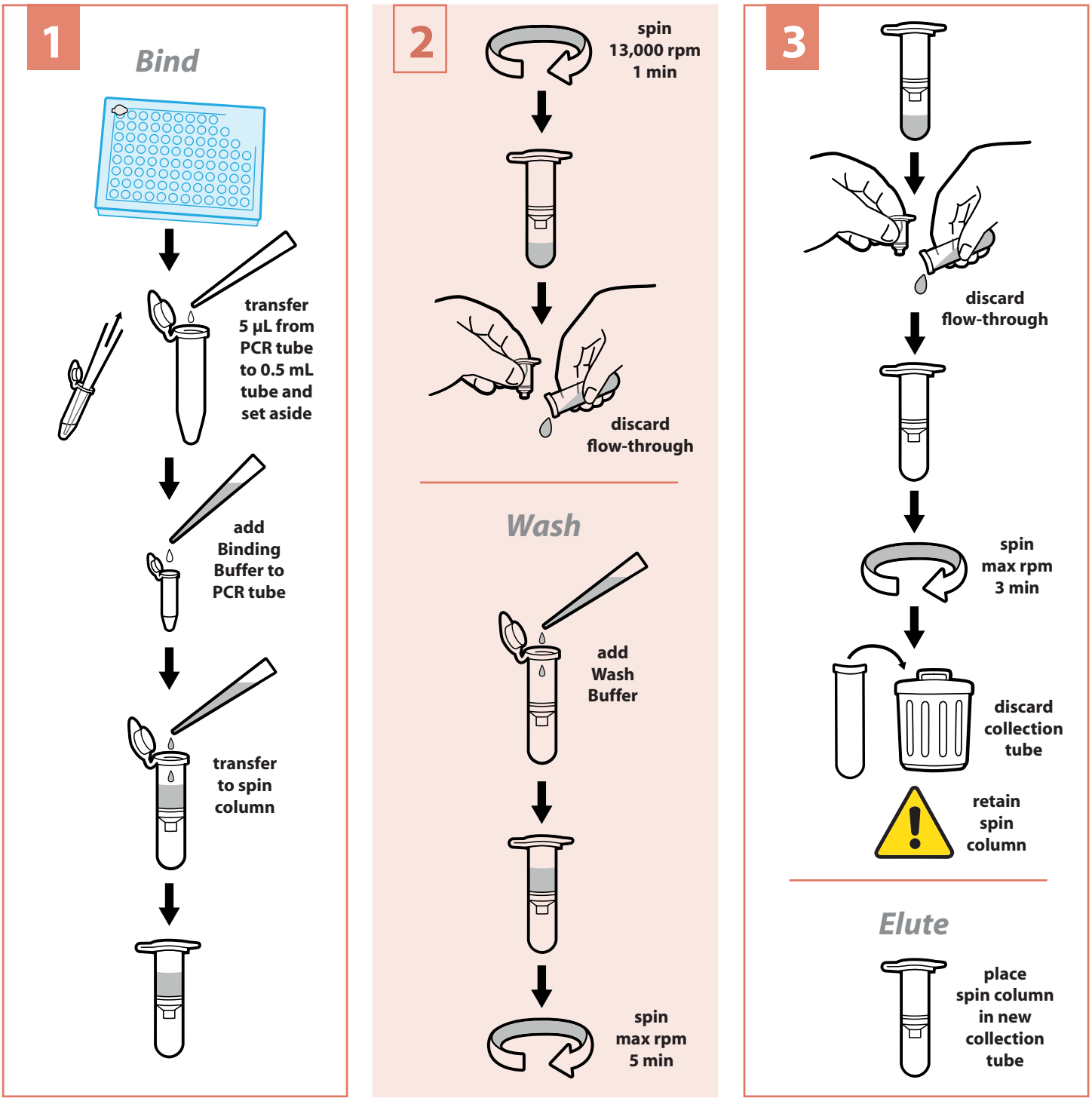
- ☐ 1. Place a small tube rack on ice.
- ☐ 2. Obtain a 200 µL PCR tube for each reaction that you will perform.
- ☐ 3. Label the top of your tubes with the appropriate specimen ID codes. Do not label the sides of the tubes.
- ☐ 4. Place the tubes in an **upper** row of a small tube rack, separated by at least one space.
- ☐ 5. Arrange the reagents shown in the table below on ice.
- ☐ 6. Using an SL-20, add the reagents in the table below to each PCR tube. Add each reagent **in the order shown below and be sure to use a fresh tip for each reagent.**

REAGENT	*VOLUME TO ADD (in µL)*
dH_2O	***8.0***
2X Master Mix	***20.0***
2.5 µM forward primer	***4.0***
2.5 µM reverse primer	***4.0***
purified gDNA	***4.0***

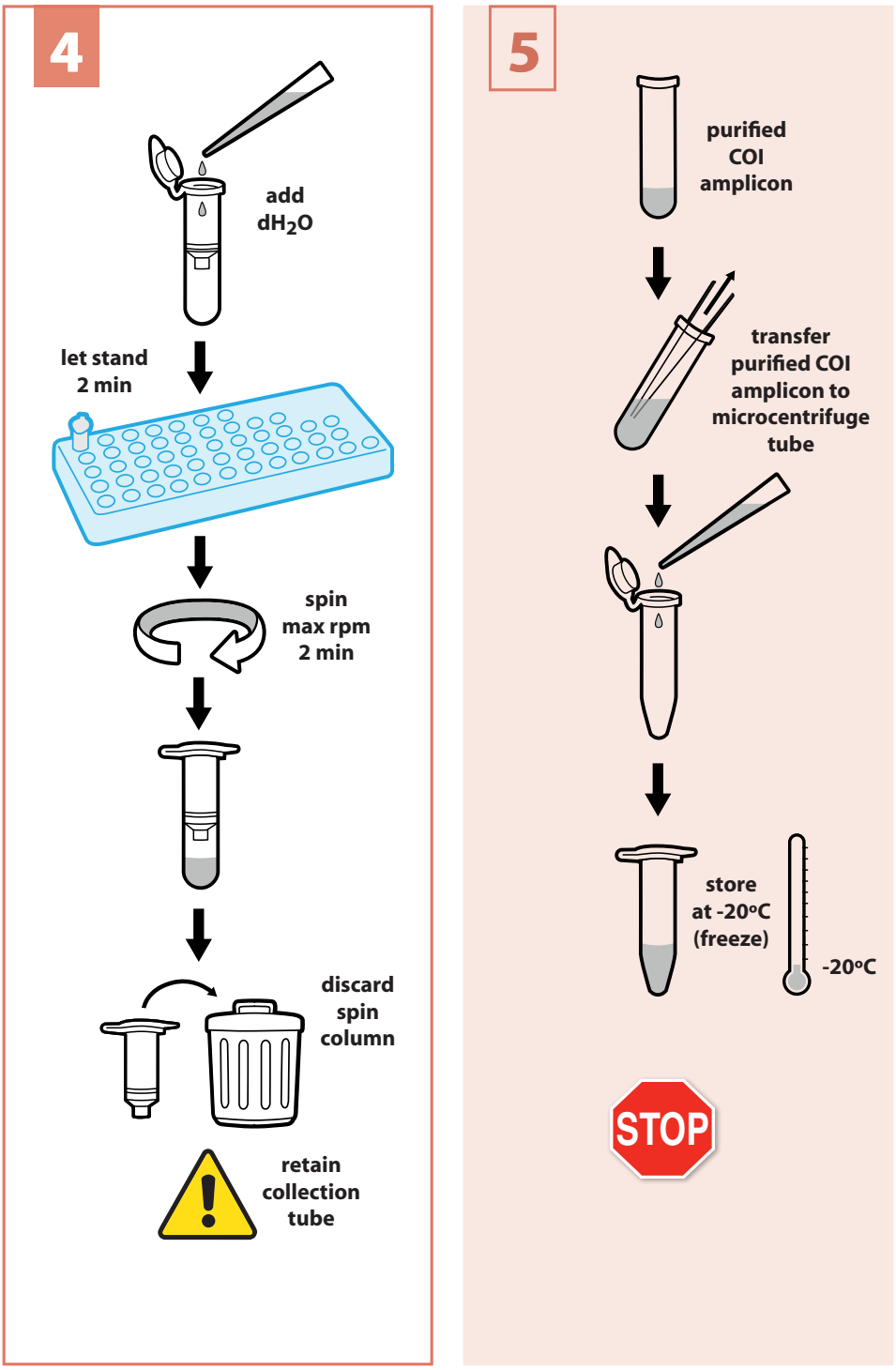
2X Master Mix contains PCR buffer, $MgCl_2$, dNTPs, and Taq polymerase

- ☐ 7. After the addition of gDNA, gently mix the contents of each tube by gently tapping with your index finger.
- ☐ 8. Spin the tubes for 5 seconds in a picofuge to bring the contents down to the bottom of the tubes.
- ☐ 9. When the entire class has completed step 8, load your samples into the thermocycler.
- ☐ 10. Your instructor will now set the thermocycler to run.

Procedural Overview



Procedural Overview



Laboratory 6: Spin-column purification of COI amplicons

Background

In Laboratory 5, you used PCR to copy and amplify a 650 bp segment of the COI gene from a gDNA template that was isolated from fish tissue. In addition to the reagents that you added to the tube at the start of the PCR reaction, your tube should now contain over a billion copies of this COI gene fragment.

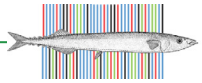
The goal of this lab is to isolate your COI gene fragment (or amplicon) from the other reagents contained in your PCR reaction tube (e.g. Taq polymerase, unused dNTPs, unincorporated primers, etc.). The presence of these other reagents will interfere with the sequencing reaction and produce poor quality sequencing data, so they must be removed from the reaction mix. To purify your COI amplicons, you will use a procedure similar to the one that you used to purify gDNA in Laboratory 2. To begin, you will add Binding Buffer to your PCR reaction tubes and transfer the resulting solution into a spin column containing a silica matrix that selectively binds DNA. When the spin column is placed in a microcentrifuge, centripetal force pulls the solution through the silica matrix; your COI amplicons bind and become trapped in the silica matrix as other materials freely pass through the matrix and into a collection tube. Once the COI amplicons are bound to the matrix, a wash step is performed to remove unbound contaminants from the matrix. Later in the protocol, a small volume of water is used to remove (elute) the COI amplicons from the column matrix. During the final centrifugation step, liberated COI amplicons will be pulled to the bottom of a clean collection tube. The solution in the collection tube will contain purified COI DNA that is now suitable for automated DNA sequencing. The process of automated DNA sequencing is based on PCR and will be discussed in a subsequent Laboratory.

Equipment/Materials

- refrigerator/freezer
- vortex mixer
- microcentrifuge
- SL-1000 micropipettes and tips
- SL-200 micropipettes and tips
- SL-20 micropipettes and tips
- fine point permanent markers
- microcentrifuge tubes
- tube racks
- 0.5 mL microcentrifuge tubes
- spin columns
- collection tubes
- Binding Buffer
- Wash Buffer
- dH_2O

Methods *(read through the entire protocol before beginning)*

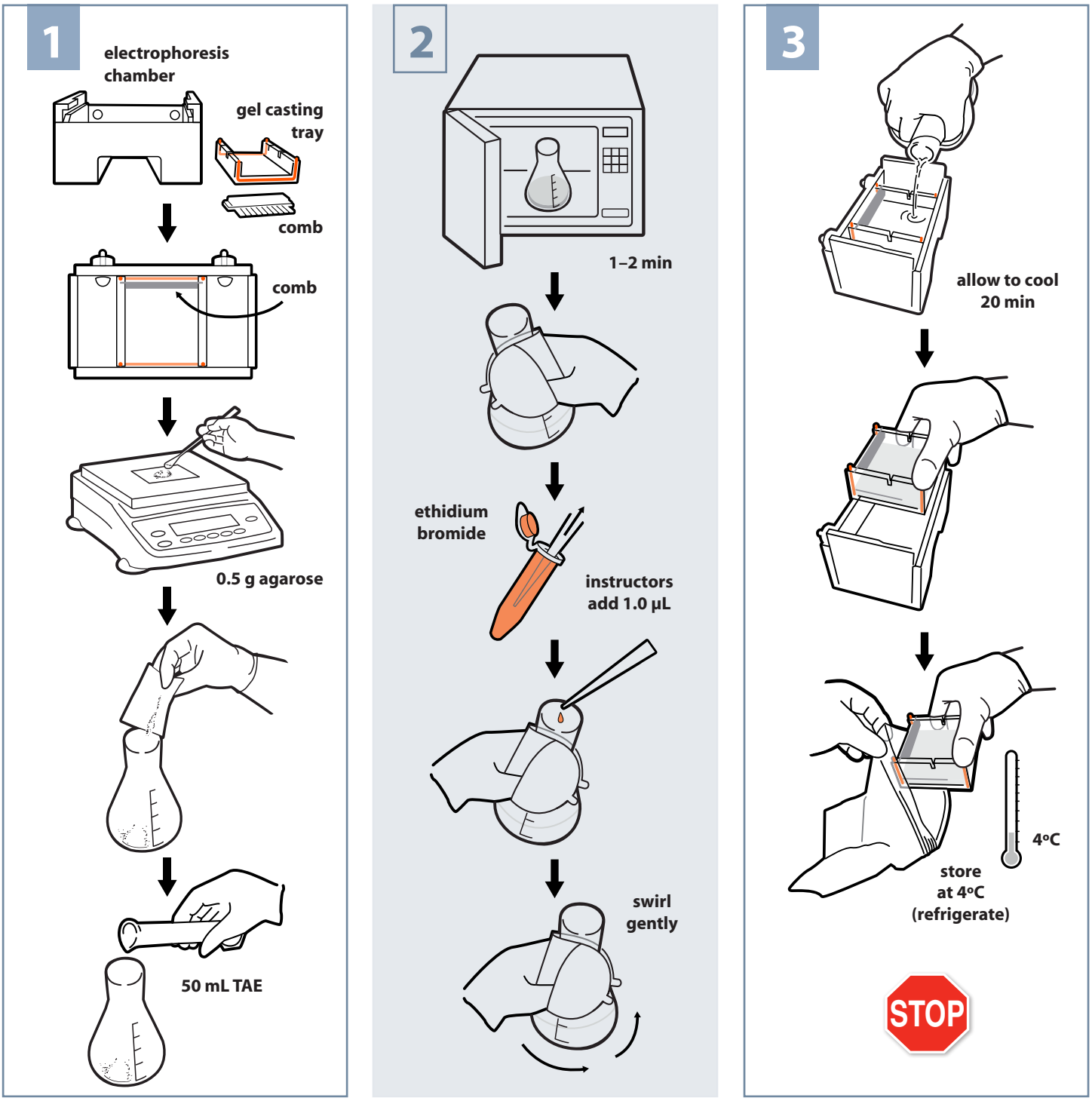
- ☐ 1. For each sample, label the lid of a 0.5 mL microcentrifuge tube with the appropriate specimen ID.
- ☐ 2. Set an SL-20 to 5 µL.
- ☐ 3. Using a clean tip, remove 5 µL from each of your PCR reaction tubes and transfer to the 0.5 mL tubes that you labeled with your specimen IDs (these aliquots will be used to verify that your PCR reaction was successful in the event that your purification yields no product). Give these tubes to your instructor before proceeding to step 4.
- ☐ 4. Set an SL-200 to 140 µL.
- ☐ 5. Using a fresh tip, add 140 µL Binding Buffer to the remainder of each PCR reaction (35 µL).
- ☐ 6. For each sample, label a spin column with the appropriate specimen ID.
- ☐ 7. Set an SL-200 to 175 µL.
- ☐ 8. Using a fresh tip, transfer the contents of each PCR tube to a separate preassembled spin column. Be sure not to touch your pipette tip to the silica matrix.
- ☐ 9. Place the spin columns containing your PCR reactions plus Binding Buffer into a microcentrifuge rotor and centrifuge for 1 minute at 13,000 rpm. During this centrifugation step, your COI gene fragments will bind the silica matrix in the spin column. Be sure to balance the tubes in the centrifuge.
- ☐ 10. Remove spin columns from the microcentrifuge, making sure that you pick up both the spin column and the bottom collection tube.
- ☐ 11. Place the spin columns in a tube rack and carry to your workstation.
- ☐ 12. Discard the contents of the collection tubes into a waste container and place the spin columns back into the same collection tube.
- ☐ 13. Set an SL-1000 to 650 µL.
- ☐ 14. Using a fresh pipette tip, add 650 µL Wash Buffer into each column.
- ☐ 15. Place the spin columns containing Wash Buffer into a microcentrifuge rotor and centrifuge for 5 minutes at max speed.
- ☐ 16. Remove spin columns from the microcentrifuge rotor, discard the contents of the collection tubes into a waste container, and place the spin columns back into the same collection tubes.



Methods *(read through the entire protocol before beginning)*

- ☐ 17. Place the **empty** spin columns into a microcentrifuge rotor and centrifuge for 3 minutes at max speed. This step is necessary to remove any residual Wash Buffer from the spin column.
- ☐ 18. Remove the spin columns from the microcentrifuge, place the empty spin columns into **new** collection tubes, and discard the used collection tubes.
- ☐ 19. Set an SL-200 to 50 µL.
- ☐ 20. Using a fresh pipette tip, add 50 µL distilled water (dH_2O) over the center of the silica matrix in each column and let stand for 2 minutes. It is essential to add the water to the **center** of the matrix. Use caution to avoid damaging the silica matrix when performing this step.
- ☐ 21. Place the spin columns containing dH_2O into a microcentrifuge rotor and centrifuge for 2 minutes at max speed. Your purified COI amplicons will be in the solution at bottom of the collection tubes, so **do not discard**.
- ☐ 22. For each sample, label a 0.5 mL microcentrifuge tube with the appropriate specimen ID followed by PCR and the date. This step can be performed while the columns are spinning in the microcentrifuge.
- ☐ 23. Carefully remove the spin columns from the microcentrifuge.
- ☐ 24. Set an SL-200 to 50 µL.
- ☐ 25. Using a fresh pipette tip, transfer the DNA solution (~ 50 µL) from the collection tubes to the 0.5 mL microcentifuge tubes that you labeled with the appropriate specimen IDs.
- ☐ 26. Store tubes containing your purified COI DNA in a freezer at -20°C until ready to proceed to Laboratory 8.

Procedural Overview



Laboratory 7: Casting an agarose gel to confirm the length of COI amplicons

Background

Recall from Laboratory 3 that agarose gel electrophoresis is a laboratory procedure that is routinely used to separate and visualize DNA fragments by their size. The primers that you used to copy and amplify the COI barcode region should produce a band of approximately 650 bp. In Laboratory 8, you will use gel electrophoresis to confirm that you generated DNA fragments of the predicted length. In preparation for this lab, you will now cast a 1% agarose gel by following the protocol below.

Equipment/Materials

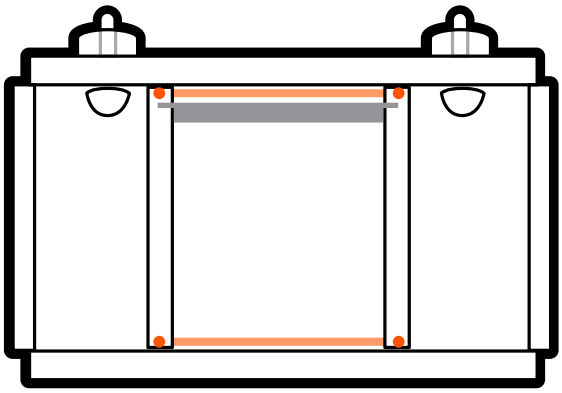
- digital balance
- microwave oven
- 4˚C refrigerator
- graduated cylinders
- electrophoresis chamber
- gel casting tray
- 10-well gel comb
- 250 mL or 500 mL flasks
- Kimwipes
- weighing paper
- rubber "hot hand" protector
- disposable gloves
- freezer bags
- fine point permanent markers
- 1X TAE buffer
- agarose powder
- ethidium bromide solution (10 mg/mL)

Methods *(read through the entire protocol before beginning)*

Important Note: Ethidium bromide is a mutagen, a suspected carcinogen, and is irritating to the eyes, skin, and mucous membranes in high concentrations. Wear a lab coat, safety goggles, and nitrile rubber gloves during this protocol.

Assembling the casting tray for OWL gel electrophoresis system:

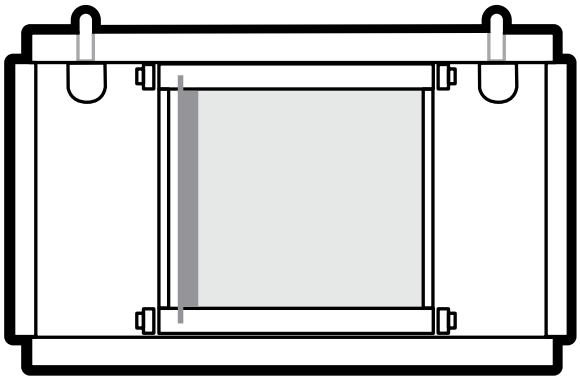
- [ ] 1. Position the gel casting tray so that its open ends are oriented perpendicular to the electrophoresis chamber.
- [ ] 2. Wet the gaskets along the edge of the open ends of the casting tray with a moist Kimwipe.
- [ ] 3. Gently press the casting tray into the electrophoresis chamber. To prevent leaks, be sure that the rubber gaskets along the open edges of the casting tray make a tight seal against the inside walls of the chamber.
- [ ] 4. Insert a 10-well comb into the grooves near the top edge of the casting tray. A figure of correctly positioned casting tray and comb is shown below:



Methods *(read through the entire protocol before beginning)*

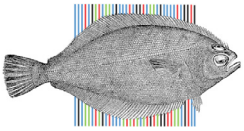
Assembling the casting tray for the Fotodyne (screw gate) gel electrophoresis system:

- [ ] 1. Position the gel casting tray so that its gates are in the up position. Loosen screws to move gates and then tighten when gates are in the proper position. It may be necessary to tape the gates to prevent leaking.
- [ ] 2. Gently position the casting tray in the electrophoresis chamber.
- [ ] 3. Insert a 10-well comb into the grooves near the top edge of the casting tray. A figure of correctly positioned casting tray and comb is shown below:



Casting agarose gels

- [ ] 1. Use a balance to weigh 0.5 g of agarose on weighing paper.
- [ ] 2. Transfer the agarose from the weighing paper to an empty 250 mL or 500 mL flask. Avoid pouring the agarose powder along the sides of the flask when transferring.
- [ ] 3. Measure 50 mL of 1X TAE buffer with a graduated cylinder and add to flask containing agarose powder.
- [ ] 4. Plug the mouth of the flask with two or three dry Kimwipes.
- [ ] 5. Place flask in a microwave and heat for 1-2 minutes on the highest setting. If the agarose isn't completely melted, microwave for an additional 15 – 30 seconds or until melted.

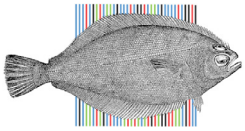


Methods *(read through the entire protocol before beginning)*

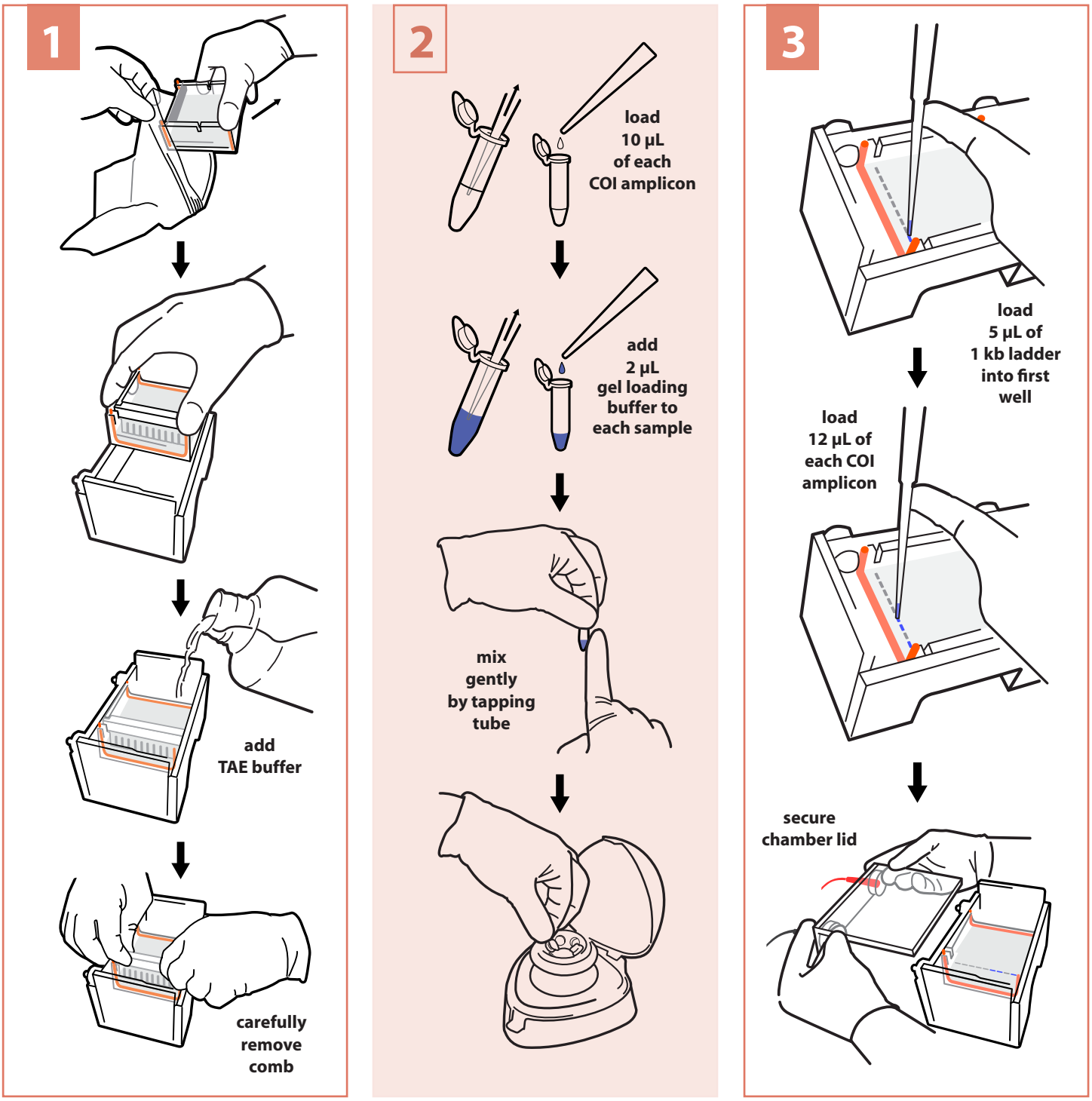
- [ ] 6. Remove the hot flask from microwave with a rubber hot hand protector or insulated gloves.
- [ ] 7. Instructors: add 1.0 µL of ethidium bromide stock solution (10 mg/mL) to each flask of melted agarose.
- [ ] 8. Gently swirl melted agarose to mix the ethidium bromide (swirl gently to avoid making bubbles).

Warning: Do not to pour hot agarose into Fotodyne electrophoresis chambers. If you are using the Fotodyne gel electrophoresis system, please allow the flask to cool for 5 minutes at room temperature approximately before proceeding to step 9.

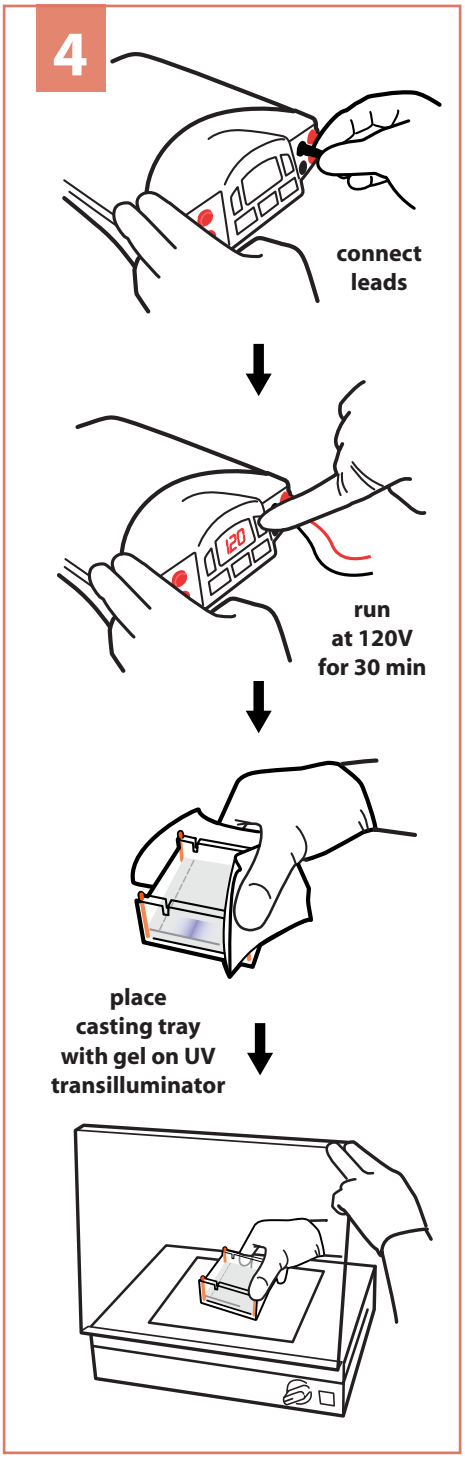
- [ ] 9. Pour the contents of the flask into the gel casting tray and allow to solidify. If any bubbles form when you pour the gel into the casting tray, simply move them to the lower part of the gel (away from the side containing comb) with a clean pipette tip.
- [ ] 10. Allow the gel to cool for 20 minutes. **Do not attempt to move the gel while it is cooling**.
- [ ] 11. When the gel is completely solidified, remove the casting tray containing the agarose gel from the electrophoresis chamber. Do not remove the comb from your gel as this may damage the wells where you will load your DNA in the next lab.
- [ ] 12. Place the entire casting tray containing your gel and comb in an air-tight freezer bag. Use a fine point permanent marker to label the bag with the date and group name. Store the bag containing your gel in a refrigerator (~ 4˚C) until you are ready to proceed to Laboratory 4.

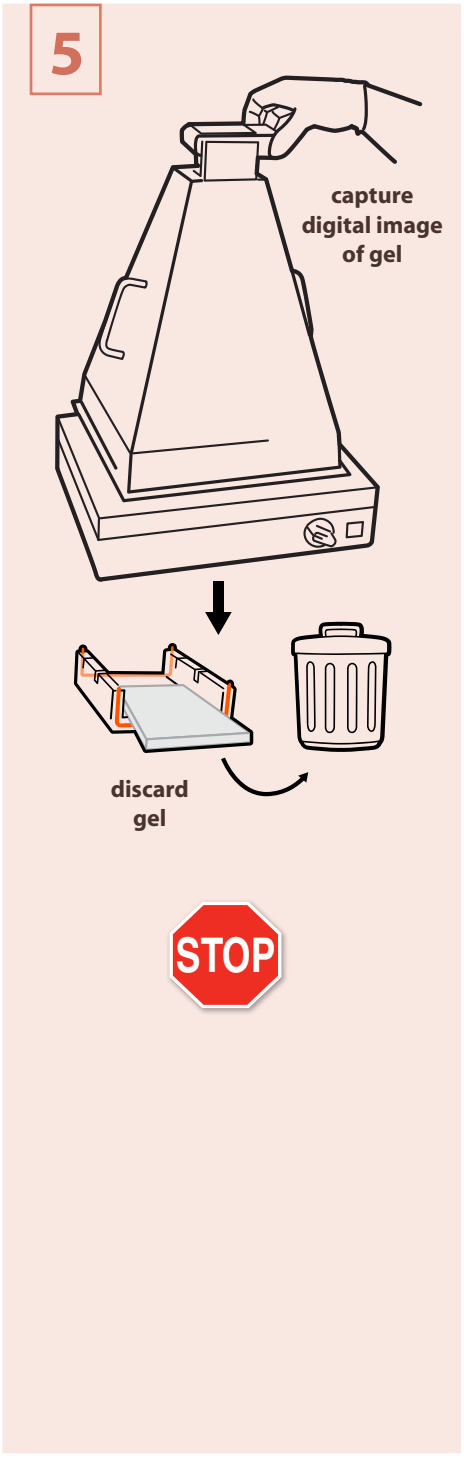


Procedural Overview



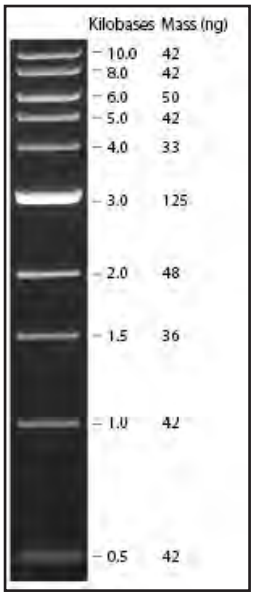
Procedural Overview



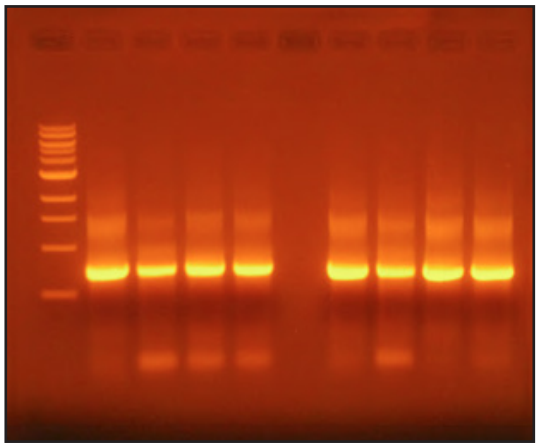


Background

Once your agarose gel is properly prepared, you will load one well (usually the first or leftmost well of the gel) with a small volume of a DNA ladder. This ladder contains DNA fragments of known sizes. You will then load the adjacent wells with a small volume (aliquot) of your purified COI DNA sample(s). As the gel runs, DNA fragments contained in the DNA ladder that was loaded into the first well will separate according to their size in a characteristic banding pattern (as shown in the figure below).



DNA ladders are purchased through various commercial vendors. Each vendor provides an image that shows the length (in bp or kb) of each DNA fragment contained in the ladder. After your gel has run, you will use the bands in the ladder lane to estimate the length of the COI amplicons that you purified in Laboratory 6. If your COI amplicons migrate at a position approximately midway between the 0.5 kb and 1.0 kb fragments in the ladder lane, then your PCR reaction was successful. Once this confirmation is made, your purified COI amplicons will be submitted for automated sequencing to generate a DNA barcode.



Equipment/Materials

SL-20 micropipette and tips

microcentrifuge

gel electrophoresis chamber and lid

DC power supply

UV transilluminator

digital camera with attached hood

agarose gel from Laboratory 7

0.5 mL microcentrifuge tubes

disposable gloves

safety goggles

gel loading buffer

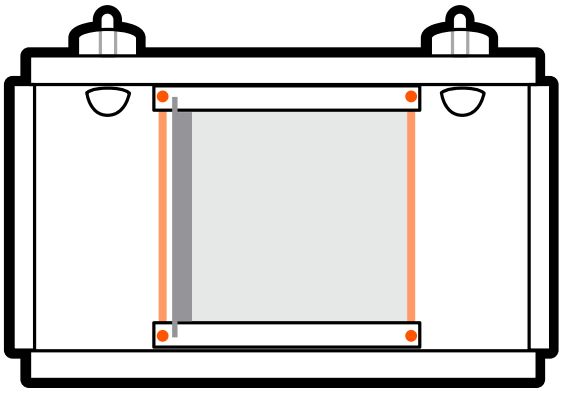
1X TAE buffer

DNA ladder

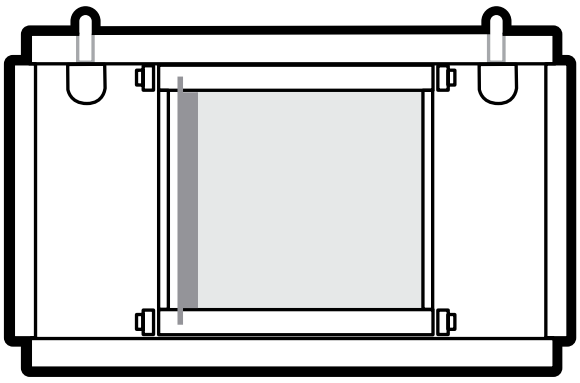
Methods *(read through the entire protocol before beginning)*

Important Note: Before getting started, thaw tubes containing purified CO1 amplicons(on ice), remove your agarose gel from the refrigerator, and allow to warm at room temperature on a lab bench. Students should wear lab coats and nitrile rubber gloves throughout the protocol below.

OWL system: Rest the casting tray containing the agarose gel and comb on the platform in the center of the electrophoresis chamber as shown in the figure below:



Fotodye (screw gate) system: Remove tape (if necessary), loosen thumb screws, lower gates, and position tray on the platform in the center of the electrophoresis chamber as shown in the figure below:



Methods *(read through the entire protocol before beginning)*

Loading agarose gels

- ☐ 1. Pour 1X TAE buffer into each reservoir of the electrophoresis chamber and completely submerge the agarose gel. The amount of buffer above the gel should be roughly equivalent to the thickness of the gel itself.
- ☐ 2. Carefully remove the comb by pulling it straight up from both sides.
- ☐ 3. Label 0.5 mL microcentrifuge tubes with the appropriate specimen IDs.
- ☐ 4. Set an SL-20 to 10 µL.
- ☐ 5. Using a fresh pipette tip, transfer 10 µL of purified (and thawed) COI PCR amplicons (from Laboratory 6) to the 0.5 mL microcentrifuge tubes that you labeled with the specimen ID codes. Instructors: be sure to store the tubes containing the remaining purified COI PCR amplicons (40 µL each) in a freezer at the end of this step. This material will be used for automated DNA sequencing if students generate bands of the predicted length and intensity.
- ☐ 6. Set an SL-20 to 2 µL.
- ☐ 7. Using a fresh tip, add 2 µL of gel loading buffer to the tubes containing 10 µL COI DNA.
- ☐ 8. Mix by gently tapping each tube with your index finger.
- ☐ 9. Briefly spin each tube in a picofuge to bring the contents down to the bottom of the tubes.
- ☐ 10. Set an SL-20 to 5 µL.
- ☐ 11. Using a fresh tip, load 5 µL of DNA ladder into the first (leftmost) well of the agarose gel.
- ☐ 12. Set an SL-20 to 10 µL.
- ☐ 13. Using a fresh tip, transfer 10 µL of each COI DNA sample containing loading buffer into a separate well of the agarose gel. Without producing bubbles in your samples, pipette each of your samples up and down two or three times immediately before loading into a well (this will help your samples sink to the bottom of the wells). If possible, skip a well between the ladder and your samples. You may also consider skipping a well between samples, if room permits. **Be sure to keep track of which wells contain your samples**.

Methods *(read through the entire protocol before beginning)*

- [ ] 14. When all of your samples are loaded, carefully secure the acrylic lid of the electrophoresis chamber by gently sliding it over the top of the chamber. Be sure that the connections are made between the outlets of the lid and the plugs of the chamber. It is very important at this stage of the protocol to avoid moving or bumping the electrophoresis chamber as this may result in DNA spilling out of its designated well and into adjacent wells.
- [ ] 15. Ensure that the positive (red) lead is located at the bottom of the gel (the side opposite the comb). If this is not the case, consult your teacher for instructions on how to proceed.
- [ ] 16. Connect the electrical leads from the lid to the power supply according to the color coding scheme.
- [ ] 17. Set the voltage on the power supply to 120V, press <RUN>, and monitor the progress of the blue indicator dye in the gel. This dye will migrate through the gel at a rate comparable to a DNA fragment that is approximately 300 bp in length.
- [ ] 18. Your gel will be ready for inspection under UV light after approximately 30 minutes. If you have sufficient time to run the gel longer, a run of approximately 40 minutes will ensure better separation of the fragments contained in the DNA ladder. Consult your instructor for guidance on how to visualize and take photographs of your gel.

Congratulations! This concludes the laboratory segment of the DNA barcoding workflow.

Record Data

10-WELL GEL ***Label this figure to document where you loaded your samples*** *(if you skip a well, place an X in the corresponding box below)*								*Group #* *Picture #*	
well 1	***well 2***	***well 3***	***well 4***	***well 5***	***well 6***	***well 7***	***well 8***	***well 9***	***well 10***