

Mission (Im)possible: Plasmid Mapping Teacher Materials

In this advanced lab, students will design and run a restriction enzyme digest to identify two unknown plasmids based on plasmid maps provided.

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Learning Goals

Student Learning Goals:

- Students will understand what a restriction enzyme is and what it does.
- Students will understand what a plasmid is and what it is used for.
- Students will understand the process of agarose gel electrophoresis.
- Students will identify a need for DNA restriction analysis.
- Students will understand how to use restriction enzyme analysis to determine a general map of a plasmid.

Student Learning Objectives:

- Students will plan a restriction enzyme analysis that will provide meaningful data.
- Students will use restriction enzymes as biotechnology tools.
- Students will perform the technique of agarose gel electrophoresis.
- Students will estimate DNA fragments sizes from agarose gel data.
- Students will analyze the results of the molecule separation by gel electrophoresis.
- Students will identify two unknown plasmids based upon their data.

Scientific Inquiry Skills:

- Students will pose questions and form hypotheses.
- Students will design and conduct scientific investigations.
- Students will use experimental data to make conclusions about the initial question and to support or refute the stated hypothesis.
- Students will follow laboratory safety rules and regulations.

Laboratory Technical Skills:

- Students will demonstrate proper use of micropipettes.
- Students will consider safety considerations when working with an electric current.
- Students will demonstrate proper use of gel electrophoresis equipment.
- Students will prepare and pour agarose gels.
- Students will perform restriction enzyme digests.

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Instructor Planning Guide

Experimental Timing:

From start to finish this lab takes 90-120 minutes. However, there are stopping points in this protocol that make it possible to complete the lab in a couple of 45-50 minute periods.

- Pre-lab discussion and student planning of their experiment (30 min)
- Restriction digest – setup (10 min) and incubation (15-30 minutes minimum). Tubes can be incubated overnight.
- *Stopping Point – Reaction tubes can be stored in the freezer after the restriction digest until you are ready to run the gel electrophoresis. Frozen tubes can be stored almost indefinitely.*
- Preparation of agarose gels and buffer (30 min)*
- Electrophoresis of DNA (20 min)**
- Visualization and interpretation of gels (10-15 min)

*Instructors may choose to prepare gels and buffer ahead of time to reduce lab time.

**Time required for electrophoresis may vary depending on the type of equipment and voltage used


Specialized Equipment need for Lab:

- p20 micropipettes
- gel electrophoresis units with power supplies (each group will run four samples plus a ladder)
- UV light or blue light source
- water bath or incubator
- centrifuge (optional)

Ordering Information:

This lab was developed using Fisher Scientific products and enzymes and plasmids from New England BioLabs. The reagents from New England BioLabs can be ordered (at no cost) by going to their website (<https://www.neb.com>). A calculation tool for ordering NEB Reagents for this lab can be found on the final page of this document.

Procedure Tips:

1. To help students (and teachers) keep track of tubes, consider purchasing a rainbow assortment of tubes. For example, all ZE tubes are blue and all Z tubes are green.
2. Before starting the experiment, ask students to check their materials list to make sure they have everything.
3.  Remind students that enzymes are heat sensitive and should be kept on ice at all times.
4. Demonstrate how to pipet very small volumes of liquid.
 - First, pipet the water into each tube.
 - After withdrawing a small amount of liquid from the stock tube, visually inspect the very end of the pipette tip. You should see a small amount of liquid.

- Insert the pipette tip containing the small amount of liquid into the tube so that it is partially submerged in the water and expel the liquid from the pipette tip.
 - Remove pipette from tube and inspect the tip again to confirm that you have successfully transferred the small volume into the tube.
 - Gently flick the tube to mix the solution. You want to avoid making bubbles.
5. Remind students to use a fresh pipette tip between each addition when setting up the restriction digest.
 6. Remind students to use a fresh pipette tip with each sample and to record the location of each of the samples as they load them into the gel.

Teaching Tips:

1. The protocol for preparing the electrophoresis gels is not included in this version of the lab. You can download the gel document from the website (<https://www.massbioed.org/educators/curriculum>), adapt it to your equipment and insert it in the lab.
2. Do not give too much guidance! Let the students choose their own enzymes, even if their choice will not allow them to distinguish between the two plasmids. Allowing your students to design an experiment that fails to give meaningful results can be more impactful than guiding them through the lab.
3. Complete maps of the two plasmids used here can be found on the final pages of these documents. New England Biolabs has many useful Interactive tools on the NEB website: <https://www.neb.com/>.

Safety Considerations:

- Gloves, lab coats and eye protection should be used whenever possible as part of good laboratory practice.
- Practice sterile techniques whenever possible, to avoid contamination of reagents.
- Exercise caution when heating and/or melting reagents during gel preparation.
- Exercise caution when working with electrical equipment.
- UV protective shields and/or glasses must be used if visualizing gels with a UV transilluminator.
- Always wash hands thoroughly after handling biological materials or reagents.
- Obtain the Material Safety Data Sheets (MSDS) available from the suppliers, and follow all safety precautions and disposal directions as described in the MSDS.
- Check with your school's lab safety coordinator about proper disposal of all reagents and gels containing DNA stains.

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Instructor Preparation Guide

Materials: This guide assumes 30 students, working in groups of two, for a total of 15 groups.

Materials for Advanced Teacher Preparation:	
1 tube	pMAL-c5X plasmid (NEB # N8108S). 1 tube will have 20 µg DNA in 40 µL solution. ▲
1 tube	pSNAPf plasmid (NEB # N9183S). 1 tube will have 20 µg DNA in 40 µL solution. ▲
1 tube	BamHI-HF restriction enzyme (NEB # R3136S). 1 tube will have 500 µL of enzyme. ▲
1 tube	EcoRI-HF restriction enzyme (NEB # R3101S). 1 tube will have 500 µL of enzyme. ▲
1 tube	HindIII –HF restriction enzyme (NEB # R3104S). 1 tube will have 500 µL of enzyme. ▲
1 tube	BstEII-HF restriction enzyme (NEB # R3162S). 1 tube will have 100 µL of enzyme. ▲
1 tube	10X concentrated CutSmart® buffer (provided with enzymes) ▲
1 tube	DNA Ladder Quick Load® Purple 1kb ladder (NEB # N0552S) 1 tube will have 1.25 mL of ladder.
1 mL	loading dye (provided with enzymes)
3 mL	distilled water
1	p20 micropipette and pipette tips
1	p200 micropipette and pipette tips
150	microcentrifuge tubes (0.5 mL or 1.5 mL)
2-3	microcentrifuge tube racks
1	centrifuge (optional)
1	ice bucket with crushed ice
1	ultrafine point permanent marker
all	reagents and equipment to prepare gels for gel electrophoresis *see Teaching Tips

Materials for each Student Workstation:	Materials for Common Workstation:
1 tube	37°C water bath or incubator UV or blue light source microcentrifuge (optional) 1X electrophoresis buffer ice buckets with restriction enzymes
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	
1 tube	

▲ **Caution: Enzymes, buffers and DNA are heat sensitive. Keep enzymes, buffers and DNA on ice at all times while working. Store in freezer long-term.**

Easy Substitutions:

- If you do not have an incubator or electric hot water bath, you can create a water bath using a larger beaker and a Styrofoam cooler. Heat two beakers of water in a microwave or on a hotplate. Place one beaker in the cooler and monitor the temperature. Add water from the second beaker of hot water as needed to maintain the temperature of your water bath.

Set-up Calendar:

2 weeks before lab:

- Check supplies and order any needed materials.
- If making any substitutions to the supply list, edit the student protocol accordingly.

1 day before lab

- Set up student lab stations with all durable materials according to the materials listed above.
- Prepare 1X TAE or similar electrophoresis buffer
- Prepare 1.0% agarose gels with DNA Stain. (Each group will run four samples plus a ladder.)

ⓘTip: Gels can be prepared ahead of time. If you pour the gels several days before the lab, they should be stored in a plastic container/bag with a damp paper towel to keep them from drying out. Gels should be stored in a cool location.

⚠ Caution: DNA stains such as GelGreen are light sensitive. Gels should be stored in an opaque container in the dark.

- Aliquot out the DNA ladder, loading dye, dH₂O, buffer.
 - Aliquot 12 μL of Quick Load® 1 Kb Ladder into tubes labeled Ladder. Prepare one tube per lab group.
 - Aliquot 40 μL of 6X loading dye into tubes labeled Loading Dye (or LD). Prepare one tube per lab group.
 - Aliquot 200 μL of distilled water into tubes labeled dH₂O. Prepare one tube per lab group.
 - Aliquot 30 μL of CutSmart® buffer into tubes labeled buffer. Prepare one tube per lab group.
 - ⚠ Caution: Enzymes, buffers and DNA are heat sensitive. Keep enzymes, buffers and DNA on ice at all times while working.
 - ⚠ Caution: Store CutSmart® buffer and DNA ladder in freezer over night.
- Label remaining tubes (to save time the morning of the lab)

⚠ Caution: You should keep the restriction enzyme tubes on ice at all times except when removing the amount needed for your reaction.

Morning of lab:

- Set up Common Workstation according to the materials list. **ⓘTip: Monitor the temperature on the hot water baths throughout the lab period to make sure that they are at the correct temperature.**

- Set up ice buckets for each student workstation.
- Aliquot 2 μL of EcoRI in tubes labeled Enz A and 2 μL of HindIII into tubes labeled Enz B. Prepare one tube of each enzyme per lab group. **⚠ Caution: Keep all tubes on ice while working with reagents.**
- Aliquot plasmid DNA into microcentrifuge tubes.
 - Aliquot 1 μL of pMAL-c5X plasmid into tubes labeled XE. Prepare one tube per lab group.
 - Aliquot 1 μL of pMAL-c5X plasmid into tubes labeled X. Prepare one tube per lab group.
 - Aliquot 1 μL of pSNAPf plasmid into tubes labeled ZE. Prepare one tube per lab group.
 - Aliquot 1 μL of pSNAPf plasmid into tubes labeled ZE. Prepare one tube per lab group.
- Set out reagents at each student workstation according to materials list.

⚠ Caution: Enzymes, buffers and DNA are heat sensitive. Keep enzymes, buffers and DNA on ice at all times.

- Prepare 3 ice buckets with 1 tube of each restriction enzyme for the common workstation (or 1 for every 5 groups)
 - Aliquot 10 μL of BamHI restriction enzyme into a tube labeled BamHI. Prepare one tube for every 5 lab groups.
 - Aliquot 10 μL of EcoRI restriction enzyme into a tube labeled EcoRI. Prepare one tube for every 5 lab group
 - Aliquot 10 μL of HindIII restriction enzyme into a tube labeled HindIII. Prepare one tube for every 5 lab group
 - Aliquot 10 μL of BstEII restriction enzyme into a tube labeled BstEII. Prepare one tube for every 5 lab group

⚠ Caution: Enzymes, buffers and DNA are heat sensitive. Keep enzymes, buffers and DNA on ice at all times.

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Answers to Student Questions

Protocol-Embedded:

p. 3:

- *The surface charge of a DNA molecule is negative.*
- *The phosphate groups (PO_4^{3-}) of DNA determine the negative charge.*
- *During gel electrophoresis, DNA molecules will migrate towards the positive pole.*

p. 4:

- *Yes SspI will cut the 50 bp DNA sequence shown. The resulting DNA fragments are:*

5' ATCGTAGGATCCTCGGAAT ATCCGCGTATATCGGAATTCGGAACTCTCTC 3'
3' TAGCATCCTAGGAGCCTTA TAAGGCGCATATAGCCTTAAGCCTTGAGAGAG 5'

p. 5:

- *How many different **linear** fragments would you have if you...*
...cut Molecule 1 with EcoRI? Three. The smallest fragment will move the furthest
...cut Molecule 1 with SspI? Two.
...cut Molecule 2 with EcoRI? Two.
...cut Molecule 2 with SspI? None

p. 7:

Answers will vary depending upon students' design.

p. 10:

- *The ladder DNA is like a molecular ruler. It will allow you to estimate the size of the bands generated by restriction digests.*
- *These controls serve as a comparison between uncut DNA and cut DNA. They will also allow you to determine if the restriction enzymes effectively cut the DNA.*
- *The X No enz control and the Z No enz control tubes would have circular DNA*
- *The smallest of these three forms will move the fastest through the agarose gel. The supercoiled plasmid form is the smallest and will migrate the fastest.*
- *The linear DNA molecules found in the X No enz control tube are linear because they have suffered a random double-stranded break. The linear DNA molecules found in the X+enz tube are all the same because they have all been cleaved at a specific restriction site.*

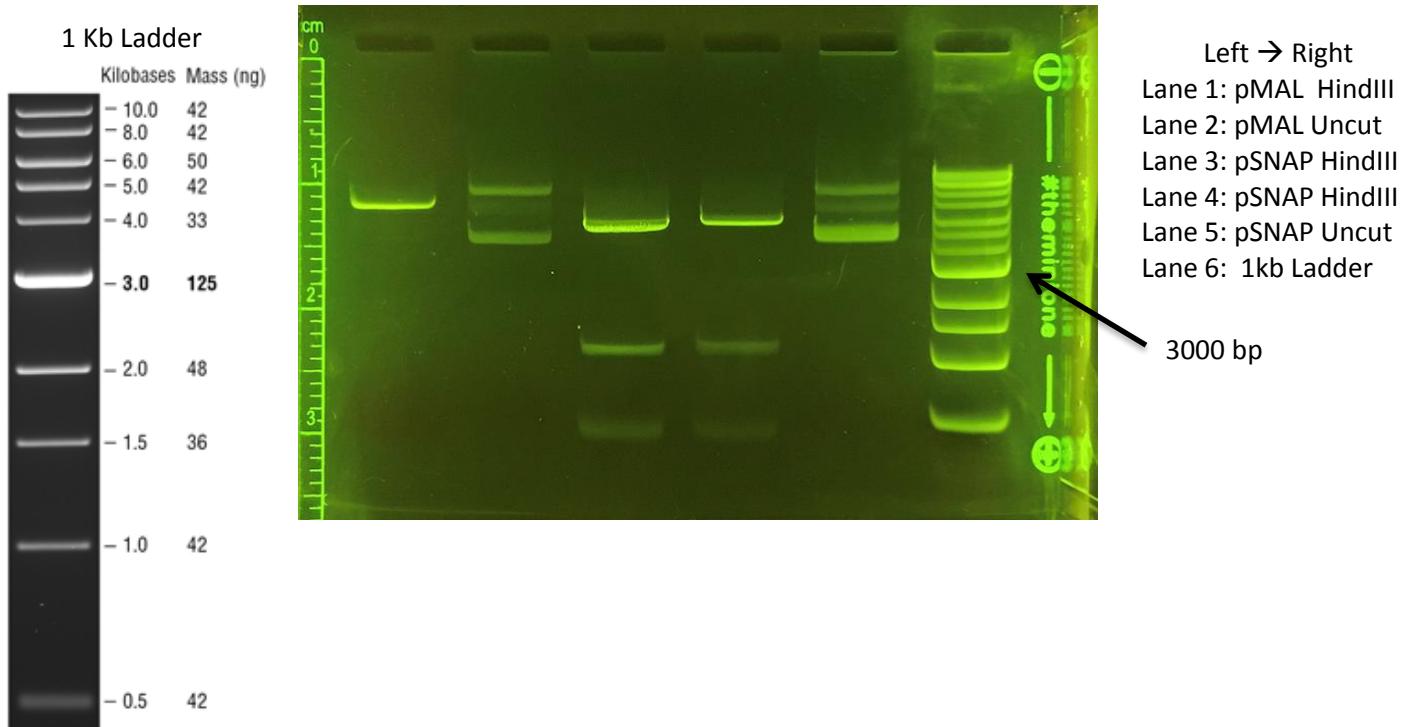
Pre-Lab:

1. A plasmid is a double-stranded, circular DNA molecule. It is naturally occurring in many bacterial cells.
2. This specific plasmid must carry a gene for ampicillin resistance. This gene encodes a protein that can break down ampicillin before it can kill the cell.
3. DNA is negatively charged.
4. If you were to compare the migration of DNA molecules in a 1% gel to the migration of the same molecules at the same voltage in a 2% gel you would find that the molecules move more slowly in the higher percentage gel. This is because high percentage gel has a more extensive crosslinking and thus has greater impact on the molecules trying to move through the matrix.
5. If enzyme Q cuts every molecule of pSNAPf at every possible restriction site and generates 4 bands,
 - then it is likely that there are 4 different sites for enzyme Q. It is possible that enzyme Q cuts in more than 4 places but produces only 4 bands if one or more of the bands on the gel is composed of two different DNA molecules of the same size.
 - if there are only four restriction enzyme sites for enzyme Q, then the fourth band would be 3949 base pairs.

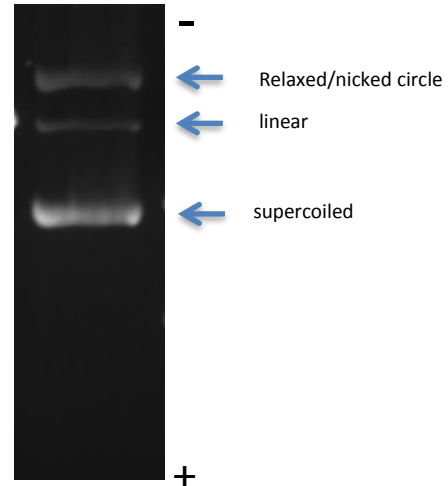
Data Collection:

Student drawings will vary depending upon the chosen digests.

- A sample gel is shown below. See following page for explanation of pattern seen in lane with uncut plasmid.



- The X and Z uncut lanes should both show multiple bands, each band represents one of the forms: supercoiled circular (the form found within cells), relaxed/nicked circular, or linear. The supercoiled circular DNA will be the fastest migrating band. If the plasmid DNA is carefully isolated from the cell, the supercoiled circular DNA will be the most abundant form of the molecule, and thus the brightest band. The relaxed circular and linear bands run very close together, but because linear DNA molecules meet less resistance, they migrate a little faster and will be closer to the positive end of the gel.



- The identity of unknown plasmid Z is pSNAPf, identity of unknown plasmid X is pMAL-5X.

Post-Lab:

- A single digest with enzymes EcoRI and BamHI are poor choices for distinguishing the two plasmids because both of these enzymes cut each plasmid once and only once. Given the size similarity, the two linear bands produced will be difficult to distinguish from each other in a 1% gel.
- Student answers will vary. May include – poor choice of enzymes for digests; partial digest of DNA produced unclear results; experimental error during set of digests or loading of gels, etc.
- If prior to restriction digest you had a million molecules of the plasmid, you would have a million molecules in each band. The total size (in base pairs) of pBR322 is $1857+1058+929+383+121 = 4348$ base pairs
- Intercalating dyes bind to DNA by inserting between the nitrogenous bases of DNA. The longer the DNA fragment, the greater the number of base pairs, and thus more dye is bound to produce a brighter signal.

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Standards Alignments

MA Science and Technology/Engineering Standards – High School (2016)

Biology

HS-LS1-1. Construct a model of transcription and translation to explain the roles of DNA and RNA that code for proteins that regulate and carry out essential functions of life.

Chemistry

HS-PS1-3. Cite evidence to relate physical properties of substances at the bulk scale to spatial arrangements, movement, and strength of electrostatic forces among ions, small molecules, or regions of large molecules in the substances. Make arguments to account for how compositional and structural differences in molecules result in different types of intermolecular or intramolecular interactions.

HS-PS1-11(MA). Design strategies to identify and separate the components of a mixture based on relevant chemical and physical properties.

HS-PS2-6. Communicate scientific and technical information about the molecular-level structures of polymers, ionic compounds, acids and bases, and metals to justify why these are useful in the functioning of designed materials.

Physics

HS-PS3-5. Develop and use a model of magnetic or electric fields to illustrate the forces and changes in energy between two magnetically or electrically charged objects changing relative position in a magnetic or electric field, respectively.

NRC Practices

- Asking questions and defining problems
- Planning and carrying out investigations
- Analyzing data
- Mathematical and computational thinking
- Constructing explanations and designing solutions
- Engaging in argument from evidence
- Obtaining, evaluating, and communicating information

Next Generation Science Standards – High School (2013)

Life Sciences

HS-LS1-1. Construct an explanation based on evidence for how the structure of DNA determines the structure of proteins which carry out the essential functions of life through systems of specialized cells.

Chemistry

HS-PS1-3. Cite evidence to relate physical properties of substances at the bulk scale to spatial arrangements, movement, and strength of electrostatic forces among ions, small molecules, or regions of large molecules in the substances. Make arguments to account for how compositional and structural differences in molecules result in different types of intermolecular or intramolecular interactions.

HS-PS2-6. Communicate scientific and technical information about the molecular-level structures of polymers, ionic compounds, acids and bases, and metals to justify why these are useful in the functioning of designed materials.

Physics

HS-PS3-5. Develop and use a model of magnetic or electric fields to illustrate the forces and changes in energy between two magnetically or electrically charged objects changing relative position in a magnetic or electric field, respectively.

Common Core State Standards Connections:

ELA/Literacy -

- RST.9-10.7** Translate quantitative or technical information expressed in words in a text into visual form (e.g., a table or chart) and translate information expressed visually or mathematically (e.g., in an equation) into words.
- RST.9-10.8** Assess the extent to which the reasoning and evidence in a text support the author’s claim or a recommendation for solving a scientific or technical problem.
- RST.11-12.1** Cite specific textual evidence to support analysis of science and technical texts, attending to important distinctions the author makes and to any gaps or inconsistencies in the account.
- RST.11-12.7** Integrate and evaluate multiple sources of information presented in diverse formats and media (e.g., quantitative data, video, multimedia) in order to address a question or solve a problem.
- RST.11-12.8** Evaluate the hypotheses, data, analysis, and conclusions in a science or technical text, verifying the data when possible and corroborating or challenging conclusions with other sources of information.
- RST.11-12.9** Synthesize information from a range of sources (e.g., texts, experiments, simulations) into a coherent understanding of a process, phenomenon, or concept, resolving conflicting information when possible.
- WHST.9-12.1** Write arguments focused on *discipline-specific content*.
- WHST.9-12.2** Write informative/explanatory texts, including the narration of historical events, scientific procedures/ experiments, or technical processes.
- WHST.9-12.5** Develop and strengthen writing as needed by planning, revising, editing, rewriting, or trying a new approach, focusing on addressing what is most significant for a specific purpose and audience.

- WHST.9-12.7** Conduct short as well as more sustained research projects to answer a question (including a self-generated question) or solve a problem; narrow or broaden the inquiry when appropriate; synthesize multiple sources on the subject, demonstrating understanding of the subject under investigation.
- WHST.9-12.9** Draw evidence from informational texts to support analysis, reflection, and research.
- SL.11-12.5** Make strategic use of digital media (e.g., textual, graphical, audio, visual, and interactive elements) in presentations to enhance understanding of findings, reasoning, and evidence and to add interest.

Mathematics -

- MP.2** Reason abstractly and quantitatively.
- MP.4** Model with mathematics.
- HSF-BF.A.1** Write a function that describes a relationship between two quantities.
- HSF-IF.C.7** Graph functions expressed symbolically and show key features of the graph, by hand in simple cases and using technology for more complicated cases.
- HSN.Q.A.1** Use units as a way to understand problems and to guide the solution of multi-step problems; choose and interpret units consistently in formulas; choose and interpret the scale and the origin in graphs and data displays.
- HSN.Q.A.2** Define appropriate quantities for the purpose of descriptive modeling.
- HSN.Q.A.3** Choose a level of accuracy appropriate to limitations on measurement when reporting quantities.
- HSS-IC.A.1** Understand statistics as a process for making inferences about population parameters based on a random sample from that population.
- HSS-IC.B.6** Evaluate reports based on data.

Calculation tool for ordering NEB Reagents for: Mission Impossible: Plasmid Mapping

Please keep in mind that NEB is a fantastic and generous partner and will provide up to \$1000 of reagents for each school. Please check with your colleagues to coordinate your ordering to ensure that your school plans ahead for ALL of the planned labs requiring NEB reagents, and please, only order as much as you need. The calculation tool below will help you determine how much of each reagent to order. *Importantly, the amount needed per group shown below includes the extra needed in case of mistakes or when aliquots are provided for each group.*

Fill out the chart below to determine how many tubes of each of the reagents you need to order. The following are important to keep in mind:

- The number of groups will vary depending on your classes and equipment.
- CutSmart Buffer and Loading Dye come with each restriction enzyme you order.

Calculation tool:

<i>Example</i>							
NEB Reagent	NEB Catalog #	Amount of Reagent In NEB Tube	Amount Needed per Group	Total Number of Groups Doing the Lab	Total Amount You Will Need	# Tubes Needed	
Reagent X	X0000	40 μL	4 μL	8	32 μL	1	
					<i>You fill this in</i>	<i>4 μL X (# groups)</i>	<i>32 μL < 40 μL</i>

NEB Reagent	NEB Catalog #	Amount of Reagent In NEB Tube	Amount Needed per Group	Total Number of Groups Doing the Lab	Total Amount You Will Need	# Tubes Needed
pSNAP _r Vector	N9183S	40 μL	2 μL			
pMAL-c5X Vector	N8108S	50 μL	2 μL			
EcoRI-HF [®]	R3101S	500 μL	5 μL			
BamHI-HF [®]	R3136S	500 μL	5 μL			
HindIII-HF [®]	R3104S	500 μL	5 μL			
BstEII-HF [®]	R3162S	100 μL	5 μL			
Quick-Load Purple 1 kb DNA Ladder	N0552S	1250 μL	12 μL *			
CutSmart Buffer [®]	Provided with restriction enzymes					
Gel Loading Dye, Purple (6X)	Provided with restriction enzymes					

*This is **per gel**. You may have more than one group per gel.

Once completed, you can submit your order here: <https://www.neb.com/forms/BioTeach>