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Applications of New Technologies to Classical Restriction Enzyme Labs

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Teaching labs employing restriction enzymes are a popular choice across the undergraduate curriculum. Long waiting periods and ethidium bromide driven safety precautions are common factors limiting the opportunities for a truly independent student experience. Recent technological advancements allow improvements of classical labs by introducing high fidelity enzymes, new generation safer DNA stains, and alternative DNA electrophoresis buffer with slower electrolyte exhaustion. The described approach can be applied to any existing restriction enzyme lab with minimal revision of lab materials as long as fast cutting versions of the used enzymes are available.

Keywords: restriction digest, DNA electrophoresis, ethidium bromide alternatives

Introduction

Restriction digest/DNA electrophoresis labs are widely used across the undergraduate curriculum as they fit well in a broad range of courses and allow acquiring/advancement of lab skills applicable to many areas of biology. Technically, a classical lab involves setting up restriction reactions and visualizing their products on agarose gel. Despite the fact that the required equipment is considered standard and most relevant tasks do not involve steep learning curves, restriction enzyme based labs rarely offer a full-scale independent student experience. Often the structure and the flow of the labs are dictated by the length of the waiting periods in the experimental protocol and not the logic of the experiment itself. Commonly, lab instructors are racing against the clock by using pre-prepared gels or gel mixes, imaging the gels after the end of the lab, and finding themselves short on time to help everyone with the analysis of restriction fragments. The use of ethidium bromide, a highly toxic DNA stain that requires cumbersome safety precautions and material disposal practices, and UV light for gel visualization further increase the complexity of the labs.

Recent technological advancements in DNA-related techniques offer some solutions of the above challenges. Taking advantage of the availability of high fidelity restriction enzymes, new DNA stains and alternative DNA electrophoresis buffer with slower electrolyte exhaustion I have redesigned our restriction enzyme module of four lab taught in the BIOL200/Cell Biology laboratory. Some aspects of the redesign were driven by the need to accommodate back to back lab sections taught by multiple instructors with minimal break time between labs. Currently, a single lab of three hours allows students to pour a gel by themselves, perform restriction digest and run their own samples with minimum handling of toxic materials. It is critical to emphasize that by their nature all compounds that bind DNA will be associated with some level of toxicity since their binding to DNA always has the potential to interfere with DNA structure. Improving the safety and efficiency of any process is continuous work in progress and its success highly depends on the characteristics of the available reagents and tools.

New Technologies and their Advantages for Teaching Labs

High Fidelity/HF Restriction Enzymes

High Fidelity/HF® (New England BioLabs, Inc.) and FastDigest® (ThermoFisher Scientific, Inc.) restriction enzymes are new generation genetically engineered versions of wild type enzymes designed to optimize restriction digest. Although initially designed and marketed for research they are excellent teaching tool

and offer numerous advantages in terms of saving time and minimizing efforts for lab preparation. The labs described below use the High Fidelity/HF® enzymes, however they can be executed equally well with FastDigest® enzymes. Both products allow a standard restriction digest of 1 μg DNA to take place within less than 15 minutes under optimal conditions, thus shortening the conventional time (two hours) for digest at least eight times. Every new generation enzyme can be used in one single universal buffer, which allows seamless double digests, as well as minimizes the time/efforts for aliquoting and the chances of mix ups during lab. Minimal star activity ensures productive overnight digestion without DNA degradation when the experimental protocol requires it. In addition, one has a choice of purchasing individual enzymes or ready to go enzyme mixes that require only the addition of DNA of interest. Pedagogically, the individual enzymes are a better option for students' first exposure to the technique, while mixes are great time saver for advanced teaching labs and research projects. Collectively, the use of new generation restriction enzymes allows restriction digest and gel electrophoresis to be completed in one 3-hour lab with minimum downtime, as well as reduction in the time for lab preparation.

DNA Stains

Until recently ethidium bromide was widely used both in research and teaching laboratories despite its toxicity. Simply, there were very limited alternatives. The rise of real-time quantitative PCR (qPCR) as a technique triggered extensive efforts to manufacture highly sensitive DNA stains. As a trend, new dyes tend to be more sensitive than ethidium bromide allowing smaller amounts to be used, and thus considered less toxic and more environmentally friendly. Another trend is to develop dyes that are not capable of penetrating the cell due to their size. The later are considered nontoxic based on their physical inability to reach the DNA in the cell. Initially, the new generation DNA stains were brought to the market as DMSO-based solutions. DMSO on its own is not a good choice for teaching labs due to its exceptional abilities to penetrate human skin which mandates the use specific type of gloves. As the field advances some dyes are becoming available as water-based solutions which is an advantage for teaching labs. Some new dyes can be imaged with blue light instead of UV, or both UV and blue light, thus creating an opportunity to further improve the safety of labs utilizing DNA stains. Many of the new products are still in process of characterization and testing or the manufacturers encourage users to refer to locally applicable regulations while deciding how to approach disposal, thus one needs to to be cautious while selecting new generation dyes. In my opinion the ideal DNA dye for a teaching lab should be highly sensitive, nonhazardous, environmentally safe, cost effective, to not require any special handing and to allow direct disposal to regular trash. In search of the perfect dye I compared side by side Ethidium bromide, SYBRSafe (Life technologies), Rediview (GeneCopoeia), SafeRed Loading dye (Genecopoeia), GelRed (Biotum) and Gelstar (Cambrex Bioscience). In addition to reviewing the information provided by each manufacturer I ran gels with various amounts of DNA (0.1-1 mucrograms /per lane) of 2 Log DNA ladder (New England Biolabs) visualizing bands ranging from 0.1 to 10 kB on TBE or Sodium Borate gels (data not shown). Fluorescence-wise all compared dyes delivered excellent DNA staining. Based on safety characteristics I found SafeRed Loading dye (Genecopoeia) and GelRed (Biotum) the closest to the idea of perfect dye, however due to the different extend of available test data the comparison is rather apples to oranges, than apples to apples. For that reason I chose to replace Ethidium bromide with GelRed and continue to use the established safety protocols based on the hazards of Ethidium bromide. It is my hope that as new dyes get fully tested dye disposal to regular trash will become a reality.

Alternative DNA Electrophoresis Buffer

Most teaching labs utilizing DNA electrophoresis use TBE (Tris/Borate/EDTA) or TAE (Tris/Acetate/EDTA) buffer which work well and are straightforward to prepare or to purchase as concentrated stock. Unfortunately, they allow fast and predictable running on very limited number of gels in the same buffer volume due to electrolyte exhaustion. In addition running multiple sections creates large volume of DNA stain-contaminated buffer that requires special handling and disposal. The handling aspect becomes rather critical when the clean-up/set up time between labs is minimal. Literature search driven from the above logistical issues identified a slow electrolyte exhaustion buffer, Sodium borate (SB) buffer (Brody and Kern, 2004) that can alleviate the experienced challenges. SB buffer was originally developed for applications requiring extended high voltage electrophoresis runs and outperforms TBE and TAE in terms of resolution. Although running gels at high voltage would save time in the teaching lab, 350 V runs are not practical since they require higher capacity (i.e. more expensive) power supplies. However due to its slow electrolyte exhaustion the SB buffer allows multiple gels to be ran without the changing the buffer, thus eliminating a tedious task executed between lab sections and reducing the total volume of DNA-stain contaminated buffer. In addition, the SB buffer is very cost effective and easy to prepare and store. The SB buffer is compatible with all DNA stains mentioned above and

does not visibly affect the quality of the staining process (data not shown).

In summary, classical restriction enzyme and DNA electrophoresis labs have been optimized using recent technological advances to offer students more extensive and safer lab experience that reflects the authentic research workflow. The student outline below focuses on the step by step instructions how to perform the labs without specific details what reagents are used. The specific reagents are addressed in the instructor's notes at the end of the article. In addition, the introductory parts of the labs have been removed in the interest of space. I would be happy to provide the complete labs upon request.

Student Outline

Restriction Enzymes and DNA Electrophoresis Lab Module

Overview

For the purpose of the next four labs we will pretend that you are newly hired employee of a biotech company that utilizes restriction enzymes as a research tool. Your initial contract is one month (in real life that period often varies between three and six months) in which you would undergo technique training and will have the opportunity to apply newly acquired knowledge to answer a small experimental question. The continuation of your contract depends on your demonstrated skills and abilities.

Specifically, first you will undergo training how to set up restriction reactions and run DNA electrophoresis. Under the guidance of your instructor you will perform a practice restriction enzyme digest with Enzyme Y-HF and Enzyme X-HF, and will resolve the products of the reactions using DNA electrophoresis. Then you will learn how to read plasmid maps, use DNA ladders and interpret DNA electrophoresis gel results. You would be asked to summarize your practice results in a write up following the format of results section of a scientific paper (graded assignment), which will conclude your new employee training.

Once trained you will be given a simple research question and design an experimental protocol to try to solve it. At the end of the third lab of the module you are expected to discuss your protocol with your lab instructor and submit a copy for grading. During Lab 8 you will perform the experiment you have planned. You need to report on your work, via several write up assignments as described in the lab schedule. A link to a virtual lab illustrating the techniques associated with this module is posted on Blackboard. Make sure to review them before coming to lab.

Lab Module Objectives:

The purpose of this lab module is to train you to design, perform and interpret experiments involving restriction digestion of DNA and DNA electrophoresis. Upon the completion of the lab module you should be able to:

- Plan and set up a restriction enzyme reaction.
- Set up, run and analyze DNA agarose gel.
- Interpret outcomes of restriction enzyme analysis of DNA.
- Generate figures and present experimental results involving restriction digest and DNA electrophoresis.

Assignments to Be Graded from This Lab Module:

- Restriction enzymes lab I results and data interpretation write up (include figure of your gel and description of your results showing all your calculations and graphing)
- Restriction enzymes lab protocol (submitted in pairs)
- Restriction enzymes lab IV results and data interpretation write up (include figure of your gel, brief description of your results, and detailed conclusions)

Available Materials, Reagents and Equipment (Please, Locate All Materials and Equipment Before You Begin):

- DNA electrophoresis station (gel box, power supply, bottle with 1Xelectrophoresis buffer; box of tips, container for gel containment, container for disposal of DNA stain-contaminated tips and tubes)
- Restriction enzymes (in the freezer)
- 10X CutSmart buffer (on ice)
- Sterile water (on the table rack)
- Agarose plates with premade wells for practice loading
- Practice loading dye (on the table rack)
- Nickel solution (needed only for the last lab in this sequence; on ice)
- Melted agarose supplemented with DNA stain (in a water bath)
- DNA plasmid (on ice)
- DNA loading dye (on the table rack)
- DNA ladder (on the table rack)
- UV illuminator
- UV protective goggles

Restriction Enzymes and DNA Electrophoresis – I (New Employee Training)

Introduction

The lab materials include short introduction structured under the sub-headers: What are restriction enzymes?; Electrophoresis of DNA restriction fragments; Visualizing DNA restriction fragments: How do DNA stains work?; and DNA ladders. In the interest of space the introduction has been omitted. Please, contact me if you wish to have a copy.

In this lab you will learn how to set up and pour agarose gel, how to set up a restriction enzyme reaction and how to analyze digested DNA using agarose gel electrophoresis. In order to avoid extensive waiting we will pour the agarose gel first and then proceed with the restriction reaction.

ATTENTION! Critical Safety Rules: *The agarose gel mix contains DNA stain, which is toxic. Please use gloves when handling the gel mix or the solidified gel!*
Gels should be handled only in the areas defined as DNA electrophoresis stations, which are lined up with white absorbent paper. Any tips that come in a contact with the gel should be disposed in the tip disposal box available at each DNA electrophoresis station. Do not dispose tips contaminated with DNA stain in the regular tip disposal.
Remember to wear UV protective goggles when observing your gels under the UV light!

I. Gel Pouring (each table needs to prepare 1 gel)

I.1. You will be given already melted agarose in proper electrophoresis buffer with added DNA stain. Please, do not remove the agarose solution from the water bath until you are ready to pour the gel.
I.2. Follow the instructor's instructions to assemble the gel bed. Make sure that your comb fits well in the gel bed and that there is sufficient space between the surface of gel bed and the comb allowing for the formation of the gel wells.
I.3. Place the assembled gel bed with comb in the container for gel containment to prevent possible leakage of the gel mix. Slowly pour the gel mix in the gel bed.
I.4. Leave the gel to solidify undisturbed (approximately 20 minutes). While the gel is solidifying, practice gel loading as demonstrated by the instructor using pre-poured agarose plates and practice loading dye found on your table.
I.5 Set up and perform Enzyme Y-HF and Enzyme X-HF restriction digest of the provided plasmid DNA (instructions below) for the rest of the waiting time.

II. Restriction Digest

Restriction enzymes are very sensitive to the environment they work in. Some of them require high salt concentrations, others are inhibited by them. For optimal digest always follow the manufacturer's recommendations found on the relevant company website and/or catalog. We will be working with several High Fidelity® or HF enzymes from New England Biolabs. Below is the manufacturer's description of the relevant experimental conditions:
Manufacturer's description of the optimal conditions for the HF restriction enzymes:
A typical restriction enzyme reaction using HF restriction enzymes takes 10 minutes at 37^{o}C, and has the following composition:

- 1X Cut Smart buffer
- 2 μg of DNA
- 2 μL of enzyme
- x μL of sterile water up to a total volume of 20 μL.

Your enzymes are very sensitive to heat. Take the enzymes out of the freezer on a chilling block. The chilling block has a green top when the temperature is the appropriate one for enzyme storage. If the chiller top turns yellow you need to return it to the freezer immediately in order to not compromise enzyme activity.

II.1 Set up the reactions outlined in the table below.
II.2 Mix all reagents carefully by flicking (instructor will demonstrate), centrifuge briefly (turn ON and then immediately OFF the switch button of the minicentrifuge) to collect everything on the bottom of the tube and add the corresponding enzyme directly in the volume of the reaction.

Reagent	Reaction 1 Negative control	Reaction 2 Enzyme X-HF digest	Reaction 3 Enzyme Y-HF digest
Sterile water	16 μL	14 μL	14 μL
Cut smart buffer, 10X	2 μL	2 μL	2 μL
DNA 1μg/μL	2 μL	2 μL	2 μL

II.3 Add 2 μL of the corresponding enzyme (reaction one is a negative control and will have no enzyme) and incubate at 37^{o}C water bath for 10 minutes. You just provided the optimal environment for your enzyme to work.
II.4. After incubation is done, store your reaction on ice until your DNA electrophoresis system is assembled.

III. Assembly of the DNA Electrophoresis System

Before proceeding further make sure that your gel is completely solidified. Then perform the following steps to prepare your gel for electrophoresis:
III.1 Take out the comb
III.2 Remove the black rubber gaskets
III.3 Lift your gel together with the gel bed and gently place it in the electrophoresis box**. Make sure that the gel wells (the empty "boxes" left after you took the comb out) are next to the negative electrode (colored in black).**
III.4 Fill the electrophoresis box with 1X electrophoresis buffer, so that the gel is completely submerged and the gel wells are full.

IV. Sample Preparation

IV.1 Obtain concentrated DNA loading dye.
IV.2 Add 5 μL of loading dye to each of the sample tubes. Mix by gently by flicking the tube. Spin the tube for 10 seconds to collect all the drops. Store the samples on your table until the gel is ready to load.

V. Loading and Running your Gel

V.1. Using a separate pipette tip for each sample, load your DNA ladder and your digested DNA samples into the gel. Gels are read from left to right. Usually, the DNA ladder is loaded first and used as a reference to orient the gel. Always make sure that you record the order and the amount of the samples you loaded.
Lane 1: DNA ladder, 10 μL
Lane 2: ……
Lane 3: ……
Lane 4: ……

V.2. Secure the lid on the gel box. The lid will attach to the base in only one orientation: red to red and black to black. Connect the electrical leads to the power supply.
V.3. Turn on the power supply and set it to 200 V. It will take approximately 30-40 minutes for your gel to run.
V.4. Once the loading dye has migrated to the 2/3 of the gel slab, turn off the power, move the gel to the UV illuminator and take a picture with your phone (instructor will demonstrate). Make sure to use UV protective goggles.

TO DO:

1. Print your gel picture as a full page and bring it to the next lab when you will be learning how to analyze restriction digest data!!!

2. Review the Analysis of Results section of the DNA electrophoresis virtual lab (Blackboard).

Restriction Enzymes and DNA Electrophoresis – II

Analysis of Digested DNA Samples

In order to proceed with your first work assignment you have to analyze your test drive results first. Plasmid map and DNA ladder information are available as laminated handout on your tables.

What are Plasmids and Plasmid Maps?

In the interest of space the introduction has been omitted. Please, contact me if you wish to have a copy.
Assignment: Using the plasmid map determine the length of the DNA fragment(s) expected from the restriction digest you have performed. Show all your calculations.

How is the Size of DNA Fragments Determined from Gel?

In the interest of space the introduction has been omitted. Please, contact me if you wish to have a copy.
Assignment: Determine the size of the DNA fragments in your gel. Compare them to your predictions.

DUE: *A write up of your restriction enzymes results and data interpretation including figure of your gel and description of your results with all calculations and graphing) is due as described in the lab schedule.*

Restriction Enzymes and DNA Electrophoresis – III (Experimental Protocol Design)

Now you are completely trained to proceed solving your first biotech company assignment:
What is the highest nickel concentration that allows 2 μg of your plasmid to be completely digested under the reaction conditions recommended by the enzyme manufacturer?

You will be provided with:
1. Description of the optimal conditions for the action of your enzyme, available from the manufacturer (see below).
2. A map of your plasmid.
3. The following reagents and materials will be available for your experiment:

- Plasmid DNA, 1μg/μL
- Enzyme Y-HF or Enzyme X-HF (one pair on each table should pick Enzyme Y-HF and the other Enzyme X-HF)
- CutSmart buffer, 10X
- 200 mM solution of Nickel salt
- Sterile water
- 37°C water bath (optimal temperature for restriction enzymes listed)
- DNA electrophoresis station and standard reagents necessary to run DNA gel and to analyze it

The experimental plan must be written in the form of a lab manual protocol (any student in the class should have enough information to carry out your experiment). If needed, please review the basic principles of experiment design discussed at the beginning of the semester.
Hints: A good experiment always has a positive and a negative control. In addition, you must have one (and only one) variable that you wish to test.

DUE: *Make sure to discuss your lab protocol with your instructor before you submit it for grading. Each pair submits only one lab protocol for grading at the end of this lab.*

Restriction Enzymes and DNA Electrophoresis – IV (Executing Your Experiment)

ATTENTION! Critical Safety Rules: *Before you begin, review the safety rules outlined at the beginning of the lab module!*

After you perform your experiment you should analyze your results and summarize your conclusions**.** While discussing your results make sure to specifically describe your findings and clearly state the outcome from your experiment, i.e. which samples contained cut DNA, which samples contained uncut DNA; did the restriction digest produce the fragments with the expected size; was the digestion complete: is all plasmid DNA cut or is there any uncut DNA left? Also you need to include the meaning of your results in the context of the problem you are solving, i.e. does nickel influence how your enzyme is working? In what way? What concentration of nickel is tolerable? Your summary should contain a photograph of your gel and the graph used to deduct DNA fragment sizes, formatted as scientific figures with appropriate figure title and legend.
DUE: *Restriction enzymes lab IV results and data interpretation write up (include figure of your gel, brief description of your results, and detailed conclusions) is due as described in the lab schedule.*

Notes for the Instructor

The described lab sequence is offered as a part of BIOL200 core course in Cell Biology required for all Biology majors. Before coming to BIOL200 students have been exposed to the concepts of restriction enzymes and DNA electrophoresis in the context of DNA fingerprinting laboratory during our Introductory Biology course for majors. Transfer students may or may not have had experience with restriction enzymes depending on the specifics of the courses they have taken. The described module of four labs allows students to get relevant hands-on training, to apply their skills toward solving a small research question and report their own results in the context of open ended, inquiry-based environment. By design the labs gradually increase in complexity and promote independent work. Rarely pairs decide to test exactly the same concentrations of nickel salt, thus every pair is "forced" to do their own concentration calculations, their own reaction composition calculations and their own data interpretation. Asking students to come to class with printed picture of their own gel ensures that every student will work with their own measurements (picture sizes are rarely exactly the same) and do their own graphing. The results and discussion write ups ask students to practice creating scientific figures of agarose gels and to polish their writing skills. A useful tool to help with student preparation for the lab is the restriction enzyme and DNA electrophoresis section of the Pearson® Molecular Biology activity from their LabBench series.

Prep-wise it is important to take into account the various options for purchasing high fidelity enzymes. A researcher would usually pick the commercial product with the highest specific activity. For the teaching lab it is more important to take into account what volume of enzyme is needed, so that students can pipet comfortably, thus making the less potent enzyme formulation better choice of purchase. The most expensive and time consuming prep item for this lab module is the plasmid DNA. We take advantage of leftover plasmid from DNA forensics lab (BioRad) taught to nonmajors. For logistical reasons the fingerprinting lab does utilize one of the provided suspect samples in the kit. Using the leftover DNA and the plasmid map in the kit manual one can select enzymes of interest. As a pretend Nickel salt we are using restriction enzyme buffer 3 from New England Biolabs, Inc. which is reasonably cheap and only requires aliquoting. While the comparison between available alternatives of ethidium bromide found out that the best available choices are SafeRed Loading dye (Genecopoeia) and GelRed (Biotum) I have replaced ethidium bromide with GelRed dye to avoid student handling of the concentrated stock solution (students are receiving gel mixes supplemented with DNA dye and thus have no access to the stock solution). In addition, students often confuse the DNA staining dye with the dyes in the gel loading buffer. Having both in one solution will complicate the matter further. For now our labs will continue to use the safety protocols established for ethidium bromide until long-term safety-related data are accumulated.

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About the Author

Boriana Marintcheva is an Associate Professor and BIOL200/Cell Biology course coordinator at Bridgewater State University in Bridgewater, Massachusetts. By training she is molecular virologist and in addition to Cell Biology, she teaches Molecular Biology and Virology courses with labs. She is also passionate about science education, science promotion and development of pedagogical tools advancing teaching and learning.

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