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Laboratory Exercise To Measure Restriction Enzyme Kinetics †

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INTRODUCTION

Catalysis by enzymes is a critical step in many biological processes. Numerous biotechnological applications also rely on enzymes, many of which have been engineered to improve their kinetics or stability (1). Given the ubiquitous role of enzymes in biology and biotechnology, enzyme kinetics is an important topic that is commonly addressed in undergraduate science coursework. Unfortunately, enzyme kinetics is often a challenging subject for students (2). Hands-on laboratory exercises provide students an opportunity to work with enzymes and visualize an enzyme-catalyzed reaction. Laboratory exercises further allow students to collect and interpret kinetic data that can provide context to understand kinetic parameters. With these goals in mind, we describe a laboratory exercise for students to measure kinetics of restriction enzymes.

Beginning in the 1950s, restriction enzymes played a transformative role in advancing molecular biology research and biotechnology by enabling the construction of recombinant DNA (3). Here, we describe a laboratory exercise where students measure restriction enzyme kinetics to couple skills in DNA technology and enzyme kinetics. The exercise offers an alternative to colorimetric enzyme assays previously described for undergraduate laboratories (4–6). Unlike other enzyme assays, our lesson does not require a spectrophotometer. Instead, our lesson accommodates laboratories that have access to a thermocycler and DNA gel electrophoresis equipment. Additional laboratory techniques used in the exercise include restriction digest, gel electrophoresis, and image analysis.

Through this exercise, students will consider the opportunities of enzyme engineering by comparing the kinetics of a restriction enzyme and an engineered version of the same enzyme. Early work to quantify restriction enzyme kinetics measured radioactivity from radio-labeled DNA excised from agarose gels (7), which is not well-suited for undergraduate laboratories. Newer methods to quantify enzyme functions have used DNA substrates with fluorescent tags (8, 9), which are advantageous compared with semi-quantitative total DNA stains. Here, we employ a fluorescent tag strategy to monitor digestion of a fluorescently-labeled PCR product by gel electrophoresis. The lab exercise can be completed in a single two-hour session. Students measure the kinetics of BsaI and BsaI-HFv2 (engineered BsaI) by monitoring the digestion of a fluorescently labeled PCR product. The exercise has the advantage of producing visual data, allowing students to view the disappearance of substrate (full-length PCR product) and the appearance of product (cleaved PCR product) in the agarose gel. DNA gel images can be quantified using the freely available software ImageJ (10) to calculate enzyme kinetic parameters for the standard and engineered enzyme.

PROCEDURE

Materials and equipment

A detailed materials list for instructors is included in Appendix 1. Student instructions to complete the laboratory exercise are included in Appendix 2. Equipment required to run the laboratory exercise include a thermocycler, microcentrifuge, gel imager, water bath or heat block, micropipettes, Nanodrop (or other DNA quantification method), and DNA gel electrophoresis apparatus.

Instructor preparation

In advance of the laboratory session, instructors prepare FAM-labeled PCR products by performing a PCR on pUC19 using primers designed to amplify a ~1-kb region that contains a BsaI restriction site (Table 1). The forward primer is synthesized with a FAM group at the 5′ end. The resulting PCR products have a single FAM tag at the 5′ end. The PCR products are then purified using a PCR purification kit.

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Laboratory session

Students are tasked with setting up restriction digest time courses of the FAM-labeled PCR products using both the standard BsaI restriction enzyme and the engineered version of the enzyme (BsaI-HFv2). Reactions are started at staggered times and halted simultaneously by the addition of a stop solution that contains EDTA, glycerol, and a loading dye. The reactions can then be loaded directly onto an agarose gel for electrophoresis. PCR product digested with BsaI yields fragments of 131 and 944 base pairs (Fig. 1).

Post-lab exercises

After data collection, students can quantify DNA bands using ImageJ (10). DNA bands can be selected using the rectangle tool and quantified by the ImageJ function Analyze Gels. Using the known amount of DNA in the undigested control (500 ng), students can calculate the fraction of PCR product (substrate) at each timepoint. From this data, students can calculate product formation and reaction velocity, which can be fit to an integrated Michaelis-Menten equation (Appendix 3), using their own code or our provided webtool to determine the K_m and maximum reaction velocity (V_{max}) of both the native and engineered enzymes (Fig. 2).

Modifications and extensions

To introduce the concept of non-specific restriction enzyme cleavage (star activity), instructors can prepare an additional PCR product from a pUC19 plasmid with a mutation in the BsaI recognition site (pUC19-ΔBsaI). In pUC19-ΔBsaI, the *bla* gene (conferring ampicillin resistance) contains a silent mutation in the codon for glycine 239 ("GGG" to "GGT"), removing the BsaI site (plasmid available upon request). Students can perform a digest on this product as a control to verify the absence of star activity (Fig. 3). Additional options for tailoring this activity to the specific needs of your curriculum include:

- Student preparation of PCR products
- Variation of experimental conditions: buffer, temperature, etc.
- Creation of models of enzyme activity using software such as MATLAB SimBiology to compare with experimental results

TABLE 1.
Primers used to generate FAM-labeled pUC19 PCR products.

Primer	Sequence	T_m (°C)
Forward	5´-/FAM/actctagcttcccggcaacaat-3´	58.7
Reverse	5´-gccagcaaaaggccaggaac-3´	59.8

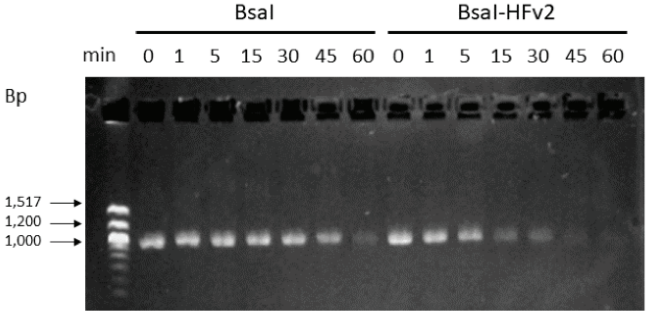

FIGURE 1. DNA gel electrophoresis of BsaI and BsaI-HFv2 time course.

Safety issues

Work for this laboratory exercise should be performed at biosafety level 1 (BSL1). Instructors should refer to the ASM BSL1 guidelines for laboratory space requirements, personal protection requirements, and standard laboratory practices. Students should be instructed on safe use of DNA electrophoresis apparatus to avoid electrical hazards. Students should also be instructed on appropriate use of the DNA gel imager. Care should be exercised with the total DNA stain and DNA gel to avoid contact with the skin or eyes. Students should follow manufacturer safety instructions when using all reagents.

CONCLUSION

Here we describe a relatively short and inexpensive enzyme kinetics laboratory exercise that gives students a hands-on experience working with restriction enzymes and kinetic data. The experiment yields data that are easily visualized and combines teaching enzyme kinetics with instruction in the techniques of restriction digest and DNA gel electrophoresis. Students also gain experience using image quantification software (ImageJ) to process the data. This exercise is appropriate for undergraduate lab courses that teach enzyme kinetics and DNA technology. This exercise may be of particular interest to students who are interested in enzyme engineering or biotechnology companies that generate these reagents. Variations or extensions to this activity could include testing buffers, temperatures, or other parameters.

SUPPLEMENTAL MATERIALS

Appendix 1: Instructor preparation protocol
Appendix 2: Student protocol
Appendix 3: Supplementary methods

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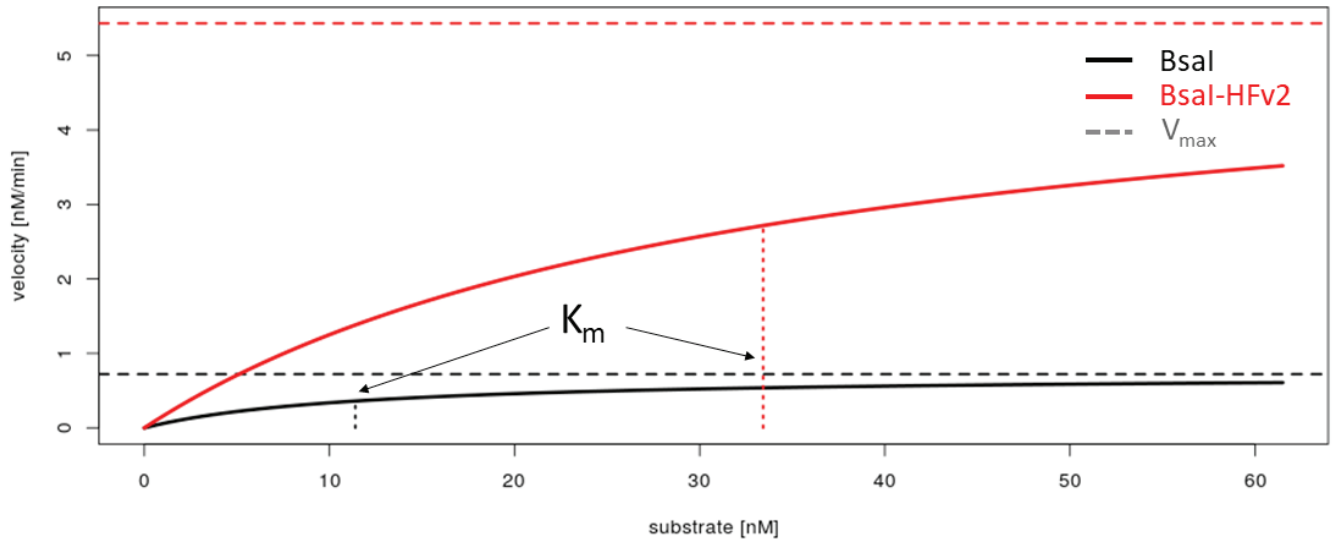

FIGURE 2. Best-fit velocity vs. substrate plots of BsaI and BsaI-HFv2 enzymes.

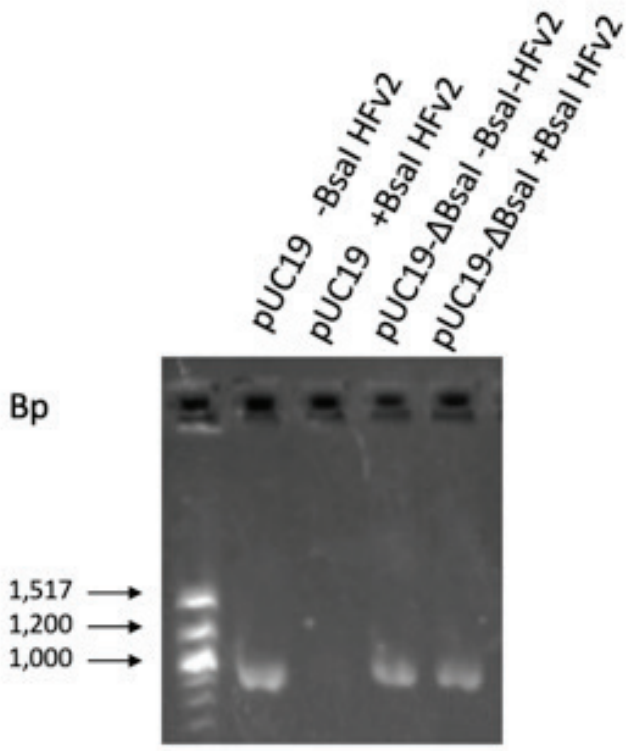

FIGURE 3. DNA gel electrophoresis of optional exercise to test BsaI-HFv2 for star activity.

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