LAB 12
PLASMID MAPPING

STUDENT GUIDE

GOAL

The objective of this lab is to perform restriction digestion of plasmid DNA and construct a plasmid map using the results.

OBJECTIVES

After completion**,** the student should be able to:

1. Perform plasmid DNA mapping.
2. Draw a plasmid map from gel electrophoresis data of the digested recombinant plasmid.
3. Explain each step in construction of a recombinant plasmid.
4. Explain how to determine if a recombinant plasmid has more than one insert.

TIMELINE

Day 1: Set up digestions of the recombinant plasmids that were constructed and isolated in the previous labs; cast 0.7% agarose gels

Day 2: Run digestions on the gel, analyze results and construct the plasmid map(s).

BACKGROUND

Mapping of DNA restriction sites is an important part of working in a molecular biotechnology lab because such maps are used to plan cloning strategy and to verify when a DNA clone has been successfully constructed. As an example, suppose that you are working with a 4,000 bp (4 kb) plasmid (a small circular piece of DNA) that has restriction sites for *Eco*R I, *Bam*H I, and *Hin*d III. When you cut the plasmid with any one of the enzymes, and run each digestion on an agarose gel, you see that one band of DNA is present and runs the same distance as the 4 kb fragment in the standard marker DNA. These data show that each enzyme has only one restriction site within the plasmid. Without further experimentation, there is no way to know where each of the three sites is located with respect to the other two. Therefore, double and even triple digestions are performed, run on a gel, and analyzed. See **Table 1** for the results of gel electrophoresis of this example plasmid.

Table 1. Results Of Gel Electrophoresis Of Example 4 kb Plasmid

Enzyme →	***Eco*R I + *Bam*H I**	***Eco*R I + *Hin*d III**	***Bam*H I + *Hin*d III**	***Eco*R I + *Bam*H I + *Hin*d III**
Fragments Produced (bp)	*2,000	500	1,500	500
		3,500	2,500	1,500
				2,000

*Indicates these two cutting sites are equal distance apart on the plasmid so that what would actually be two bands appears as a single band on the gel.

The data are very much like puzzle pieces that have to be fitted together with a lot of trial and error. Remember that we are dealing with a circle of DNA that is very likely not symmetrical. The sequence of bases from some starting point on the circle is unique, all the way around. Begin by numbering each base pair of the plasmid consecutively, and arbitrarily assign one restriction endonuclease cutting site at zero on the circle. By analyzing these data, you may be able to see that two of the restriction sites are opposite one another and that the third cuts between the other two. But does the third enzyme cut the right half of the circle or the left half of the circle? Without more information, it is impossible to tell. Thus, you must draw two possible maps for this plasmid (**Figure 2**).

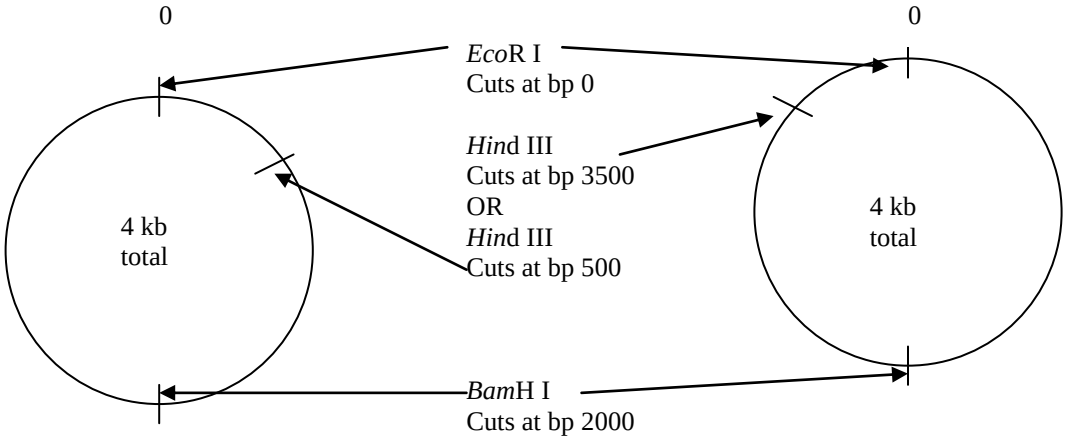


Figure 2. Two Possible Map Orientations of a 4 kb Plasmid

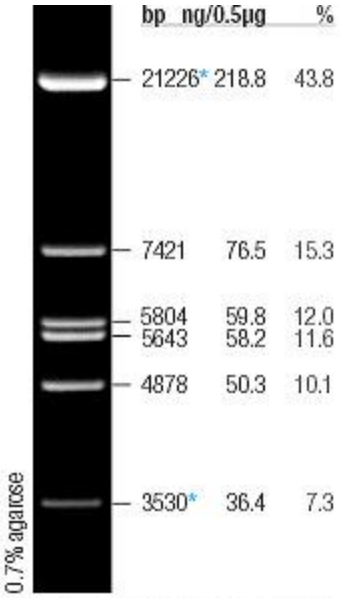
DNA Restriction with Multiple Enzymes

Using plasmid DNA restriction results to map the restriction sites on a plasmid can be easy compared to setting up the reactions and then deciphering the results from an agarose gel. When restriction sites for two different endonucleases are to be cut simultaneously, the buffer concentrations for each must be conducive to cutting. Endonuclease suppliers formulate buffers for each enzyme and also give information about buffer compatibility. At the end of this lab there are copies of pages from a supplier's catalogue that identifies the cutting ability of their enzymes in different buffers. The goal in selecting the buffers is to cut the DNA with the highest efficiency possible. On rare occasions, the DNA is cut with one enzyme, the buffer is adjusted with the lab's own buffers to the correct concentrations for the second enzyme, and then the second enzyme is added and the DNA incubated a second time.

The Importance of Stoichiometry in Analysis of Restriction Results

Stoichiometry is "the methodology and technology by which the quantities of reactants and products in chemical reactions are determined," (*New College Edition of The American Heritage Dictionary of the English Language*). When analyzing restriction digestions, the relative intensity of each band must be observed to determine if one band could represent fragments of DNA of similar but unequal size. In **Figure 3**, the two gel results are for the same DNA cut with the same enzyme and yet, the results appear to be different. If gel B were run longer, the third band from the top would separate out into

two bands as in gel A. There could also be fragments of DNA from different parts of the plasmid that run together on the gel because they are the same size. In either case, this "double" band would appear brighter than expected, relative to the other fragments on the gel.



0.5µg of the Marker was run on a 20cm length of 0.7% agarose gel in 1X TAE buffer at 3V/cm 18hrs (until bromophenol blue dye reached the bottom of the gel).

Lambda DNA/EcoRI Marker, 1#SM0281
Range: 6 fragments (in bp): 21226*, 7421, 5804, 5643, 4878, 3530*.

Catalogue Number: 1LLE-500
Volume: 5 x 1 ml for 500 loadings
Batch Number: Marked on tube

The λ *EcoR*I DNA Ladder is prepared by restriction digestion of Lambda DNA to completion with EcoRI, followed by heat inactivation of the enzyme.

The resulting 6 fragments range from 3.53 kilobases to 21.23 kilobases in size. (3530, 4878, 5643, 5804, 7421, 21226 bp)

The λ *EcoR*I DNA Ladder is supplied ready to use in loading buffer containing MZ Blue Dye.

Load 10 to 15 µl onto the gel (340 to 500 ng)

Recommended concentration of agarose 0.7 to 1.7%.

LABORATORY OVERVIEW

In this lab, the relative location of restriction sites on a plasmid will be mapped. Each team will set up digestions and then run them on an agarose gel with standard DNA markers. The size of all resulting fragments will be estimated using the standard markers, and the plasmid map drawn from the estimated sizes.

SAFETY GUIDELINES

Hot or Boiling agarose can cause burns. Be sure to wear heat resistant gloves when removing hot flasks and bottles from the microwave or autoclave if the media is not already prepared for you.

The **electric current** in a gel electrophoresis chamber is extremely dangerous. Never remove a chamber lid or touch the buffer once the power is turned on. Make sure that the counter where the gel is being run is dry.

Ethidium bromide is a strong mutagen and a possible carcinogen. Gloves must always be worn when handling gels or buffers containing this chemical.

UV light from the transilluminator, used to illuminate DNA dyed with ethidium bromide, is dangerous. Protect your eyes by wearing UV blocking glasses or UV blocking face shield.

MATERIALS:

PLASMID DNA RESTRICTION

per class

Water bath set at 37°C with floatees
Ice buckets with ice
Thawed recombinant plasmid DNA
Molecular grade water
Nalgene ice tray for restriction enzymes
Eco RI endonuclease
Xba I endonuclease
*Hin*d III endonuclease
Restriction enzyme reaction buffers
Universal buffer for double digestion
1.5 ml microcentrifuge tubes
microcentrifuge tube racks
personal microcentrifuges
Sharpie permanent markers
TE buffer (to be used for dilution of uncut plasmid DNA)

GEL CASTING

Per class

balance, spatula and weigh boats
microwave oven
hot gloves
250 ml flask per group
dH_2O
agarose
horizontal gel electrophoresis rigs

GEL ELECTROPHORESIS OF DIGESTED PLASMID DNA

Per class

metric rulers
Molecular weight marker DNA (NOT High mass ladder – 0.5 and 0.1 bp markers are required)
PUC18 vector DNA
automatic micropipetters and tips
used tip containers
gloves
Kim wipes
microfuge tube racks
Sharpie marking pens
D.C. power supply – one per 2 groups
UV Transilluminator
UV Camera and film
UV safety goggles and face shields
DNA InstaStain sheets
10 ml serological pipette
One 50 ml beaker per group
1x TAE electrophoresis buffer (dilute from 50x, if needed)
10x gel loading solution

PROCEDURE

PART I. PLASMID DNA DIGESTION

NOTE: Set up one set of digestions per team. (Each person on the team can set up two.) Buffers used in double digestions must be compatible, i.e., both restriction enzymes should cut when combined with a particular buffer. Consult the Promega enzyme buffer chart on the last two pages of this lab to determine the type of buffer to use in the double digestions.

1. Thaw your plasmid DNA from the minipreps performed previously. Each must be thoroughly thawed before use. Place all on ice after they have thawed.

2. Label a 1.5 mL microfuge tube for each of the digestions, below.
   *Eco*R I
   *Eco*R I + *Hin*d III
   *Eco*R I + *Xba* I
   *Hin*d III
   *Hin*d III + *Xba* I

3. Determine which restriction buffer to use with each digestion by using the buffer compatibility chart at the end of this lab. Each student should calculate the reagents required for each of the four digestions using the following guide. Verify your volumes with the instructor before proceeding to the next step.

Molecular grade H_2O	___ µl
miniprep DNA (150 ng)	___ µl
restriction enzyme buffer [10 X]	___ µl
Restriction enzyme(s)	0.5 µl (each)
Total volume	**20.0 µl**

4. Each team should set up five digestions, in the order given above. Use a new tip for each reagent. Set up each reaction with all reagents except the restriction enzyme in each. Then get the Nalgene ice tray with the enzymes and add the enzyme(s) to each reaction.

5. Pulse spin in a microcentrifuge to mix.

6. Place all mixed digestions into the 37°C water bath for two hours or overnight. Your instructor will remove the tubes from the incubator and place them in the freezer until the next lab period if the digestions are left overnight, so make sure labels on the tubes are legible.

PART III. GEL ELECTROPHORESIS OF PLASMID DIGESTS

Gels may need to be cast. Consult the instructor to determine what type of gel will be used.

1. Retrieve the digestion reactions from the freezer and thaw. Pulse-spin to move all contents to the bottom of the tube.

2. If E-gels are used, pre run for 2 minutes with the comb in. Remove the comb.

3. Dilute samples in 1.5 ml tubes, as given, and load the gel in the following order: (NOTE: make sure the molecular weight ladder used has 0.5 and 0.1 fragments.

Lane 1: Empty
Lane 2: Uncut plasmid DNA (1 µl + 15 µl ddH_2O)
Lane 3: 20 µL *Eco*R I digestion
Lane 4: 20 µL *Eco*R I + *Hin*d III digestion
Lane 5: 20 µL *Eco*R I + *Xba* I digestion
Lane 6: 20 µL *Hin*d III digestion
Lane 7: 20 µL *Hin*d III + *Xba* I digestion
Lane 8: **10 ml molecular weight DNA ladder (diluted to 20 µl)**
Lane 9: pUC
Lane 10-12: Empty

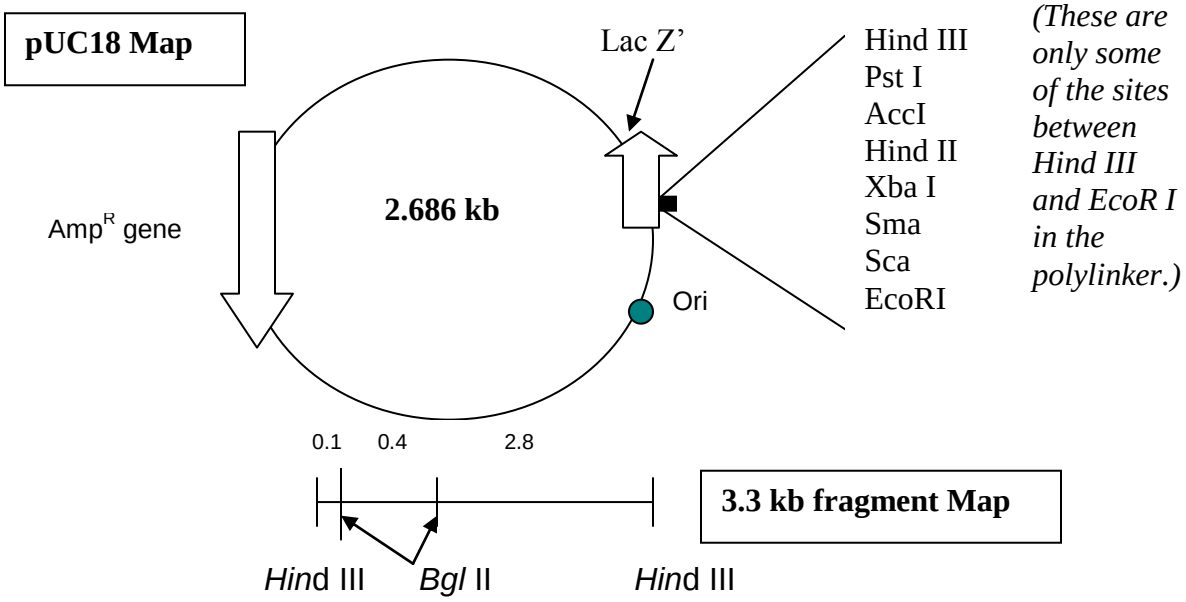
4. Add 10 µl of ethidium bromide stock (10 mg/ml), if using a gel that was cast. Run a 0.7% agarose gel for only 3-4 cm so that any small fragments will not run off. Run the E-gel for 15 minutes. Document the results with the lab camera. (NOTE: While the gel is running, work on the DATA ANALYSIS section of the lab.)

DATA ANALYSIS

Use the Internet or any other source available to you (except another student) to construct a linear restriction map of Lambda DNA that has the following enzyme restriction sites: *Eco*R I, *Hin*d III and *Xba* I. Based on the fragment of Lambda DNA that you gene cleaned, ligated and transformed, draw a recombinant plasmid map. If it is possible that you could have more than one recombinant plasmid, draw the map for each. Construct a data table that lists the different size fragments of DNA resulting when this (these) recombinant plasmid(s) are cut with the restriction enzymes used in this lab. Once you have gel electrophoresis results, analyze them to determine which Lambda DNA fragment was inserted into the pUC18 plasmid.

QUESTIONS

1. The map of pUC18 used to construct a recombinant plasmid is shown, below. It was originally digested with *Hin*d III for ligation of the 3.3 kb fragment taken from another plasmid. The linear fragment has two *Bgl* II sites, one that is 0.1 kb from one end and the other 0.4 kb away from the other. Given this information, draw a recombinant plasmid map.



2. For the recombinant plasmid in question 1, predict what size fragments will appear on the gel for each of the following digestions: *Eco*R I; *Hin*d III; *Bgl* II; *Bgl* II + *Eco*R I; and *Bgl* II + *Hin*d III.
3. What would you expect the results of the *Eco*R I digestion to be if there were two inserts ligated into one vector? If there were two vectors ligated together with one insert?
4. Can there be more than one possible orientation of this plasmid?
5. Based on what you have learned over the last 4 labs, write a summary of the steps one would use to clone a gene taken from another plasmid or from Lambda into a plasmid vector and to verify that it is the plasmid of the correct orientation.

For more practice drawing plasmid maps, go to the Blackboard website for the class. Plasmid mapping problems are in the course documents folder.

From the Promega Catalogue

Technical and Legal References

Technical Reference

Restriction Enzyme Activity in Promega 10X Buffers.

The 10X Buffer supplied with each restriction enzyme is optimized to give 100% activity at the 1X concentration. In many cases, good activity is also obtained using one of Promega's 4-CORE® 10X Buffers. This table may be used to select the best buffer for digestions with multiple restriction enzymes. Enzyme activity is expressed as a percent of the activity obtained with the optimized buffer for each enzyme in a one-hour digest. Refer to the table on the previous page for the composition of each Restriction Enzyme 10X Buffer.

Promega Enzyme	Buffer Supplied with Enzyme	A	B	C	D	MULTI-CORE™	Enzyme Assay Temperature
Aat II	J	50–75%	10–25%	<10%	<10%	<10%	37°C
Acc I	G	50–75%	25–50%	25–50%	10–25%	25–50%	37°C
Acc III	F	<10%	10–25%	25–50%	25–50%	<10%	65°C
*Acc*65 I	D	10–25%	50–75%	75–100%	100%	100%	37°C
*Acc*B7 I	E	10–25%	50–75%	100%*	<10%	100%	37°C
Age I	K	25–50%	25–50%	25–50%	50–75%	100%	37°C
Alu I	B	75–100%	100%	75–100%	10–25%	10–25%	37°C
*Alw*26 I	C	10–25%	25–50%	100%	10–25%	75–100%	37°C
*Alw*44 I	C	<10%	25–50%	100%	25–50%	100%	37°C
Apa I	A	100%	50–75%	50–75%	<10%	75–100%	37°C
Ava I	B	10–25%	100%	50–75%	25–50%	<10%	37°C
Ava II	C	50–75%	50–75%	100%	25–50%	25–50%	37°C
Bal I	G	10–25%	<10%	<10%	<10%	<10%	37°C
*Bam*H I	E	75–100%*	75–100%	75–100%	50–75%	75–100%	37°C
Ban I	G	25–50%	25–50%	10–25%	<10%	100%	50°C
Ban II	E	75–100%	75–100%	75–100%	25–50%	100%	37°C
Bbu I	A	100%	75–100%	75–100%	<10%	100%	37°C
Bcl I	C	10–25%	75–100%	100%	50–75%	10–25%	50°C
Bgl I	D	10–25%	25–50%	75–100%	100%	100%	37°C
Bgl II	D	25–50%	75–100%	75–100%	100%	<10%	37°C
*Bsa*M I	D	10–25%	25–50%	50–75%	100%	25–50%	65°C
*Bsa*O I	C	10–25%	50–75%	100%	25–50%	100%	50°C
*Bsp*1286 I	A	100%	50–75%	25–50%	10–25%	75–100%	37°C
*Bsr*BR I	H	10–25%	50–75%*	100%*	50–75%	100%	65°C
*Bsr*S I	D	10–25%	25–50%	10–25%	100%	100%	65°C
*Bss*H II	H	75–100%	50–75%	75–100%	50–75%	75–100%	50°C
*Bst*71 I	D	10–25%	25–50%	25–50%	100%	10–25%	50°C
*Bst*98 I	D	<10%	10–25%	10–25%	100%	25–50%	37°C
*Bst*E II	D	25–50%	50–75%	50–75%	100%	100%	60°C
*Bst*O I	C	10–25%	25–50%	100%	25–50%	<10%	60°C
*Bst*X I	D	<10%	10–25%	25–50%	100%	10–25%	50°C
*Bst*Z I	D	<10%	<10%	10–25%	100%	10–25%	50°C
*Bsu*36 I	E	<10%	25–50%	50–75%	25–50%	50–75%	37°C
Cfo I	B	75–100%	100%	75–100%	25–50%	100%	37°C
Cla I	C	75–100%	75–100%	100%	75–100%	100%	37°C
Csp I	K	<10%	10–25%	25–50%	50–75%	10–25%	30°C
*Csp*45 I	B	25–50%	100%	50–75%	25–50%	50–75%	37°C
Dde I	D	25–50%	25–50%	50–75%	100%	25–50%	37°C
Dpn I	B	50–75%	100%	75–100%	50–75%	100%	37°C
Dra I	B	75–100%	100%	75–100%	50–75%	25–50%	37°C
*Ecl*HK I	E	<10%	<10%	75–100%	10–25%	50–75%	37°C
*Eco*47 III	D	<10%	25–50%	50–75%	100%	25–50%	37°C
*Eco*52 I	L	<10%	<10%	10–25%	25–50%	<10%	37°C
*Eco*ICR I	B	10–25%	100%	75–100%	<10%	100%	37°C
*Eco*R I	H	25–50%	50–75%	50–75%	50–75%	100%*	37°C
*Eco*R V	D	10–25%	25–50%	50–75%	100%	100%	37°C
Fok I	B	75–100%	100%	75–100%	25–50%	50–75%	37°C
Hae II	B	50–75%	100%	50–75%	10–25%	100%	37°C
Hae III	C	75–100%	75–100%	100%	50–75%	100%	37°C
Hha I	C	50–75%	75–100%	100%	50–75%	75–100%	37°C

*Not recommended due to potential star activity.

(continued on next page)

From the Promega Catalogue

Technical and Legal References

Technical Reference

Restriction Enzyme Activity in Promega 10X Buffers (continued).

Promega Enzyme	Buffer Supplied with Enzyme	A	B	C	D	MULTI-CORE™	Enzyme Assay Temperature
Hinc II	B	25–50%	100%	25–50%	50–75%	100%	37°C
Hind III	E	25–50%	100%	75–100%	10–25%	50–75%	37°C
Hinf I	B	50–75%	100%	75–100%	75–100%	50–75%	37°C
Hpa I	J	25–50%	50–75%	25–50%	10–25%	100%	37°C
Hpa II	A	100%	50–75%	50–75%	10–25%	100%	37°C
*Hsp*92 I	F	10–25%	75–100%	50–75%	25–50%	10–25%	37°C
*Hsp*92 II	K	10–25%	25–50%	25–50%	<10%	<10%	37°C
I-*Ppo* I	NA	10–25%	25–50%	25–50%	25–50%	—	37°C
Kpn I	J	100%*	25–50%	25–50%	<10%	75–100%	37°C
Mbo I	C	10–25%	75–100%	100%	50–75%	<10%	37°C
Mbo II	B	10–25%	100%	50–75%	75–100%	100%	37°C
Mlu I	D	10–25%	25–50%	50–75%	100%	10–25%	37°C
Msp I	B	75–100%	100%	75–100%	25–50%	25–50%	37°C
*Msp*A1 I	C	25–50%	100%*	100%	10–25%	100%	37°C
Nae I	A	100%	50–75%	25–50%	<10%	50–75%	37°C
Nar I	G	75–100%	50–75%	75–100%	25–50%	50–75%	37°C
Nci I	B	100%*	100%	25–50%	25–50%	50–75%	37°C
Nco I	D	50–75%	75–100%	75–100%	100%	75–100%	37°C
Nde I	D	<10%	<10%	25–50%	100%	25–50%	37°C
Nde II	D	<10%	<10%	10–25%	100%	25–50%	37°C
*Ngo*M IV	MULTI-CORE™	100%*	100%*	100%*	<10%	100%	37°C
Nhe I	B	75–100%	100%	75–100%	10–25%	100%	37°C
Not I	D	<10%	10–25%	25–50%	100%	25–50%	37°C
Nru I	K	<10%	<10%	<10%	50–75%	10–25%	37°C
Nsi I	D	10–25%	50–75%	50–75%	100%	10–25%	37°C
Pst I	H	10–25%	50–75%	50–75%	50–75%	25–50%	37°C
Pvu I	D	10–25%	25–50%	50–75%	100%	<10%	37°C
Pvu II	B	25–50%	100%	50–75%	25–50%	50–75%	37°C
Rsa I	C	75–100%	75–100%	100%	<10%	<10%	37°C
Sac I	J	75–100%	25–50%	25–50%	<10%	100%	37°C
Sac II	C	100%	50–75%	100%	50–75%	<10%	37°C
Sal I	D	<10%	10–25%	25–50%	100%	<10%	37°C
*Sau*3A I	B	25–50%	100%	75–100%	<10%	100%	37°C
*Sau*96 I	C	25–50%	25–50%	100%	50–75%	50–75%	37°C
Sca I	K	<10%	100%*	50–75%	75–100%	10–25%	37°C
Sfi I	B	75–100%	100%	75–100%	25–50%	75–100%	50°C
Sgf I	C	25–50%	25–50%	100%	<10%	<10%	37°C
Sin I	A	100%	75–100%	50–75%	10–25%	100%	37°C
Sma I	J	<10%	<10%	<10%	<10%	100%	25°C
*Sna*B I	B	50–75%	100%	50–75%	<10%	100%	37°C
Spe I	B	75–100%	100%	75–100%	75–100%	100%	37°C
Sph I	K	75–100%	75–100%	100%*	75–100%	10–25%	37°C
Ssp I	E	10–25%	50–75%	50–75%	75–100%	50–75%	37°C
Stu I	B	75–100%	100%	75–100%	50–75%	50–75%	37°C
Sty I	F	25–50%	75–100%	75–100%	75–100%	<10%	37°C
Taq I	E	10–25%	25–50%	50–75%	50–75%	100%	65°C
*Tru*9 I	F	75–100%	50–75%	75–100%	25–50%	25–50%	65°C
*Tth*111 I	B	50–75%	100%	75–100%	25–50%	100%	65°C
Vsp I	D	<10%	25–50%	75–100%	100%	<10%	37°C
Xba I	D	50–75%	75–100%	75–100%	100%	100%	37°C
Xho I	D	25–50%	75–100%	75–100%	100%	10–25%	37°C
Xho II	C	25–50%	25–50%	100%	10–25%	<10%	37°C
Xma I	B	50–75%	100%	25–50%	<10%	50–75%	37°C
Xmn I	B	75–100%	100%	75–100%	10–25%	75–100%	37°C

*Not recommended due to potential star activity.