HiPer® Restriction Mapping Teaching Kit

Product Code: HTBM021

Number of experiments that can be performed: 5/20

Duration of Experiment

Protocol: 1.5 hours
Observation and result: 30 minutes

Storage Instructions

- The kit is stable for 6 months from the date of receipt
- Store Plasmid DNA, Restriction Enzymes, 10X Assay Buffer and 1 kb DNA ladder at -20°C
- Store 6X Dye at 2-8°C
- Other kit contents can be stored at room temperature (15-25°C)

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Aim:

To determine the restriction endonuclease sites of two restriction enzymes on a circular plasmid and construct a restriction map of the plasmid.

Introduction:

Restriction mapping is the process through which one can obtain the complete structural information of a plasmid by using restriction endonucleases. It involves the digestion of DNA with a series of restriction enzymes and then performing agarose gel electrophoresis method to separate the digested DNA fragments and measure the molecular size of the same.

Principle:

Negatively charged DNA molecules are separated on agarose gel matrix according to their molecular weight upon electrophoresis. The position of DNA in the agarose gel is visualized by staining the gel with low concentration of a fluorescent intercalating dye like Ethidium bromide. Smaller molecules move faster and migrate farther than larger ones because the migration rates of DNA molecules are inversely proportional to the logarithms of the molecular weights. This method is frequently performed to determine the size of an unknown DNA fragment by comparing it with DNA ladders of known size. A standard curve can be obtained by plotting the molecular size of the fragments of the marker against the reciprocal of their respective mobility. The relative mobility (R_f) of the DNA ladder depends upon the log of its relative molecular weight. The R_f value can be determined after dividing the distance traveled by the DNA by distance traveled by tracking dye. The molecular size of the test sample can be obtained from the standard curve. The mobility rate of DNA molecules vary from one experiment to another. So, the control DNA ladder should always be loaded onto the same gel.
To make a restriction map of a circular plasmid, (with three restriction sites for restriction enzyme X and two restriction sites for restriction enzyme Y) it has to be digested with two restriction enzymes separately as well as together and after that agarose gel electrophoresis has to be performed to determine the size of the digested fragments as shown in figure 1.

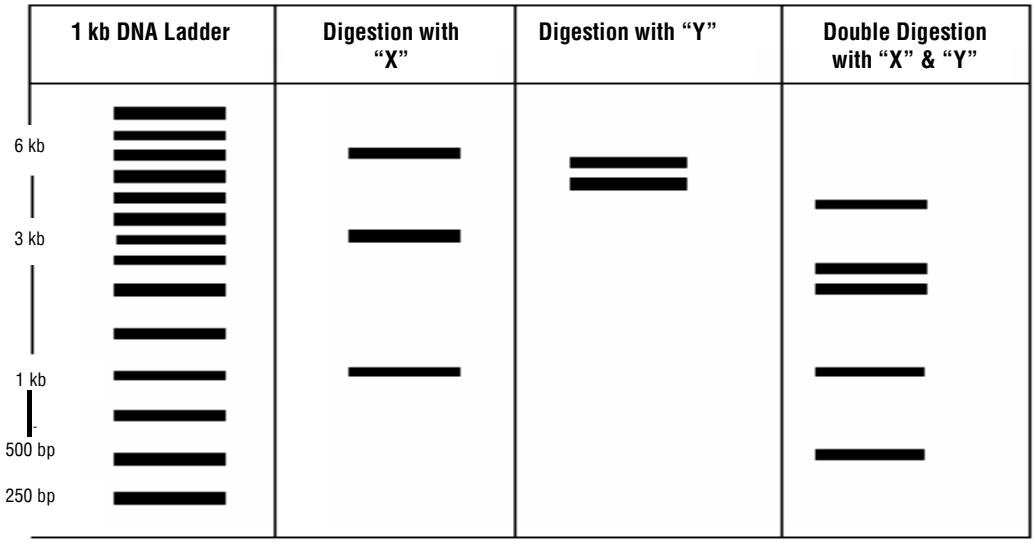


Fig 1: The pattern of the digested DNA after agarose gel electrophoresis

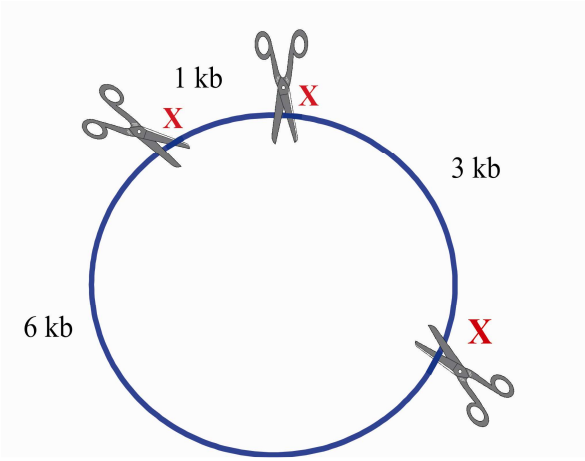
From the gel picture the following can be inferred:
After restriction digestion with X, the sizes of the fragments are: 6 kb, 3 kb and 1 kb.
After restriction digestion with Y, the sizes of the fragments are: 5.5 kb and 4.5 kb.
After restriction digestion with both X and Y, the sizes of the fragments are: 4 kb, 2.5 kb, 2 kb, 1 kb a.d 0.5 kb.

With the above data, the restriction map of the plasmid can be done in the following way:

a. First draw a circle
b. Add up the fragments from each of the restriction profiles. Each should add upto the same number.

X = 6 kb+ 3 kb + 1 kb = 10 kb
Y = 5.5 kb + 4.5 kb = 10 kb
X & Y = 4 kb + 2.5 kb + 2 kb + 1 kb + 0.5 kb =10 kb

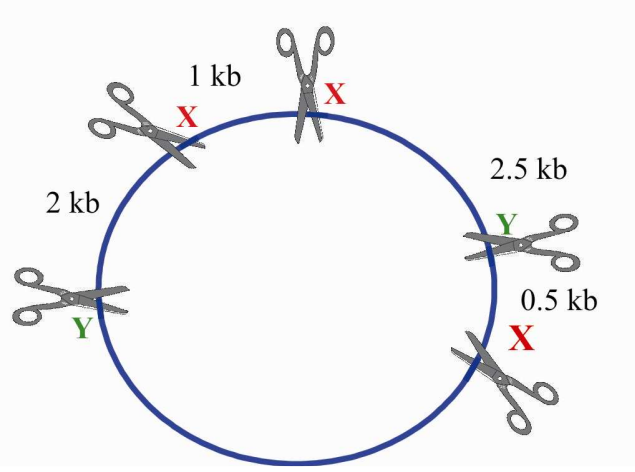
c. Put in the first site at 12 O' clock position.
d. Put in the remaining restriction enzyme (X) sites according to their sizes.
e. From the gel digest, there are three fragments which means there are 3 sites as marked below:



f. Put in sites for the second enzyme Y and map these sites relative to the X sites.

The second enzyme generates two fragments 5.5 kb and 4.5 kb, so there must be two sites.

The combination of the two enzymes X and Y generates fragments of 4 kb, 2.5 kb, 2 kb, 1 kb and 0.5 kb.
The final map that we obtain as follows:



4 kb

Kit Contents:

The kit provides material sufficient for 5/20 preparations.

Table 1: Enlists the materials provided in this kit with their quantity and recommended storage

Sr. No.	Product Code	Materials Provided	Quantity		Storage
			5 expts	20 expts	
1	TKC187	Plasmid DNA	0.12 ml	0.42 ml	-20°C
2	MBRE001	Restriction Enzyme: *Eco*RI	0.015 ml	0.045 ml	-20°C
3	MBRE003	Restriction Enzyme: *Hin*dIII	0.015 ml	0.045 ml	-20°C
4	TKC188	10 X Assay Buffer	0.1 ml	0.35 ml	-20°C
5	TKC189	1 kb DNA ladder	0.02 ml	0.07 ml	-20°C
6	TKC294	6X Dye	0.2 ml	0.7 ml	2-8°C
7	ML024	Molecular Biology Grade Water	0.75 ml	3.0 ml	R T
8	ML016	50X TAE	60 ml	210 ml	R T
9	MB002	Agarose	3 g	11 g	R T
10	CG281	Polypropylene Tube (0.5 ml)	18 Nos.	63 Nos.	R T

Materials Required But Not Provided:

Glass wares: Measuring cylinder, Beaker
Reagents: Ethidium bromide (10 mg/ml)
Other requirements: Electrophoresis apparatus, UV Transilluminator, Heating block or Water Bath, Vortex Mixer, Micropipettes, Tips, Adhesive tape, Crushed ice, Microwave/ Hotplate/ Burner

Storage:

HiPer® Restriction Mapping Teaching Kit is stable for 6 months from the date of receipt without showing any reduction in performance. On receipt, store Plasmid DNA, Restriction Enzymes, 10 X Assay Buffer and 1 kb DNA ladder at -20°C and 6X Dye at 2-8°C. Other reagents can be stored at room temperature (15-25°C).

Important Instructions:

1. Read the entire experiment carefully before starting the experiment.
2. The restriction enzymes are temperature sensitive and should always be placed on ice during the experiment.
3. While performing the experiment place the assay buffers and restriction enzymes on ice.
4. Use fresh tip while adding different solution to the tube.
5. While preparing the reaction mixture the enzymes should always be added at last.

Procedure:

1. Before starting the experiment, crush ice and place the vials containing plasmid DNA, Restriction Enzymes and Assay Buffers onto it.
2. In this experiment plasmid DNA is digested with two restriction enzymes EcoRI and HindIII separately as well as together.
3. Set up the reaction mixture as follows:

Reaction 1 (EcoRI digestion)

1. Plasmid DNA	– 5.0 µl
2. 10X Assay Buffer	– 5.0 µl
3. Milli Q water	– 39.0 µl
4. *Eco*RI	–1.0 µl
Total	**50 µl**

Reaction 2 (HindIII digestion)

1. Plasmid DNA	– 5.0 µl
2. 10X Assay Buffer	– 5.0 µl
3. Milli Q water	– 39.0 µl
4. *Hin*dIII	–1.0 µl
Total	**50 µl**

Reaction 3 (EcoRI & HindIII digestion)

1. Plasmid DNA	– 10.0 µl
2. 10X Assay Buffer	– 5.0 µl
3. Milli Q water	– 33.0 µl
4. *Hin*dIII	–1.0 µl
5. *Eco*RI	– 1.0 µl
Total	**50 µl**

4. After preparing the three reaction tubes, mix the components by gentle pipetting and tapping.
5. Incubate the tubes at 37°C for 2 hours.
6. After incubation, immediately add 10 µl of 6X dye to each tube.
7. Run the samples on agarose gel as given below.

Agarose Gel Electrophoresis:

1. Prepare gel tray by sealing the ends with adhesive tape. Place comb in gel tray about 1 inch from one end of the tray and position the comb vertically such that the teeth are about 1-2 mm above the surface of the tray.

2. To prepare 50 ml of 1.0 % agarose solution, measure 0.5 g agarose into a glass beaker or flask and add 50ml 1X TAE buffer. Heat the mixture on a microwave or hot plate. Swirl the glass beaker/ flask

occasionally, until agarose is dissolved completely (Ensure that the lid of the flask is loose to avoid buildup of pressure).

3. Allow the solution to cool down to about 55-60°C. Add 2 µl Ethidium bromide (10 mg/ml), mix well and pour the gel solution into the gel tray to a depth of about 5mm. Allow the gel to solidify for about 30 minutes at room temperature.

4. To start the run, carefully remove the adhesive tape from both the ends of the gel tray, place the tray in electrophoresis chamber, and fill the chamber (just until wells are submerged) with 1X TAE electrophoresis buffer and gently remove the comb.

5. Load 3 µl of the ready to use DNA ladder onto well 1. Load 15 µl of each DNA samples from Reaction I and Reaction 2 onto wells 2 and 3. Load 30 µl of DNA samples from reaction 3 onto well 4.

6. Connect the power cord to the electrophoretic power supply according to the convention: Red- Anode and Black- Cathode. Electrophorese at 100-120 volts and 90 mA current until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized.

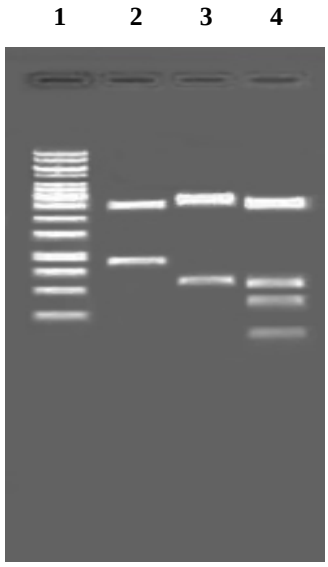
7. Electrophoresis apparatus should always be covered to protect against electric shocks. Avoid use of very high voltage which can cause trailing and smearing of DNA bands in the gel, particularly with high-molecular-weight DNA.

8. Monitor the temperature of the buffer periodically during the run. If the buffer becomes heated, reduce the voltage. Melting of an agarose gel during electrophoresis is a sign that the voltage is too high, that the buffer may have been incorrectly prepared or has become exhausted during the run.

9. Switch off the power supply once the tracking dye from the wells reaches 3/4th of the gel which takes approximately 45 minutes. Observe the gel on a UV transilluminator.

Observation and Result:

Perform Agarose Gel Electrophoresis. Visualize the DNA bands using UV transilluminator.



Lane 1: 1 kb Ladder

Lane 2: *Eco*RI digest

Lane 3: *Hin*dIII digest

Lane 4: *Eco*RI + *Hin*dIII digest

Interpretation:

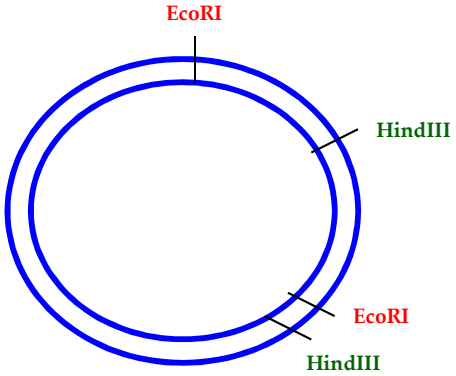
From the gel image the sizes of the fragments observed are as follows:

For EcoRI: 1 kb and 2.7 kb

For HindIII: 0.7 kb and 3.0 kb

For EcoRI + HindIII: 0.1 kb, 0.4 kb, 0.6 kb and 2.6 kb

Using these data the restriction map can be constructed as follows:



Troubleshooting Guide:

Sr.No.	Problem	Possible Cause	Solution
1	Partial or no digestion	Insufficient incubation time	Incubate the samples for longer time at 37°C (60-120 minutes)
		Improper addition of restriction enzyme	Always add the restriction enzyme at the end of the reaction mixture and add appropriate amount as given in the protocol
		Components of the reaction mixture not mixed properly.	Ensure that all the components are thoroughly mixed by gentle pipetting after preparing the reaction mixture
		Degradation of restriction enzymes	Always place the vials containing restriction enzymes on ice as they are temperature sensitive.
2	Star activity	Improper restriction enzyme addition	Add exact amount of restriction enzyme as per the procedure, avoid pipetting error
3	Improper resolution of bands on agarose gel	Gel not run for sufficient duration	Run the gel for longer period of time till the bands are separated properly

Technical Assistance:

At HiMedia we pride ourselves on the quality and availability of our technical support. For any kind of technical assistance, mail at mb@himedialabs.com