HiPer®Restriction Fragment Length Polymorphism (RFLP) Teaching Kit

Product Code: HTBM026
Number of experiments that can be performed: 5/25

Duration of Experiment

Protocol:3.5 hours
Agarose Gel Electrophoresis:1 hour

Storage Instructions:

- The kit is stable for 12 months from the date of receipt
- Store DNA samples, 1 kb DNA ladder, Restriction Enzymes, 6X Dye and Buffers at-20°C
- Other reagents can be stored at room temperature (15-25°C)

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Aim:

To learn the process of DNA fingerprinting following Restriction Fragment Length Polymorphism (RFLP) method by restriction digestion of DNA and analysis of the digested fragments on agarose gel.

Introduction:

Restriction fragment length polymorphism (RFLP) method in molecular biology was evolved for detecting variation at the DNA sequence level of various biological samples. The principle of this method is based upon the comparison of restriction enzyme cleavage profiles following the existence of a polymorphism in a DNA sequence related to other sequence. In RFLP, DNA of individuals to be comparedis digested with one or more restriction enzymes and the resulting fragments are separated according to molecular size using gel electrophoresis along with a molecular weight marker. Through this approach two individuals can present different restriction profiles.

Principle:

Restriction fragment length polymorphism (RFLP) analysis isextensively used in molecular biology for detecting variation at the DNA sequence level. Theprinciple of this analysis is to compare restriction digestion profiles of DNA samplesisolated from different individuals. RFLP functions as a molecular marker as it is specific to a single clone/restriction enzyme combination.Most RFLP markers are co-dominant and highly locus-specific. In molecular biology, restriction fragment length polymorphism, or RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologousDNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated.

RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and digest DNA wherever a specific short sequence occurs, in a process known as restriction digestion. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis. Molecular markers are used to estimate the fragment size. RFLP is specific to a single clone/restriction enzyme combination and it occurs when the length of a detected fragment varies between individuals.

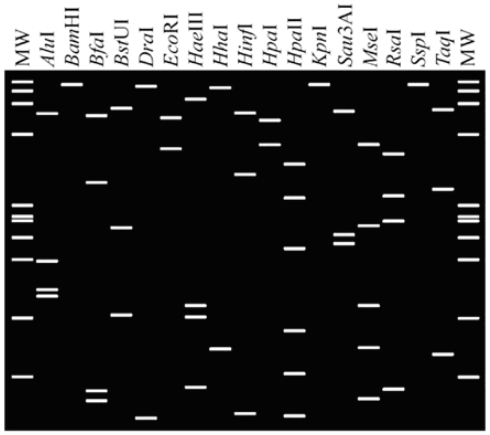


Fig 1: A typical RFLP profile of a certain individual

RFLP analysis was the first DNA profiling technique for genetic fingerprinting,genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

Presence and absence of fragments resulting from changes in recognition sites are used for identification of species or populations.

Application of RFLP in mapping genetic disease: For the detection of sickle cell anemia, DNA from the hemoglobin gene from each family member is digested with a particular restriction endonuclease. Since the hemoglobin gene is polymorphic, there is more than one DNA sequence encoding for this gene. Hb A is the wild type allele, and Hb S is the allele that codes for the sickling of red blood cells. RFLP's are produced using this polymorphic DNA sequence and the resulting fragments are separated by agarose gel electrophoresis and as shown in Figure 2:

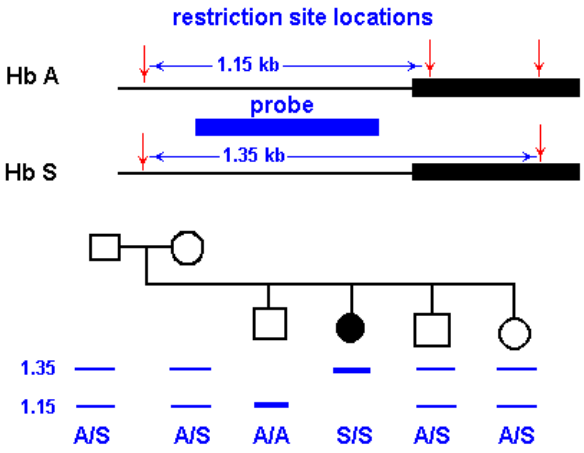


Figure 2: RFLP pattern for the detection of sickle cell anemia

The wild-type hemoglobin gene, Hb A shows a band at 1.15 kb, while the sickled hemoglobin gene, Hb S, shows a band at 1.35 kb. A person homozygous for sickle cell anemia (S/S) shows only one RFLP at 1.35 kb, while people heterozygous for this disease (A/S) have RFLP's at 1.35 kb and 1.15 kb. People who have not inherited this gene (A/A) show one RFLP at 1.15 kb. Therefore, by studying the RFLP pattern one can detect the presence of a genetic disease in a certain individual.

Kit Contents:

This kit can be used to teach RFLP method by comparing the restriction profile of one unknown sample withthree reference samples.

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	25 expts	
1	TKC290	Reference Sample 1	0.08 ml	0.4 ml	-20^{O}C
2	TKC291	Reference Sample 2	0.08 ml	0.4 ml	-20^{O}C
3	TKC292	Reference Sample 3	0.08 ml	0.4 ml	-20^{O}C
4	TKC293	Test Sample	0.08 ml	0.4 ml	-20^{O}C
5	TKC189	1 Kb DNA Ladder	0.03 ml	0.135 ml	-20^{O}C
6	MBRE001	Restriction Enzyme: *Eco*RI	0.025 ml	0.11 ml	-20^{O}C
7	MBRE011	Restriction Enzyme: *Pst*I	0.025 ml	0.11 ml	-20^{O}C
8	TKC188	10X Assay Buffer	0.07 ml	0.32 ml	-20^{O}C
9	ML024	Molecular Biology Grade Water	0.55 ml	2.5 ml	RT

10	ML016	50X TAE	60 ml	270 ml	RT
11	MB002	Agarose	3 g	11 g	RT
12	TKC294	6X Dye	0.12 ml	0.55 ml	2-8°C
13	CG281	Polypropylene Tubes (0.5 ml)	24 Nos.	108 Nos.	RT

Materials Required But Not Provided:

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

Storage:

HiPer® RFLP Teaching Kit is stable for 12 months from the date of receipt without showing any reduction in performance. On receipt store the DNA samples, Restriction Enzymes, Assay Buffers, 1 kb DNA ladder and 6X Dye at -20°C. All 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 DNA samples, restriction enzymes and assay buffers onto it.
2. In this experiment three reference DNA samples and the test sample are digested simultaneously with two restriction enzymes *Eco*RI and *Pst*I.
3. Set up four separate reaction mixtures as follows:

DNA sample	– 15.0 µl
10X Assay Buffer	– 3.0 µl
Milli Q water*	– 10.0 µl
*Eco*RI	–1.0 µl
*Pst*I	– 1.0 µl
Total	**30µl**

*Molecular biology grade water is recommended (Product code: ML024).

4. After preparing the four reaction tubes, mix the components by gentle pipetting and tapping.
5. Incubate the tubes at 37°C for 2-3 hours.
6. After incubation, immediately add 5 µl of 6X Dye to each tube.
7. Run the samples on agarose gel as given below:

Agarose Gel Electrophoresis:

Preparation of 1X TAE: To prepare 500 ml of 1X TAE buffer add 10 ml of 50X TAE Buffer to 490 ml of sterile distilled water*. Mix well before use.

Preparation of agarose gel: To prepare 50 ml of 1.5 % agarose gel, measure 0.75 g agarose in a glass beaker or flask and add 50ml 1X TAE buffer. Heat the mixture on a microwave or hot plate or burner, swirling the glass beaker/flask occasionally, until agarose dissolves completely (Ensure that the lid of the flask is loose to avoid buildup of pressure). Allow the solution to cool to about 55-60°C. Add 2 µl Ethidium bromide (10 mg/ml), mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature.

NOTE: Ethidium bromide is a powerful mutagen and is very toxic. Appropriate safety precautions should be taken by wearing latex gloves; however, use of nitrile gloves is recommended.

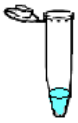
Loading of the DNA samples: Load 5 µl of ready to use DNA Marker into the well 1. Load 30 µl of each DNA samples (reference samples) onto wells 2, 3 and 4. Load 30 µl of test DNA sample well 5.

Electrophoresis: 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 onthe size of DNA to be visualized. Switch off the power supply once the tracking dye from the wells reaches 3/4th of the gel which takes approximately 45 - 60 minutes. Observe the gel under a UV transilluminator.

Flowchart:

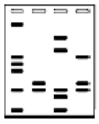
Keep all the components on ice

Prepare reaction mixture for two restriction enzymes



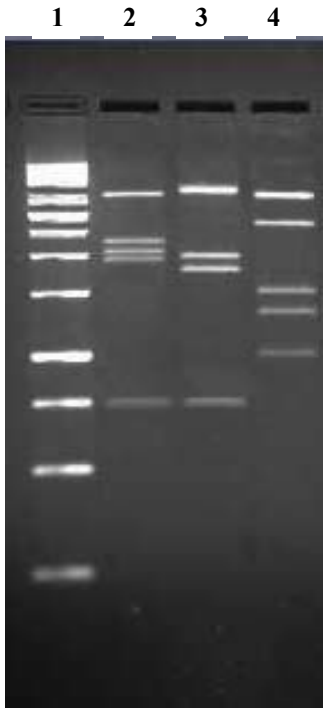
Mix gently and incubate at 37°C for

Visualize the digested bands after electrophoresing on



Observation and Result:

Perform Agarose Gel Electrophoresis. Visualize the DNA bands using UV transilluminator. Compare the restriction profile of unknown sample with that of the reference samples.



Lane 1: 1 kb DNA ladder
Lane 2: Reference Sample 1
Lane 3: Reference Sample 2
Lane 4: Reference Sample 3

Interpretation:

From all the restriction profiles obtained one can observe the restriction profile of given unknown sample matches with which reference sample. As the restriction enzymes recognize and digest a particular sequence, any slight change in that results in different restriction profile of a particular sample.

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	Reaction mixture incubated for longer time than specified in the protocol	Do not exceed the incubation time beyond 1 and a half hour
		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