G-Biosciences ♦ 1-800-628-7730 ♦ 1-314-991-6034 ♦ technical@GBiosciences.com

A Geno Technology, Inc. (USA) brand name

DNA Restriction Digestion Analysis

Teacher's Guidebook

(Cat. # BE-307)

MATERIALS INCLUDED

This kit has enough materials and reagents for 24 students (six groups of four students).

- 1 vial DNA: Plasmid 1
- 1 vial DNA: Plasmid 2
- 1 vial Res. Enz.: R.E. Buffer 2 (4X)
- 6 vials HindIII Enzyme
- 6 vials EcoRI Enzyme
- 1 vial Sterile Water
- 1 bottle TAE Buffer (50X)
- 1 vial LabSafe™ Nucleic Acid Stain
- 1 vial DNA Loading Buffer (6X)
- 1 vial DNA Ladder (1kb)
- 1 pack Agarose
- 60 2ml Centrifuge Tubes

SPECIAL HANDLING INSTRUCTIONS

- Store plasmids, restriction enzymes and buffers, DNA Loading Buffer and DNA Ladder frozen at -20°C
- Store LabSafe™ Nucleic Acid Stain at 4°C.
- All other reagents can be stored at room temperature.
- Briefly centrifuge all small vials before opening to prevent waste of reagents.

ADDITIONAL EQUIPMENT REQUIRED

- Agarose Electrophoresis Equipment
- Waterbath or beaker and thermometer
- Washing trays 12cm x 12cm
- UV Light box or transilluminator

TIME REQUIRED

- **Day 1**: 3 hours

OBJECTIVES

- Learn to perform digestions with restriction enzymes.
- Digest DNA Plasmids with unique restriction enzymes.
- Resolve digested fragments on agarose gel.
- Perform restriction map analysis.

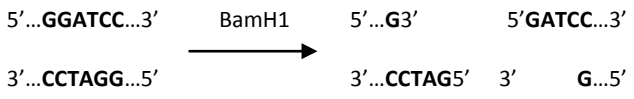
BACKGROUND

In 1970, Hamilton Smith published a paper on the discovery and purification of the first restriction enzyme, or endonuclease, HindII; for which he was awarded the Noble Prize in medicine. Restriction endonucleases are important enzymes that cleave the backbone of DNA molecules at specific sites or sequences. The use of restriction endonucleases has proved invaluable in molecular biology, cloning, genetic engineering and a multiple of other scientific disciplines.

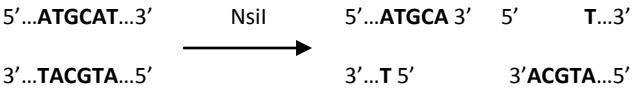
The majority of restriction endonucleases are isolated from bacteria, where they are thought to play a role in host defense by cutting and destroying foreign DNA, for example, of invading viruses. To ensure the bacteria's own genome is not degraded, each restriction endonuclease has a complementary methylating enzyme that methylates the restriction endonuclease site on the genomic DNA preventing digestion. The combined role of these two enzymes is known as the restriction:modification system.

Each restriction endonuclease recognizes a specific sequence of nucleotides, normally ranging from 4-8 base pairs in length. The shorter the recognition sequence the more frequently the site will appear in the DNA sequence. For example a six base pair sequence will appear, on average, every 4^6 or 4096bp, where as a four base pair site will appear every 256bp. Some restriction endonucleases share the same sites as other restriction endonucleases, these are known as isoschizomers.

There are three major patterns of digestions that are achieved by restriction endonucleases. The first is the generation of a 5' overhang. This occurs when the enzyme cuts at a different place on each strand of DNA leaving one of the strands longer than the other producing a sticky end. This is seen when DNA is digested with BamHI and EcoRI.



The second pattern is the 3' overhang, as generated by the restriction enzyme NsiI:



The 5’ and 3’asymmetric ends generated by enzymes are known as sticky ends or cohesive ends as they readily stick, or anneal, together with their complimentary base pairs.

The third pattern is known as the blunt ends, these occur when the enzyme cuts the both strands of DNA in the same place resulting in no overhang. An example is the restriction enzyme EcoRV:

Another feature of restriction endonucleases is that some are unambiguous and others are ambiguous. For example, BamHI is unambiguous as it recognizes 6 specific, defined nucleotides (GGATCC); HinFI, an ambiguous enzyme, recognizes a 5 base pair sequence, which starts with GA and ends in TC, but can have any base as the middle base pair.

There are several important factors to consider when using restriction enzymes. These include buffer composition, incubation temperature, DNA methylation and star activity.

There is no universal digestion buffer for restriction enzymes as different enzymes have different preferences for ionic strength (salt concentration) and major cations (sodium or potassium). There are 3-4 commonly used buffers that are generally suitable for most enzyme conditions. For pH, enzymes commonly work around pH8.0, however some enzymes are more particular and have specific buffers. Use of the wrong buffer leads to poor digestions.

Most of the restriction enzymes available have optimal activity at 37°C, but, as with the buffers there are many exceptions. Enzymes that have been isolated from thermophiles, bacteria that grow in high temperature environments, have optimal activity at 50-65°C, whereas some enzymes have very short half lives at 37°C and are used at 25°C.

DNA methylation has an inhibitory effect on some restriction enzymes. DNA methylation has two functions: 1) Protection from host restriction enzymes that target foreign (i.e. viral DNA) in an immune response; 2) A gene regulation control step. Almost all strains of *E.coli* contain two site specific DNA methylases that methylate specific sequences. Researchers must check information of their restriction enzyme of choice and the effects of methylation.

A final consideration is that some enzymes under *non-standard conditions* will cleave DNA at sites different from their specific recognition sequence. Non-standard conditions include high pH (>8.0) or low ionic strength, high glycerol concentrations (enzymes usually supplied in 50% glycerol), extremely high enzyme concentration or the presence of organic solvents (ethanol, DMSO) in the reaction.

For molecular cloning, researchers have specifically designed multiple cloning sites, or MCS, into plasmids. A MCS is a short DNA sequence that contains numerous unique restriction enzyme sites. All the enzymes that cut within the MCS do not cut anywhere else on the plasmid. The MCS is the site on a plasmid where new DNA fragments are inserted.

This kit is designed to use HindIII and EcoRI restriction endonucleases to cut two plasmids. One plasmid contains a gene of interest and this is excised from the Plasmid, the other Plasmid will be cut within its MCS, so that later the gene of interest can be inserted. The resulting fragments can be used in subsequent kits for further cloning.

TEACHER'S PRE EXPERIMENT SET UP

Preparation of agarose gel

Each group of 4 students will have 4 DNA samples, so for 6 groups, there needs to be 24 wells for the samples, plus a well for the reference 1kb DNA ladder. For optimal results, the capacity of each well should be equal to or greater than 30µl.

Make 1-2 hours before the experiment.

Wear heat protective gloves throughout the agarose melting and pouring procedure.

1. Prepare running buffer: In a clean two-liter container, add the entire contents of the TAE buffer (50X) and add two liters of ultra pure water to make a 1X TAE buffer solution. Stir until thoroughly mixed.

2. Prepare agarose: In a clean, glass 1000ml container add the entire contents of the agarose pack and add 500ml of the 1X TAE buffer from step 1.

3. Heat the solution in a microwave on full power, using 10 second bursts, or use a boiling waterbath. Check to see if all the agarose has dissolved. Continue until agarose has dissolved.

   
   DO NOT BOIL. The agarose gets very hot, very quickly and can cause severe burns. Wear heat protective gloves throughout the melting and pouring procedure.

4. Once the agarose has cooled to the point it can be held comfortably in your hand, add the entire contents of the LabSafe™ Nucleic Acid Stain to the agarose and swirl to mix.

5. Pour the agarose into the gel casting mould as per the manufacturer's instructions. You will need 25 wells that each holds 30µl for each group, use an appropriate size comb.

6. Once the gels have set, remove the comb, transfer to the running apparatus and cover with the running buffer until ready to use.

Prepare the reference markers

1. Add 25µl ultra pure water to the lyophilized DNA ladder (1kb), dissolve by gently pipetting up and down 5-6 times.

2. Add 5µl DNA Loading Buffer (6X), mix by gently pipetting up and down 5-6 times.

3. Load 10µl into each reference well.

Reconstitute DNA & Aliquot Reagents

 *Once the reagents have been thawed and/or resuspended they must be kept on ice. The reagents must remain on ice throughout the experiment.*

1. Transfer 250µl sterile water to the Plasmid 1. Resuspend the plasmid by gently pipetting up and down.

2. Label six tubes with "Plasmid 1". Transfer 40µl Plasmid 1 from step 1 in to the bottom of each tube. Supply each group with a single tube.

3. Transfer 250µl sterile water to the Plasmid 2. Resuspend the Plasmid by gently pipetting up and down.

4. Label six tubes with "Plasmid 2". Transfer 40µl Plasmid 2 from step 3 in to the bottom of each tube. Supply each group with a single tube.

5. Label six tubes with "4X Buffer". Transfer 40µl R.E. Buffer 2 (4X) into the bottom of each tube. Supply each group with a single tube.

6. Supply each group with a single tube of EcoRI and HindIII enzyme.

7. Label six tubes with "Sterile Water". Transfer 25µl Sterile Water into the bottom of each tube. Supply each group with a single tube.

MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 40µl Plasmid 1
- 40µl Plasmid 2
- 40µl 4X R.E. Buffer 2
- 15µl HindIII
- 15µl EcoRI
- 25µl Sterile Water
- 4 Centrifuge Tubes (2ml)
- 1 vial DNA Loading buffer (to be shared with class)

PROCEDURE

1. Each group will set up four tubes, two restriction digests and two undigested controls. Label four tubes with your group name and numbers 1 through 4 to represent the following:

   a. Plasmid 1 uncut

   b. Plasmid 1 digested with HindIII and EcoRI

   c. Plasmid 2 uncut

   d. Plasmid 2 digested with HindIII and EcoRI

2. Place the tubes in the ice bucket containing the rest of the components for the experiment.

   All the components and the reactions must remain on ice during the set up of the experiment.

3. Using a clean pipette tip for every reagent or solution, set up the following reactions on ice to avoid cross contamination:

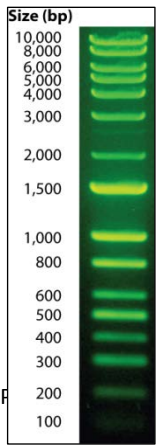
	Tube 1	Tube 2	Tube 3	Tube 4
Plasmid 1	20µl	20µl	None	None
Plasmid 2	None	None	20µl	20µl
HindIII	None	5µl	None	5µl
EcoRI	None	5µl	None	5µl
4X Buffer	10µl	10µl	10µl	10µl
Sterile Water	10µl	None	10µl	None

4. Mix the contents of each tube by gently pipetting 4-5 times.

5. Place each tube in a waterbath or incubator at 37°C for one hour.

6. After the one-hour incubation the DNA is visualized by agarose electrophoresis on a 1% agarose gel. Add 10µl DNA loading buffer (6X) to each tube and load 30µl of each sample.

7. The agarose gels should have been prepared by your teacher/supervisor. Each student takes turn in loading a sample into



a well. Your teacher/ supervisor will load a reference lane to determine the size of the DNA (see image for reference markers).

8. Once the samples are all loaded, apply a current and migrate at 12-15V/cm. For an 8cm long gel run at 96-120 volts.

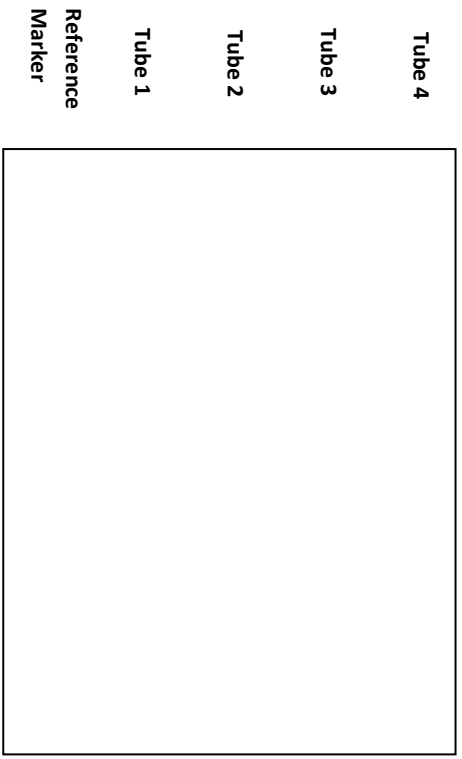
9. Once the blue dye front has migrated ¾ the length of the gel, turn off the power and carefully transfer the gel to a UV Light box.

 *CAUTION: Wear UV Safety Specs when working around the UV Light Box.*

 *Following electrophoresis, if the fragments are to be used for subsequent cloning experiments then use the "Purification of a Gene" kit. For cloning, use the Plasmid 1 band of ~700bp and Plasmid 2 band of ~2,600bp.*

RESULTS, ANALYSIS & ASSESSMENT

1. Draw a representation of your results in the box below:



2. Describe the differences between the uncut Plasmids and the Plasmids treated with the enzymes.

Uncut Plasmid 1= ~3300bp

Cut Plasmid 1= ~2600bp and 700bp

Uncut Plasmid 2= ~2600bp

Cut Plasmid 2= ~2600bp

If multiple bands are seen in the uncut lanes: This is due to the circular DNA running as supercoiled, nicked and linearized. Each form migrates differently.

www.GBiosciences.com

G-Biosciences ♦ 1-800-628-7730 ♦ 1-314-991-6034 ♦ technical@GBiosciences.com

A Geno Technology, Inc. (USA) brand name

DNA Restriction Digestion Analysis

Student's Handbook

(Cat. # BE-307)

OBJECTIVES

- Learn to perform digestions with restriction enzymes.
- Digest DNA Plasmids with unique restriction enzymes.
- Resolve digested fragments on agarose gel.
- Perform restriction map analysis.

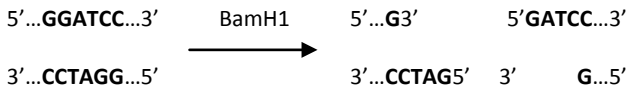
BACKGROUND

In 1970, Hamilton Smith published a paper on the discovery and purification of the first restriction enzyme, or endonuclease, HindII; for which he was awarded the Noble Prize in medicine. Restriction endonucleases are important enzymes that cleave the backbone of DNA molecules at specific sites or sequences. The use of restriction endonucleases has proved invaluable in molecular biology, cloning, genetic engineering and a multiple of other scientific disciplines.

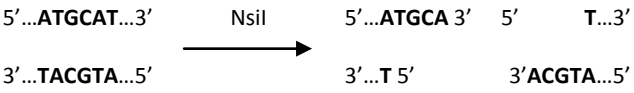
The majority of restriction endonucleases are isolated from bacteria, where they are thought to play a role in host defense by cutting and destroying foreign DNA, for example, of invading viruses. To ensure the bacteria's own genome is not degraded, each restriction endonuclease has a complementary methylating enzyme that methylates the restriction endonuclease site on the genomic DNA preventing digestion. The combined role of these two enzymes is known as the restriction:modification system.

Each restriction endonuclease recognizes a specific sequence of nucleotides, normally ranging from 4-8 base pairs in length. The shorter the recognition sequence the more frequently the site will appear in the DNA sequence. For example a six base pair sequence will appear, on average, every 4^6 or 4096bp, where as a four base pair site will appear every 256bp. Some restriction endonucleases share the same sites as other restriction endonucleases, these are known as isoschizomers.

There are three major patterns of digestions that are achieved by restriction endonucleases. The first is the generation of a 5' overhang. This occurs when the enzyme cuts at a different place on each strand of DNA leaving one of the strands longer than the other producing a sticky end. This is seen when DNA is digested with BamHI and EcoRI.



The second pattern is the 3' overhang, as generated by the restriction enzyme NsiI:



The 5’ and 3’asymmetric ends generated by enzymes are known as sticky ends or cohesive ends as they readily stick, or anneal, together with their complimentary base pairs.

The third pattern is known as the blunt ends, these occur when the enzyme cuts the both strands of DNA in the same place resulting in no overhang. An example is the restriction enzyme EcoRV:

Another feature of restriction endonucleases is that some are unambiguous and others are ambiguous. For example, BamHI is unambiguous as it recognizes 6 specific, defined nucleotides (GGATCC); HinFI, an ambiguous enzyme, recognizes a 5 base pair sequence, which starts with GA and ends in TC, but can have any base as the middle base pair.

There are several important factors to consider when using restriction enzymes. These include buffer composition, incubation temperature, DNA methylation and star activity.

There is no universal digestion buffer for restriction enzymes as different enzymes have different preferences for ionic strength (salt concentration) and major cations (sodium or potassium). There are 3-4 commonly used buffers that are generally suitable for most enzyme conditions. For pH, enzymes commonly work around pH8.0, however some enzymes are more particular and have specific buffers. Use of the wrong buffer leads to poor digestions.

Most of the restriction enzymes available have optimal activity at 37°C, but, as with the buffers there are many exceptions. Enzymes that have been isolated from thermophiles, bacteria that grow in high temperature environments, have optimal activity at 50-65°C, whereas some enzymes have very short half lives at 37°C and are used at 25°C.

DNA methylation has an inhibitory effect on some restriction enzymes. DNA methylation has two functions: 1) Protection from host restriction enzymes that target foreign (i.e. viral DNA) in an immune response; 2) A gene regulation control step. Almost all strains of *E.coli* contain two site specific DNA methylases that methylate specific sequences. Researchers must check information of their restriction enzyme of choice and the effects of methylation.

A final consideration is that some enzymes under *non-standard conditions* will cleave DNA at sites different from their specific recognition sequence. Non-standard conditions include high pH (>8.0) or low ionic strength, high glycerol concentrations (enzymes usually supplied in 50% glycerol), extremely high enzyme concentration or the presence of organic solvents (ethanol, DMSO) in the reaction.

For molecular cloning, researchers have specifically designed multiple cloning sites, or MCS, into plasmids. A MCS is a short DNA sequence that contains numerous unique restriction enzyme sites. All the enzymes that cut within the MCS do not cut anywhere else on the plasmid. The MCS is the site on a plasmid where new DNA fragments are inserted.

This kit is designed to use HindIII and EcoRI restriction endonucleases to cut two plasmids. One plasmid contains a gene of interest and this is excised from the Plasmid, the other Plasmid will be cut within its MCS, so that later the gene of interest can be inserted. The resulting fragments can be used in subsequent kits for further cloning.

MATERIALS FOR EACH GROUP

Supply each group with the following components.

- 40µl Plasmid 1
- 40µl Plasmid 2
- 40µl 4X R.E. Buffer 2
- 15µl HindIII
- 15µl EcoRI
- 25µl Sterile Water
- 4 Centrifuge Tubes (2ml)
- 1 vial DNA Loading buffer (to be shared with class)

PROCEDURE

1. Each group will set up four tubes, two restriction digests and two undigested controls. Label four tubes with your group name and numbers 1 through 4 to represent the following:

    a. Plasmid 1 uncut

    b. Plasmid 1 digested with HindIII and EcoRI

    c. Plasmid 2 uncut

    d. Plasmid 2 digested with HindIII and EcoRI

2. Place the tubes in the ice bucket containing the rest of the components for the experiment.

     *All the components and the reactions must remain on ice during the set up of the experiment.*

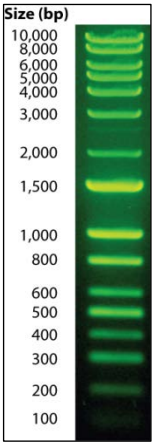
3. Using a clean pipette tip for every reagent or solution, set up the following reactions on ice to avoid cross contamination:

	Tube 1	Tube 2	Tube 3	Tube 4
Plasmid 1	20µl	20µl	None	None
Plasmid 2	None	None	20µl	20µl
HindIII	None	5µl	None	5µl
EcoRI	None	5µl	None	5µl
4X Buffer	10µl	10µl	10µl	10µl
Sterile Water	10µl	None	10µl	None

4. Mix the contents of each tube by gently pipetting 4-5 times.

5. Place each tube in a waterbath or incubator at 37°C for one hour.

6. After the one-hour incubation the DNA is visualized by agarose electrophoresis on a 1% agarose gel. Add 10µl DNA loading buffer (6X) to each tube and load 30µl of each sample.



7. The agarose gels should have been prepared by your teacher/supervisor. Each student takes turn in loading a sample into a well. Your teacher/ supervisor will load a reference lane to determine the size of the DNA (see image for reference markers).

8. Once the samples are all loaded, apply a current and migrate at 12-15V/cm. For an 8cm long gel run at 96-120 volts.

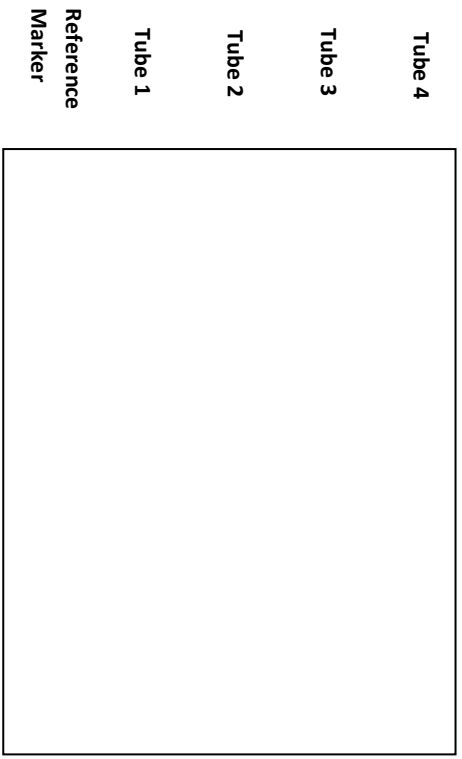
9. Once the blue dye front has migrated ¾ the length of the gel, turn off the power and carefully transfer the gel to a UV Light box.

CAUTION: Wear UV Safety Specs when working around the UV Light Box.

Following electrophoresis, if the fragments are to be used for subsequent cloning experiments then use the "Purification of a Gene" kit. For cloning, use the Plasmid 1 band of ~700bp and Plasmid 2 band of ~2,600bp.

RESULTS, ANALYSIS & ASSESSMENT

1. Draw a representation of your results in the box below:



2. Describe the differences between the uncut Plasmids and the Plasmids treated with the enzymes.

_____

_____

_____

_____

_____

_____

www.GBiosciences.com