High Fidelity restriction enzyme Protocol

Reagents:

- Ice
- DNA
- Buffer
- Distilled water
- Restriction enzyme
- Tube to put the mixture

Materials:

- Micropipettes
- Eppendorf

Equipment:

- Microcentrifuge

Summarized Protocol (Considering a total reaction volume of 50 µl)

1. Prepare a mixture containing: 1 µg of DNA, 5µl of 10X NeBuffer, and 1 µl of enzyme (this element should be the last component added to reaction).
2. Mix the components by pipetting the reaction mixture up and down, or snapp the reaction tube. Then with a quick touch spin-down in a microcentrifuge. **Do not vortex the reaction.**
3. **Incubate for 1 hour (this time** can be decreased by using an excess of enzyme).

Specific Protocol

1. In a 1.5mL tube combine the following:
    a. DNA
    b. Restriction Enzyme
    c. Buffer
    d. BSA (if recommended by manufacturer)
    e. Distilled water up to total volume
2. Specific quantities:
    a. **DNA:** the amount of DNA required for molecular cloning **is 1-3 µg of DNA**. The total reaction volume usually varies from 10-50

µL depending on the application and the volume of DNA to be cut.

b. **Restriction Enzyme (Add this element at the end)**: One unit of enzyme will cut 1 µg of DNA in a 50 µL reaction in 1 hour. Using this ratio, it can be calculated the minimum amount of enzyme for the reaction, but is better to use a little more enzyme because the restriction enzyme activity is determined under ideal conditions with very clean DNA.
c. Reactions are often performed with 0.2-0.5 µL this volume has already considered an extra amount of the enzyme needed.
d. **Buffer:** the required quantity should be specified in the instructions of the enzyme.
   - For a double digest (digesting two enzymes at the same time), choose a buffer that works for both enzymes. Página para poner las dos enzimas a utilizar: https://nebcloner.neb.com/#!/protocol/re/double/HindIII-HF,AccI

3. Mix all the elements gently by pipetting
4. Incubate tube at 37 °C for 1 hour (temperature should be specified by the manufacturer's instructions). For digests with a quantity greater than 1 µg of DNA (used for cloning), it is recommended to **digest for at least 4 hours.**

   a. If the digested DNA will be used for another application (for example a digestion with another enzyme in a different buffer), but will not be gel purifying it, it may be needed to inactivate the enzymes following the digestion reaction. This may involve incubating the reaction at 70 °C for 15 mins, or purifying the DNA via a purification kit. Observe the enzyme manufacturer's instructions for more details.
5. To observe results of digest conduct a gel electrophoresis.

A typical restriction digestion reaction looks like this:

- 1 µg DNA
- 1 µL of each Restriction Enzyme
- 3 µL 10x Buffer
- 3 µL 10x BSA (if recommended)
- x µL dH_2O (to bring total volume to 30µL)

or like:

- 1 µg DNA
- 1 µL of each Restriction Enzyme
- 5 µL 10x Buffer
- total reaction volume 50 µL

Additional information:

- The amount of DNA to be cut, depends on the application:
    - Diagnostic digests typically involve ~500 ng of DNA
    - Molecular cloning often requires 1-3 µg of DNA.
    - The total reaction volume usually varies from 10-50 µL depending on application and is largely determined by the volume of DNA to be cut.
- **Restriction enzyme:** the quantity of restriction enzyme for a given digestion depends on the amount of DNA that is going to be cut. By definition: one unit of enzyme will cut 1 µg of DNA in a 50 µL reaction in 1 hour. Using this ratio, it can be calculated the minimum amount of enzyme for the reaction, but is better to use a little more enzyme because the restriction enzyme activity is determined under ideal conditions with very clean DNA. Reactions are often performed with 0.2-0.5 µL this volume has already considered an extra amount of the enzyme needed.
- **Incubation time:** it depends on the application and the amount of DNA in the reaction, it can range from 45 minutes to overnight. For a diagnostic digest, 1-2 hours is enough.
    - Can be decreased to 5-15 minutes by using a Time-Saver™ Qualified enzyme.

Important tips to consider

Enzyme

- Keep on ice when not in the freezer
- In general, 5–10 units of enzyme per µg of DNA are recommend, and 10–20 units for genomic DNA in a 1 hour digest.

DNA

- Should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents or excessive salts. Extra wash steps during purification are recommended.
- Methylation of DNA can inhibit digestion with certain enzymes.

Buffer

- Use at a 1X concentration.

- Supplement with SAM (S-Adenosyl methionine) to the recommended concentration if required.

Reaction Volume

- A 50 µl reaction volume is recommended for digestion of 1 µg of substrate.
- Enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol.
- Additives in the restriction enzyme storage buffer (e.g., glycerol, salt) as well as contaminants found in the substrate solution (e.g., salt, EDTA, or alcohol) can be problematic in smaller reaction volumes.
- The following guidelines can be used for techniques that require smaller reaction volumes.

	Restriction enzyme*	**DNA**	**10X NEBuffer**
10 µl rxn**	1 unit	0.1 µg	1 µl
25 µl rxn	5 units	0.5 µg	2.5 µl
50 µl rxn	10 units	1 µg	5 µl

- * Restriction Enzymes can be diluted using the recommended diluent buffer when smaller amounts are needed.
- ** 10 µl rxns should not be incubated for longer than 1 hour to avoid evaporation.