May 12, 2022

Sequential Double Digest

Forked from Sequential Double Digest

DOI
dx.doi.org/10.17504/protocols.io.6qpvr6m72vmk/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.6qpvr6m72vmk/v1

External link: https://www.neb.com/protocols/2014/05/07/double-digest-protocol-with-standard-restriction-enzymes

Protocol Citation: Larissa de Clauser 2022. Sequential Double Digest. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.6qpvr6m72vmk/v1**

Protocol status: Working
We use this protocol and it's working

Created: May 04, 2022

Last Modified: May 12, 2022

Protocol Integer ID: 61958

Keywords: restriction enzyme, sequential double digest protocol with standard restriction enzyme, sequential double digest, sequential double digest protocol, sequential digest, standard restriction enzyme, enzyme, protocol, buffer

Abstract

This is the Sequential Double Digest Protocol with Standard Restriction Enzymes. If there is no buffer in which the two enzymes exhibit > 50% activity, this sequential digest can be performed.

Guidelines

Digesting a DNA substrate with two restriction enzymes simultaneously (double digestion) is a common timesaving procedure. Over 200 restriction enzymes are 100% active in **CutSmart™ Buffer**, making double digestion simple. If you are using an enzyme that is not supplied with CutSmart Buffer, the **Performance Chart for Restriction Enzymes** rates the percentage activity of each restriction endonuclease in the four standard NEBuffers.

NEB's online tools, **Double Digest Finder** and **NEBcloner** will help guide your reaction buffer selection when setting up double digests.

Setting up a Double Digestion
Double digests with NEB's restriction enzymes can be set up in CutSmart Buffer. Otherwise, choose an NEBuffer that results in the most activity for both enzymes. If **star activity** is a concern, consider using one of our **High Fidelity (HF™) enzymes**.

Set up reaction according to recommended **protocol**. Enzyme volume should not exceed 10% of the total reaction volume to prevent **star activity** due to excess glycerol. For example, in a 50 µl reaction, the total amount of enzyme added should not exceed 5 µl.

If two different incubation temperatures are necessary, choose the optimal reaction buffer and set up reaction accordingly. Add the first enzyme and incubate at the desired temperature. Then, heat inactivate the first enzyme, add the second enzyme and incubate at the recommended temperature.
Depending on an enzyme's activity rating in a non-optimal NEBuffer, the number of units or incubation time may be adjusted to compensate for the slower rate of cleavage.

Setting up a Double Digestion with a Unique Buffer (designated "U")
NEB currently supplies three enzymes with unique buffers: **EcoRI**, **SspI** and **DpnII**. In most cases, DpnII requires a sequential digest. Note that EcoRI has an HF version which is supplied with CutSmart Buffer.

Setting up a Sequential Digestion
If there is no buffer in which the two enzymes exhibit > 50% activity, a sequential digest can be performed. Set up a reaction using the restriction endonuclease that has the lowest salt concentration in its recommended buffer and incubate to completion. (For reference, 1 X CutSmart and NEBuffer 1.1 do not contain any salt. 1X NEBuffer 2.1 contains 50 mM NaCl and 1X NEBuffer 3.1 contains 100 mM NaCl.)

Adjust the salt concentration of the reaction (using a small volume of a concentrated salt solution) to approximate the reaction conditions of the second restriction endonuclease.
Add the second enzyme and incubate to complete the second reaction.
Alternatively, a spin column can be used to isolate the DNA prior to the second reaction.

Troubleshooting

Before start

NEB's online tools, **Double Digest Finder** and **NEBcloner** will help guide your reaction buffer selection when setting up double digests.

1 Set up the following reaction using the restriction endonuclease that has the lowest salt concentration in its recommended buffer (total reaction volume **50 µl**).

Note

A 50 µl reaction volume is recommended for digestion of 1 µg of substrate.

Note

The enzyme should be the last component added to reaction

2 Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

3 Quick ("touch") spin-down in a microcentrifuge. **Do not vortex the reaction.**

4 Incubate for 1 hour at the enzyme-specific appropriate temperature.

5 Adjust the salt concentration of the reaction (using a small volume of a concentrated salt solution) to approximate the reaction conditions of the second restriction endonuclease.

Note

Alternatively, a spin column can be used to isolate the DNA prior to the second reaction.

6 Add the second enzyme.

7 Mix components by pipetting the reaction mixture up and down, or by "flicking" the reaction tube.

8 Quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

9 Incubate for 1 hour at the enzyme-specific appropriate temperature.