Restriction Enzyme Digestion – NEB Protocol
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Digesting genomic, vector, or PCR product DNA with restriction endonucleases can be used for specifically combining multiple pieces of DNA in a specific order, removing DNA fragments of interest, or as a means of verifying the sequence of DNA. One can combine multiple restriction endonucleases in the same DNA digestion as long as they are compatible in the same buffer and active at the same temperature.

Timeline

Reaction set-up:	5-10 min
Incubation:	15 – 60 min

Reaction Set-Up (50.0 µL)

Component	V (µL, 1 RE used)	V (µL, 2 RE used)
Nuclease free water	43.0	42.0
Restriction enzyme 10× buffer[A]	5.0	5.0
DNA, 1 µg/µL[B]	1.0	1.0
Restriction enzyme[C]	1.0	2.0

Incubate for up to 1 hour at the appropriate temperature listed for the enzyme(s) used.

A: We use NEB enzymes, check the website (https://www.neb.com/products/restriction-endonucleases) to determine which buffer will enable the highest rate of activity for the enzyme you plan on using. If using two enzymes in the same reaction, the double digest finder (https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder) makes it easy to select a buffer that will yield optimal activity for both restriction enzymes.
B: Your plasmid is probably not this concentrated, change this volume accordingly.
C: This should be the very last component added to the reaction. Look up the specific enzymes you will be using on NEB. Read about them. NEB provides useful information that can save you time!

Enzyme	Cat #	Temp	Supplied NEBuffer	Supplements	% Activity in NEBuffer			
				SAM	1.1	2.1	3.1	CutSmart
Acc65I Yes dcm	R0599	37°C	NEBuffer 3.1	no	10	75*	100	25
HindIII-HF® Yes	R3104	37°C	CutSmart® Buffer	no	10	100	10	100

In this example from the double digest finder, you can see everything you need to know to use these restriction enzymes. Pay attention to the symbols next to the enzyme names.

 indicates that this is a "time saver" enzyme, meaning that it can digest the recommended amount of DNA in 5-15 minutes under recommended conditions, and also that it is safe to leave reacting overnight.

indicates that the enzyme can be heat inactivated at either 65 °C or 80 °C at 20 minutes, check the enzyme's page to find learn specifically what temperature.

indicates that the enzyme is blocked by dcm or dam methylation of substrate DNA. The source of your DNA matters! PCR product is not methylated; XL1-Blue cells have these methylation enzymes; BL21 DE3 cells are dcm deficient.

Notice the reported relative activities on the right side of the above table. Ideally, one could employ a buffer in which all enzymes in the reaction have 100% of their relative activity, however this rarely happens and it's best to choose the buffer that yields the greatest amount of activity total. In the above case, that's with buffer 2.1 *but* notice the asterisk next to 75 for the Acc65I activity; this indicates that the enzyme may exhibit star cutting in enzyme 2.1. Avoid star cutting as much as possible as this is when an enzyme cleaves DNA at a somewhat similar sequence to its native target. Read more at on NEB's website (https://www.neb.com/tools-and-resources/usage-guidelines/star-activity).

One could perform the double digest with Acc65I and HindIII-HF in Cut Smart buffer for at least one hour, however it may be better to perform these digestions sequentially so both enzymes may act at their greatest activity.