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FAQs for Restriction Endonucleases

1. **How is NEB's new buffer system going to help me?**
   Although the old buffer system worked well, NEB is continuously looking for ways to enhance the convenience and performance of its products for our customers. By adding BSA to the buffer, we were able to offer even more enzymes that cut in a single buffer (>200). This improves ease of use, especially when performing double digests. In addition, it eliminated the need to add BSA when setting up restriction enzyme digests.

2. **Has the conversion to the new buffer system altered any of the properties of the Restriction Enzyme itself?**
   The nature of the restriction enzymes is the same. However, for a handful of enzymes, BSA enhances activity. As a result, to keep the enzyme titer within specification for these products, the protein concentration may have been adjusted slightly.

3. **If I have an old tube of Restriction Enzyme, what NEBuffer should I use?**
   All NEB Restriction Enzymes have color-coded labels for the appropriate NEBuffer; this system can either be used with the previously supplied NEBuffer or with the newly recommended buffer. We have maintained the color-coding of our buffer and enzyme tubes so you can always match an enzyme to its recommended buffer.

4. **I currently have an old tube of Restriction Enzyme - is it still active in the new buffer?**
   Provided it is still prior to the product's expiry date, yes. The new buffers are mostly identical, except that BSA has been added directly to the buffer and DTT has been removed. BSA will not harm the reaction and may even enhance it in some cases. Extensive testing has shown that DTT is not required.

5. **I currently have an old tube of NEBuffer - can I still use it or should I throw it away?**
   You can use the old tube of NEBuffer until the expiration date. Please remember to follow the original recommendations for use with the old buffer, which may include the addition of BSA for some enzymes.

6. **My enzyme used to come with another NEBuffer, but CutSmart Buffer is now recommended? Why?**
   Reformulation of the NEB buffers has allowed some restriction enzymes to now also work well in CutSmart buffer. This enabled us to offer >200 restriction enzymes in a single buffer, greatly increasing convenience, especially for double digests.

7. **Why aren't all your enzymes available in CutSmart Buffer?**
   Some restriction enzymes require high salt and CutSmart Buffer is a low salt buffer. Over 200 restriction enzymes are supplied with CutSmart Buffer, but the remaining enzymes performed more optimally in another buffer. Please refer to the Performance

Chart available at neb.com for more details.

8. **The previous Activity/Performance Chart stated that activity in NEBuffer 4 is 50% yet the enzyme is now supplied with CutSmart Buffer. What has changed?**
   Reformulation of NEBuffer system, including the addition of BSA, allowed some enzymes to work more optimally in CutSmart Buffer. This enabled us to offer >200 restriction enzymes that work in a single buffer, greatly increasing convenience for our customers.

9. **How do I perform a double digest with an enzyme that comes with the new buffer and an enzyme that comes with the old buffer system?**
   We recommend using the Double Digest Finder (available by clicking the Tools and Resources tab at neb.com) and use the buffer it recommends. For example, you have old buffer NEBuffer 1 with one of the enzymes and CutSmart Buffer with the second enzyme. If Double Digest Finder recommends to use NEBuffer 1.1, then you can use NEBuffer 1 + BSA for the double digest. Alternatively, if it recommends CutSmart Buffer, then you can simply use the new buffer.

10. **Why did you add BSA into all the restriction enzyme reaction buffers?**
    As an added convenience for our customers, NEB has included BSA in all its restriction enzyme buffers, eliminating the need to add it as a separate step. This makes digestion reactions simpler, set-up time even faster, and eliminates the number of tubes to store.

11. **Why did you remove DTT from your restriction enzyme buffers?**
    DTT was originally included in the buffer formulation for historical reasons. That is, DTT is commonly used is many molecular biology enzyme buffers and thus was also included in these restriction enzyme buffers, when they were first developed. However, after extensive testing, we have found that it is not necessary for restriction enzyme activity. In addition, BSA in the presence of DTT can on occasions form a white precipitate after repeated freeze/thaws.

12. **What effect does BSA have on the performance of NEB's restriction enzymes when included in the new buffers?**
    NEB has not determined any negative effects when including BSA in the reaction buffer. In some instances, activity may be enhanced.

13. **My restriction enzyme used to be available at a lower concentration. Why does it now come at a higher concentration of 10,000 u/ml?**
    NEB has standardized many of its restriction enzymes to 10,000 u/ml. This contributes to ease of use and often results in improved performance by having less glycerol in the digest.

14. **I see you have renamed NEBuffer 1 to 1.1, NEBuffer 2 to 2.1, and NEBuffer 3 to 3.1. Why isn't there NEBuffer 4.1?**
    When BSA was added and DTT was removed, NEB changed the name of NEBuffer 4 to CutSmart Buffer. We felt that the name better represented the >200 restriction

enzymes that are available in this new buffer. Furthermore, this buffer also which works well with many of our downstream cloning enzymes (see chart on neb.com).

15. I found the color-coding of the restriction enzyme buffers (yellow, blue, red, green) to be very useful. Will the color-coding be maintained?
Yes. NEB will retain the same color-coding as used with the old buffer system. NEBuffer 1.1 will be yellow; NEBuffer 2.1 will be blue; NEBuffer 3.1 will be red; CutSmart Buffer will be green.

16. I don't want to use BSA in my buffer. What are my options?
NEB continues to offer the old buffers, which do not contain BSA in their formulation, and thus can be purchased separately. Please note that BSA has always been included in the formulation of the enzymes' storage buffer.

17. The product page on www.neb.com states that my restriction enzyme comes with the new buffer, but when it arrived, it came with the old buffer. Why?
We are currently in the process of transitioning all of our restriction enzymes between the old and new buffer systems. The product pages on neb.com were updated to include the new buffer information mid March, 2013. Replacement of product inventory containing the new buffers will happen gradually over the next few months. We are working as quickly as we can to replace stock, but there may be a short period where you may receive an enzyme pack that contains the old buffer. If you would like to receive new buffer please contact info@neb.com. Information regarding performance in the old buffer system is still available at www.neb.com.

18. When will all the restriction enzymes be available with the new buffer system?
NEB is working to make sure all enzymes are switched to the new buffer system by late May, 2013.

19. Will the old restriction enzyme buffers (without BSA) still be available for sale?
Yes. Old buffer formulations will be available with the same catalog numbers.

20. Will BSA still be available as a separate product for sale?
Yes. NEB offers BSA, Molecular Biology grade (NEB #B9000).

21. How do NEB modifying enzymes perform in CutSmart Buffer?
NEB has tested many modifying enzymes involved in the cloning workflow and has confirmed most have 100% functional activity in CutSmart Buffer. Please refer to chart on neb.com.

22. My enzyme is no longer Time-Saver qualified. What happened?
The Time-Saver definition originally referred to any restriction enzyme that could cut substrate DNA rapidly (in 5-15 minutes). We have recently made the definition more stringent; Time-Saver qualified enzymes can also safely be used overnight without any negative effects. If your enzyme was Time-Saver qualified, but no longer is, it will still cut DNA in 5-15 minutes. However, it may not be able to be incubated overnight and is

usually accompanied by a note explaining why. A full list of enzymes that digest DNA in 5-15 minutes, as well as Time-Saver qualified enzymes, can be found at www.neb.com.

23. Do I have to set-up digests with Time-Saver qualified enzymes for 5-15 minutes? Can I digest longer?
NEB's Time-Saver enzymes have the benefit of working fast (5-15 minutes), but are also designed and qualified to withstand overnight digestions.

24. How can I search for a restriction enzyme by sequence, overhang or name?
Enzyme Finder, an interactive tool available on our website under "Tools & Resources", can be used to search for restriction enzymes by name, sequence, overhang or type. NEB enzymes include enzyme properties, icons and are displayed as links.

25. How should I stop my restriction digest?
If no further manipulations of the digested DNA are planned, the reaction can be terminated by adding a stop solution. At NEB, we use Orange or Blue Gel Loading Dye (NEB #B7022 or NEB #B702). If further manipulations of the digested DNA are required, heat inactivation (raising the temperature to 65°C or 80°C for 20 minutes) is the simplest method of stopping a reaction. Since this method does not work for all restriction enzymes, refer to the product information for the particular enzyme(s) you are using. Phenol/chloroform extraction is another means of inactivating a restriction enzyme.

26. How stable is a particular restriction enzyme?
All enzymes are assayed for activity every 3-6 months; the expiration date is given on the label attached to each vial of enzyme. After thirty-five years of experience with restriction enzymes, we have found that most are very stable when stored at -20°C in the recommended storage buffer. Exposure to temperatures above -20°C should be minimized whenever possible.

27. What does HF refer to following the name of a restriction enzyme?
HF stands for high fidelity. Many restriction endonucleases are capable of cleaving sequences which are similar but not identical to their defined recognition sequence. This altered or relaxed specificity has been termed star activity or "off target cleavage". HF restriction endonucleases have been engineered to cleave with higher fidelity than the wild type enzyme, hence exhibiting less star activity. Screens using increased glycerol concentration, increased reaction time and high enzyme concentration were used to identify novel enzymes that would offer the highest fidelity over a wide range of conditions.

28. When should I choose the HF version of an enzyme?
The HF version of the enzyme has the same cleavage specificity as the wild type enzyme, and should be chosen if star activity is a concern or if the recommended buffer is more convenient for double digestion or other multi-step protocol. There is no disadvantage to using the HF version and it costs the same as its non-HF counterpart.

29. When is star activity a concern?

Star activity is of concern if extra banding can cause misinterpretation of results in genotyping and mutational analysis procedures or when cloning into a specific site sequence. Experimental design can promote star activity. Small reaction volumes are more likely to contain glycerol concentrations of 5% or greater, a condition known to increase star activity. A 5% glycerol concentration occurs when setting up a double digest in a 20 µl reaction using 1 µl of each enzyme. Overnight digests are more likely to generate star activity.

30. What does it mean to be Time-Saver™ qualified?

Time-Saver qualified enzymes will digest 1µg of substrate DNA in 15 minutes, and be flexible enough to digest overnight without degradation of DNA.

31. What are the advantages of using a RE-Mix Restriction Enzyme Master Mix?

RE-Mix master mixes provide a fast and simple way to perform restriction digests. The master mix contains all the elements necessary to perform restriction digests in 5-15 minutes. Just add DNA and water and incubate the reaction at the recommended temperature.

32. How should I set up a restriction digest?

Most researchers follow the general rule that 10 units of restriction endonuclease is sufficient to overcome variability in DNA source, quantity and purity. Generally, 1 µl of enzyme is added to 1 µg of purified DNA in a final volume of 50 µl of the appropriate 1X NEBuffer followed by incubation for 1 hour at the recommended temperature. If an excess of enzyme is used, the length of incubation can often be decreased to save time. Alternatively, you can productively digest with fewer units of enzyme for up to 16 hours with many restriction enzymes.

To keep glycerol concentration at less than 5% in a reaction, the restriction enzyme, which is supplied in 50% glycerol, should not exceed 10% of the total reaction volume.

An extremely important, yet often overlooked, element of a successful restriction digest is mixing. The reaction must be thoroughly mixed to achieve complete digestion. We recommend gently pipetting the reaction mixture up and down or "flicking" the reaction tube. Follow with a quick ("touch") spin-down in a microcentrifuge. Do not vortex the reaction.

33. I don't see any cleavage after my restriction digest. What factors can interfere with cleavage?

The preparation of DNA to be cleaved should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents, or excessive salts, all of which can interfere with restriction enzyme activity. DNA methylation is also an important element of a restriction digest.

If you are having difficulty cleaving your DNA substrate, we recommend the following control reaction.: Incubate experimental DNA in reaction buffer <u>without</u> restriction enzyme (degradation of DNA indicates contamination in the DNA preparation or reaction buffer) and control DNA (DNA with multiple known sites for the enzyme, e.g. lambda or adenovirus-2 DNA) <u>with</u> restriction enzyme to more accurately judge

whether or not the reaction went to completion. If the control DNA is cleaved and the experimental DNA resists cleavage, the two DNAs can be mixed to determine if an inhibitor is present in the experimental sample. If an inhibitor (often salt, EDTA or phenol) is present, the control DNA will not cut after mixing.

34. How can I generate a restriction enzyme site map for my sequence?
NEBcutter, a computer program for restriction enzyme site mapping is available on the NEB web site under "Tools & Resources". The program accepts sequences retrieved from a local file or GenBank. It also accepts an input sequence pasted into a designated field. When presented with a sequence, NEBcutter will find the largest open reading frames within the sequence and indicate those restriction enzymes that could be used to excise the gene. It also locates the positions of all restriction enzymes that cut only once within the sequence chosen. NEBcutter is also fully aware of the information on the methylation sensitivity of restriction enzymes and alerts a user to overlapping methylation by *dam*, *dcm*, etc.

35. What information is available in the Restriction Enzyme Database (REBASE)?
The Restriction Enzyme Database (REBASE), found under "Tools & Resources" on our web site, is a comprehensive database of information about restriction enzymes and related proteins. It contains published and unpublished references, recognition and cleavage sites, isoschizomers, commercial availability, methylation sensitivity, crystal and sequence data. DNA methyltransferases, homing endonucleases, nicking enzymes, specificity subunits and control proteins are also included. Most recently, putative DNA methyltransferases and restriction enzymes, as predicted from analysis of genomic sequences, have been added.

36. Is extended digestion (incubation times > 1 hour) recommended?
The unit definition of our restriction enzymes is based on a 1 hour incubation (although most will work in 5-15 minutes). Incubation time may be shortened if additional units of restriction enzyme are added to the reaction. Conversely, longer incubation times are often used to allow a reaction to proceed to completion with fewer units of enzyme. This is contingent on how long a particular enzyme can survive (maintain activity) in a reaction. Some enzymes survive for long periods (> 16 hours) while others survive only an hour or less in a reaction. For each restriction enzyme, we report the minimum number of units (1.0, 0.5, 0.25 or 0.13) required to digest 1 µg of substrate DNA in 16 hours. Enzymes that require less than 1 unit can be used at lower concentrations for extended incubation times. Note that DNA substrates are digested at varying rates, the actual number of units required for a complete digestion will change from substrate to substrate. Check individual restriction enzyme information before extending reaction times, as those that exhibit star activity should be used under recommended conditions to inhibit noncanonical cleavage.

37. Do degenerate recognition sites need to be palindromic?
Most Type II restriction enzyme recognition sites are palindromic and include only specified base pairs (i.e., EcoRI recognizes 5'GAATTC). However, some enzymes have degenerate sites, meaning that they contain one or more base pairs that are not specifically defined (i.e., BsrFI recognizes 5' RCCGGY, where R= A or G and Y= C or T). For degenerate enzymes, any base represented by the single letter code may be

present at either location in the recognition site for cleavage to occur. For example, BsrFI recognizes all of the following sequences: 5′ ACCGGC, 5′ ACCGGT, 5′ GCCGGC, and 5′ GCCGGT.