Table of Contents

DEPHOSPHORYLATION

1. Does the DNA need to be purified after a restriction digest and prior to the dephosphorylation step?

2. Does the DNA need to be purified after the dephosphorylation step and prior to the ligation step?

3. Which alkaline phosphatase, CIP, Antarctic or rSAP, works best?

4. Are the alkaline phosphatases active in NEBuffers?

5. Why do some protocols recommend using CIP at 50°C and some recommend 37°C?

PHOSPHORYLATION

6. What factors can cause incomplete phosphorylation when using T4 Polynucleotide Kinase?

7. How can the rate of phosphorylation be improved when using T4 Polynucleotide Kinase?

8. How much substrate can be phosphorylated in a standard reaction?

9. How many units of T4 Polynucleotide Kinase should be used for a typical reaction?

10. How do I inactivate T4 Polynucleotide Kinase?

11. Can I use T4 Polynucleotide Kinase and T4 DNA Ligase in the same reaction buffer?

BLUNTING

12. I've blunted my DNA with the Quick Blunting Kit and now need to dephosphorylate the reaction with an alkaline phosphatase. Do I need to purify the reaction?

13. Does the PCR product need to be purified before blunting with the Quick Blunting Kit? What methods can be used to purify the product?

14. I've digested my DNA and now want to blunt directly with the Quick Blunting Kit without purifying, can I add the blunting reagents directly to the restriction digest?

15. I've accidentally skipped the heat-kill step after the blunting reaction with the Quick Blunting Kit. Will the ligation still work?

16. I've blunted my sonicated gDNA with the Quick Blunting Kit for 15 minutes instead of the recommended 30 minute incubation time, will the reaction still work?

17. What enzymes can be used for removing 3' overhangs and filling in 5' overhangs (3' recessed ends)?

18. Can T4 DNA Polymerase be used in other NEBuffers?

19. What are the main causes of blunting reaction failure using T4 DNA Polymerase?

20. Is T4 DNA Polymerase active at room temperature?

21. Are the nucleotides needed to remove a 3' overhang using T4 DNA Polymerase or DNA Polymerase I, Large (Klenow) Fragment?

22. What are the main causes for blunting reaction failure using DNA Polymerase I, Large (Klenow) Fragment?

23. Can DNA Polymerase I, Large (Klenow) Fragment be used in other NEBuffers?

24. What enzyme can I use to remove 5′ overhangs?

25. Will Mung Bean Nuclease degrade double stranded DNA?

26. What adjustments in protocol are recommended when using Mung Bean Nuclease for blunting?

27. Is Mung Bean Nuclease active in other NEBuffers?

28. Can Mung Bean Nuclease be heat inactivated?

DNA A-TAILING

29. What enzymes can be used for A-Tailing?

30. Does the DNA need to be purified after the PCR amplification and prior to the tailing reaction?

FAQs for DNA End Modification

DEPHOSPHORYLATION

1. **Does the DNA need to be purified after a restriction digest and prior to the dephosphorylation step?**
   If the enzymes used in the restriction digest are heat inactivatable, then a purification step is not needed. If the enzymes used are not heat-inactivatable, then a clean-up step between the restriction digest and the dephosphorylation step is recommended.

2. **Does the DNA need to be purified after the dephosphorylation step and prior to the ligation step?**
   If you use rSAP or Antarctic Phosphatase, which are heat-inactivatable, then a clean-up step is not necessary. If the alkaline phosphatase used is CIP, which is not heat-inactivatable, then a clean-up step is necessary.

3. **Which alkaline phosphatase, CIP, Antarctic or rSAP, works best?**
   rSAP (NEB #M0371) is a superior choice because it combines the advantages of both CIP (NEB #M0290) and Antarctic Phosphatase (NEB #M0289). rSAP has a very high specific activity comparable to CIP, but CIP cannot be completely heat-inactivated. rSAP is heat-inactivated in 5 minutes, as is Antarctic Phosphatase, but rSAP is preferred over Antarctic Phosphatase because it does not require added zinc or any other co-factors. As a result, rSAP can be added directly to restriction enzyme digests. Additionally, after heat-inactivation of rSAP, it is not necessary to purify vector DNA prior to the ligation reaction.

4. **Are the alkaline phosphatases active in NEBuffers?**
   rSAP: rSAP is active in all restriction enzyme NEBuffers 1.1, 2.1, 3.1 and CutSmart™ Buffer, and can be added directly to digested DNA.

   Antarctic Phosphatase: Antarctic phosphatase has a strict requirement for Zinc and has optimal activity at pH 6.0. Antarctic Phosphatase can be used in NEBuffers 1, 2, 3 or 4 as well as the unique NEBuffers for EcoRI and BamHI only when supplemented with 10X Antarctic Phosphatase Reaction Buffer to a final concentration of 1X.

   CIP: CIP will work in NEBuffers 2, 3, or 4, as well as the unique NEBuffer EcoRI. NEBuffer 3 gives optimum activity. Add 2X more CIP if the buffer contains less than 50 mM salt.

5. **Why do some protocols recommend using CIP at 50°C and some recommend 37°C?**

It has been shown that DNA fragments with 3´ extensions or blunt ends are slightly more difficult to dephosphorylate with CIP than those fragments with 5´ extensions. CIP works slightly better on 3´ extensions and blunt-ends at 50°C. We have found that the difference in efficiency is about 20-25%. Some protocols also recommend the addition of more CIP for these difficult ends. For the sake of simplicity, we recommend 37°C and an enzyme concentration of 0.5 unit/µg in a 10 µl reaction for all types of ends.

PHOSPHORYLATION

6. What factors can cause incomplete phosphorylation when using T4 Polynucleotide Kinase?

The most likely cause of incomplete phosphorylation is oxidized DTT in the T4 Polynucleotide Kinase (PNK) reaction buffer (DTT oxidation occurs naturally and is accelerated by repeated freeze/thaw cycles or excessive heating). Use fresh buffer (< 1 year) or add fresh DTT to 5 mM using a 1M stock.

Other factors include:
An excess of salt, phosphate or ammonium ions present: PNK is inhibited by high levels of salt (50% inhibition by 150 mM NaCl), phosphate (50% inhibition by 7 mM phosphate) and ammonium ions (75% inhibited by 7 mM $(NH_4)_2SO_4$). NEB's ThermoPol reaction buffer contains 10 mM $(NH_4)_2SO_4$ which must be removed before performing a kinase reaction. DNA should not be precipitated in the presence of ammonium ions prior to phosphorylation. Drop dialyze or use a commercially available spin column to remove salt from the sample.

7. How can the rate of phosphorylation be improved when using T4 Polynucleotide Kinase?

If working with 5´-recessed ends, heat the reaction mixture for 10 min at 70°C, chill rapidly on ice before adding the ATP (or Ligase buffer containing ATP) and enzyme, then incubate at 37°C.

Add PEG to the reaction. The addition of PEG 8000 to a 5% final concentration (w/v) can improve the results.

Add spermidine. Spermidine will enhance the reactions approximately 20-30%, but it is not used in the unit determination and is not required for full activity.

Use an alternative buffer for the exchange reaction. Higher levels of incorporation in the exchange reaction can be attained by using a buffer containing 50 mM imidazole-Cl (pH 6.4), 10 mM $MgCl_2$, and 5 mM DTT (Sambrook, J. et al. (1989) Molecular Cloning, second edition, pp 10.59-10.67, 11.31-11.33, Cold Spring Harbor Laboratory, Cold Spring Harbor). This buffer is not supplied by NEB.

8. How much substrate can be phosphorylated in a standard reaction?

Up to 350 pmol of 5´ termini for cold phosphorylation and up to 50 pmol for hot phosphorylation.
Note: 1 µg of a 20 mer = 150 pmol of 5´ termini.

Example, to calculate the number of pmol in 1 µg of a 30 mer: 150 pmol X (20 mer/30 mer) = 100 pmol.

9. How many units of T4 Polynucleotide Kinase should be used for a typical reaction?
Typically, 10 units for a cold reaction and 20 units for a hot labeling reaction. Since the [substrate] and the [ATP] are below the K_m in a hot reaction, more units are required. The unit determination is done under ideal conditions where the substrate and ATP concentrations are above their K_m's.

10. How do I inactivate T4 Polynucleotide Kinase?
Incubation for 20 minutes at 65°C completely inactivates T4 Polynucleotide Kinase.

11. Can I use T4 Polynucleotide Kinase and T4 DNA Ligase in the same reaction buffer?
Yes, for cold phosphorylations, but not for hot labeling reactions since the cold ATP in T4 Ligase buffer will interfere with labeling.

BLUNTING

12. I've blunted my DNA with the Quick Blunting Kit and now need to dephosphorylate the reaction with an alkaline phosphatase. Do I need to purify the reaction?
Yes, the reaction will need to be purified prior to dephosphorylation with Antarctic Phosphatase, rSAP, CIP or any other commercially available phosphatase. The reaction can be purified using a commercial purification kit, phenol extraction/ethanol precipitation or gel electrophoresis.

13. Does the PCR product need to be purified before blunting with the Quick Blunting Kit? What methods can be used to purify the product?
Yes, PCR reactions should be purified before blunting. Any PCR column purification will work.

14. I've digested my DNA and now want to blunt directly with the Quick Blunting Kit without purifying, can I add the blunting reagents directly to the restriction digest?
We recommend to heat inactivate the restriction enzyme after a restriction digest and prior to the blunting reaction. If the restriction digest has been carried out with enzymes that can be inactivated by a heat treatment, then after the heat inactivation step, add 0.1 volume of dNTPs and 1 ul of Blunting Enzyme and incubate at room temperature for 15 minutes. If the restriction enzymes used are not inactivated by a heat treatment, then the reaction will need to be purified using a commercial

purification kit, phenol extraction/ethanol precipitation or gel electrophoresis prior to the blunting step.

15. I've accidentally skipped the heat-kill step after the blunting reaction with the Quick Blunting Kit. Will the ligation still work?
Yes, but there may be a higher background since the polynucleotide kinase will still be active and can phosphorylate the vector.

16. I've blunted my sonicated gDNA with the Quick Blunting Kit for 15 minutes instead of the recommended 30 minute incubation time, will the reaction still work?
A slightly lower transformation efficiency is seen with shorter incubations but the reaction will still work. In general, 1.5 X fewer transformants are seen when PCR product or sheared/nebulized DNA is incubated for 15 minutes instead of 30 minutes. If a 15 minute incubation time is desired, increase blunting mix to 2 µl in the reaction.

17. What enzymes can be used for removing 3' overhangs and filling in 5' overhangs (3' recessed ends)?
DNA Polymerase I, Large (Klenow) Fragment (NEB# M0210) and T4 DNA Polymerase are the best choices for this application. DNA Polymerase I, Large (Klenow) Fragment can be used at 25°C or room temperature, but T4 DNA Polymerase must be used at 12°C due to its robust exonuclease. Both work well in a wide variety of buffers.

18. Can T4 DNA Polymerase be used in other NEBuffers?
When supplemented with dNTPs (not included), T4 DNA Polymerase is fully active in NEBuffers 2.1 and CutSmart, as well as NEBuffers 2 and 4 when supplemented with BSA. It is also active in T4 DNA Ligase Reaction Buffer when supplemented with BSA. There is an approximate 25% loss of activity in NEBuffers 1.1 and 3.1 as well as NEBuffers 1 and 3 when supplemented with BSA.

19. What are the main causes of blunting reaction failure using T4 DNA Polymerase?
The following conditions can cause the exonuclease to overwhelm the polymerase activity causing recessed instead of blunt ends:
*Adding too much enzyme
*Incubating for too long
*Incubating at greater than 12°C
*Failure to add nucleotides or the nucleotide level is too low
*Heat inactivating without EDTA

20. Is T4 DNA Polymerase active at room temperature?
We suggest 12°C. The DNA ends "breathe" at higher temperatures allowing the exonuclease to remove nucleotides past blunt.

21. Are the nucleotides needed to remove a 3' overhang using T4 DNA Polymerase or DNA Polymerase I, Large (Klenow) Fragment?

Yes, T4 DNA Polymerase will remove bases past blunt if the nucleotide concentration is less than 100 µM. In the absence of dNTPs DNA Polymerase I, Large (Klenow) Fragment will remove more nucleotides than necessary to blunt.

22. What are the main causes for blunting reaction failure using DNA Polymerase I, Large (Klenow) Fragment?

The following conditions can cause the exonuclease to overwhelm the polymerase activity causing recessed instead of blunt ends:
*Adding too much enzyme
*Incubating for too long
*Incubating at temperatures greater than 25°C
*Failure to add nucleotides or the nucleotide level is too low
*Heat inactivating without EDTA.

23. Can DNA Polymerase I, Large (Klenow) Fragment be used in other NEBuffers?

Yes, Klenow is active in all NEBuffers (1, 1.1, 2, 2.1, 3, 3.1, 4, CutSmart) and T4 DNA Ligase Buffer, but optimal activity is observed in NEBuffer 2. All NEBuffers must be supplemented with dNTPs.

24. What enzyme can I use to remove 5′ overhangs?

Mung Bean nuclease can remove 5′ overhangs. However this enzyme is a very active nuclease, so some optimization may be required to use it in this application.

25. Will Mung Bean Nuclease degrade double stranded DNA?

When used at temperatures higher than the 30°C recommended, or for longer periods, degradation of the substrate DNA will result. Further, since the endonucleic activity is known to occur at 'bubbles' on the DNA helix, which transiently form along the length of the molecule, substrates which are already nicked, or are of high AT content are more susceptible to this endonucleic activity.

26. What adjustments in protocol are recommended when using Mung Bean Nuclease for blunting?

The recommended temperature of incubation is room temperature, since higher temperatures may cause sufficient breathing of the dsDNA ends that the enzyme may degrade some of the dsDNA sequence. The number of units to be used and time incubation may be determined empirically to obtain best results.

27. Is Mung Bean Nuclease active in other NEBuffers?

Mung Bean Nuclease is active in NEBuffers 1, 2, and 4. Longer incubations may be required when using these buffers.

28. Can Mung Bean Nuclease be heat inactivated?

DO NOT ATTEMPT TO HEAT INACTIVATE! Although Mung Bean Nuclease can inactivated by heat, this is not recommended because the DNA begins to "breathe" before the Mung Bean Nuclease is inactivated and undesirable degradation occurs at breathing sections. Purify DNA by phenol/chloroform extraction and ethanol precipitation or spin column purification.

DNA A-TAILING

29. What enzymes can be used for A-Tailing?

Taq DNA Polymerase and Klenow exo-, are good choices for this application.

30. Does the DNA need to be purified after the PCR amplification and prior to the tailing reaction?

If the amplification has been carried out with a high fidelity polymerase, it is essential that the DNA be purified prior to the tailing reaction. The presence of any active high fidelity polymerase will result in the removal of any non-templated nucletiodes added.