FAQs for DNA End Modification: Phosphorylation

1. 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.

2. 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.

3. 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.

4. 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.

5. How do I inactivate T4 Polynucleotide Kinase?

Incubation for 20 minutes at 65°C completely inactivates T4 Polynucleotide Kinase.

6. 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.