LABELING THE 5' ENDS OF DNA WITH T4 POLYNUCLEOTIDE KINASE

Forward Reaction - Using DNA Molecules with Protruding 5' Termini as Templates

1. Mix:

dephosphorylated DNA, 5' ends	1-50 pmoles
10X kinase buffer I	10 µl
[g-^{32}P]ATP (sp. act.=3000 Ci/mmole)	50pmoles(150 µCi)
T4 polynucleotide kinase	10-20 units
H_20	to 50 µl

Incubate at 37^{o}C for 30 minutes.

10X Kinase buffer I
0.5 M Tris Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM DTT
1 mM spermidine
1 mM EDTA

2. Add 2 µl of 0.5 M EDTA. Extract once with phenol/chloroform, and precipitate the DNA with ethanol.

3. Redissolve the DNA in 50 µl of TE (pH 7.9).

4. Separate the labeled DNA from unincorporated [g-^{32}P]ATP by chromatography on or centrifugation through small columns of Sephadex G-50.

Notes

i. 1 mole of 5' ends=0.5 mole of DNA.
For example:
1 mole of linear pBR322 DNA=3.2 X 10^6 g
1 mole of 5' ends of linear pBR322 DNA=1.6 X 10^6 g
1 pmole of 5' ends of linear pBR322 DNA=1.6 µg

ii. Spermidine stimulates incorporation of [g-^{32}P]ATP and inhibits a nuclease present in some preparations of polynucleotide kinase.

iii. The ATP concentration in the reaction should be at least 1 µM.

iv. The dephosphorylated DNA should be rigorously purified by gel electrophoresis or density gradient centrifugation in order to free it from low-molecular-weight nucleic acids. Although such contaminants may make up only a small fraction of

the weight of the nucleic acid in the preparation, they provide a much larger proportion of the 5' ends. Unless steps are taken to remove them, contaminating low-molecular-weight DNAs and RNAs can be the predominant species of nucleic acid that are labeled in polynucleotide kinase reactions.

v. Ammonium ions are strong inhibitors of polynucleotide kinase. Therefore DNA should not be dissolved in or precipitated from buffers containing ammonium salts prior to kinasing.

Forward Reaction - Using DNA Molecules with Blunt Ends or Recessed 5' Termini as Templates

DNA molecules with blunt ends or recessed 5' ends are labeled less efficiently with polynucleotide kinase than molecules with protruding 5' ends. The efficiency of labeling may be improved as follows:

1. Mix:

dephosphorylated DNA, 5' ends		1-50 pmoles
0.2 M Tris Cl (pH 9.5)		
a solution of 10 mM spermidine	4 µl	
1 mM EDTA		
H_20		to 40 µl

--*DENATURING SOLUTION*--
for 1 ml:
200 µl 1 M Tris pH 9.5
10 µl 1M spermidine
2 µl 0.5 M EDTA
788 µl H2O

Heat to 70^{o}C and chill quickly on ice.

2. Add 5 µl of 10X blunt-end kinase buffer.

10X Blunt-end kinase buffer
0.5 M Tris Cl (pH 9.5)
0.1 M $MgCl_2$
50 mM DTT
50% glycerol

--BLUNT-END KINASE BUFFER--
for 1 ml:

250 µl 2 M Tris pH 9.5
100 µl MgCl2
50 µl 1 M DTT
500 µl glycerol
100 µl H2O

3. Add at least 50 pmoles of [g-^{32}P]ATP (sp. act.=3000 Ci/mmole) in a volume of 5 µl.

4. Add 20 units of T4 polynucleotide kinase.

5. Mix and incubate at 37^{o}C for 30 minutes.

6. Add 2 µl of 0.5 M EDTA.

7. Extract once with phenol/chloroform.

8. Add 5 µl of 3 M sodium acetate (pH 5.2) to the aqueous phase and precipitate the DNA with ethanol.

9. Redissolve the DNA in 50µl of TE (pH 7.6).

10. Separate the labeled DNA from unincorporated [g-^{32}P]ATP by chromatography or centrifugation through small columns of Sephadex G50.

Forward Reaction - Using Synthetic Linkers as Templates

1. Dissolve 1 OD_{260} of linkers (as supplied by the manufacturer) in 100 µl of TE (pH 7.6).

2. Mix:

linkers (0.5 mg/ml)	4 µl (~2 µg)
[g-^{32}P]ATP (sp. act. >3000 Ci/mmole)	6.6 pmoles=20 µCi
10X Linker-kinase buffer	1 µl
H_20	to 9 µl

Add 1 µl (10 units) polynucleotide kinase.

10X Linker-kinase buffer
0.7 M Tris Cl (pH 7.6)
0.1 M $MgCl_2$
50 mM DTT

3. Incubate at 37°C for 15 minutes.

4. Add:

10X linker-kinase buffer	1 µl
10 mM ATP	1 µl
H_20	7 µl
polynucleotide kinase	1 µl (10units)

Incubate for a further 30 minutes at 37^{o}C. Store the kinased linkers at -20^{o}C.

5. Check that the kinased linkers can be ligated according to the following steps.

a. Mix:

kinased linkers	1 µl	
10X ligation buffer		1 µl
H_20		7 µl
T4 DNA ligase		1 µl

Incubate at 4°C for 4 hours.

10X Ligation buffer
0.66 M Tris Cl (pH 7.6)
50 mM $MgCl_2$
50 mM DTT
mM ATP

b. Heat to 65°C for 15 minutes (to inactivate ligase).

c. Remove 5 µl of the kinased, ligated linkers to a fresh tube. Add:

H_20		10 µl
10X restriction buffer	2.5 µl	
restriction enzyme		10 units

Incubate at 37°C for 1 hour.

d. Analyze unligated, kinased linkers (2 µl), ligated, kinased linkers (5 µl), and ligated, kinased, restricted linkers (5 µl) by electrophoresis through an 8% acrylamide gel, case and run in 0.5X TBE. Run the gel until the bromophenol blue has migrated half the length of the gel.

e. Cover the gel with Saran Wrap and expose for autoradiography at -70°C.

Procedure from Maniatis cloning manual.

Reference: Maxam and Gilbert. 1980. Methods Enzymol. 65, 499.