Source:

Avian Myeloblastosis Virus particles

Description:

A DNA polymerase which catalyzes the polymerization of DNA, using DNA, RNA or DNA: RNA hybrids and templates. Besides possessing 5 '-3' DNA polymerase activity, the enzyme also possesses some RNase H activity, which breaks apart RNA: DNA hybrids. It is used primarily for the synthesis of first and second strand cDNA and primer extensions. To a lesser extent, it is used for RNA sequencing and the preparation of probes for hybridization.

Unit Definition:

One unit of the enzyme is the amount required to catalyze the incorporation of 1nmol of dTTP into acid-insoluble form in 10 minutes at 37 ^{O}C in a buffer containing:

- 50mM Tris, pH 8.3
- 7mM MgCl2
- 40mM KCl
- 1mM DTT
- 0.1mg/ml BSA
- 0.5mM radiolabeled dTTP
- 35ug/ml rA_{400}:dT_{50}

The enzyme is supplied in a buffer which contains:

- 0.1M Potassium phosphate, pH 7.2
- 0.2% Triton X-100
- 2mM DTT
- 5-0% Glycerol

Each lot is rigorously tested for:

- Activity 10U/ul
- Electrophorectiv purity >95%
- DNase activity <1%
- RNase activity <3%

Function Autoradiographic analysis of synthesized 1.2Kb cDNA

Supplied as a 10U/ul solution. Each 200U vial is packaged with 5x reaction buffer which contains:

- 250mM Tris, pH 8.3
- 50mM MgCl2
- 250mM KCl
- 2.5mM Spermidine
- 50mM DTT

Notes:

The enzyme requires the presence of either Mg^{2+} or Mn^{2+} ions to function. There is also evidence of Mn^{2+} activating DNA endonuclease activity in the enzyme. The enzyme 's activity is also strongly inhibited by the presence of rRNA and tRNA. The optimal reaction temperature is 42 ^{O}C. Inactivation of the enzyme can be accomplished by the addition of EDTA or by heating to 70 ^{O}C for 10 minutes.

Storage:
Freezer (-15 ^{O}C to -20 ^{O}C). For long term storage, maintain at -70 ^{O}C.

Sample Protocol:
I. Conversion of mRNA to cDNA [1]

1. Prepare >10ug poly (A+) mRNA (concentration should be >1ug/ul).
2. Heat mRNA for 5 montues at 65 ^{O}C (use tightly sealed microcentrifuge tube to prevent loss). Place tube on ice.
3. Prepare (in a separate tube) the following buffer:
4. 20ul 5mM dNTP 's (500uM final each)
   40ul 5x buffer (supplied with enzyme)
   10ul 200mM DTT (10mM final)
   20ul 0.5mg/ml oligo (dT) 12-18 (50ug/ml final)
   60ul H_2O
   10ul (10U) RNase inhibitor (50U/ml final)

Mix by vortexing, and briefly centrifuge to concentrate contents in tube. Transfer this buffer to the tube containing the RNA (from step 2.)

5. Add 20ul (200U) AMV reverse Transciptase (1,000 U/ml final). Mix as above.
6. If quantitation of incorporation is desired, see the provided steps below.
7. Incubate tube(s) at room temperature for 5 minutes and then at 42 ^{O}C for 1.5 hr.
8. Add 4ul 0.5M EDTA, pH 8.0 and 200ul buffered phenol. Vortex well and microcentrifuge for 1 minute. Transfer the upper (aquesous) phase to a new tube.
9. To the tube containing the lower organic (phenol) phase, add 100ul TE vortex and microcentrifuge for 1 minute. Remove upper layer as before and combine with original upper aqueous phase. This back extraction of the phenol layer has been shown to increase yield. The lower organic layer can be discarded at this point.
10. To the tube containing the combined aqueous phases, add 1ml phenol:chloroform mixture, vortex and microcentrifuge for 1 minute. Transfer upper aqueous layer to a separate tube. Add 100ul TE to lower organic phase as before, vortex, microcentrifuge, and combine aqueous phases.
11. To the combined aqueous phase, add 125ul 7.5M ammonium acetate and 950ul of 95% ethanol. Place on a dry ice/ethanol bath for 15 minutes, warm to 4 ^{O}C and microcentrifuge for 10 minutes at 4 ^{O}C. This will yield a pellet of nucleic acids, which may be visible as a small yellow/white pellet.
12. Carefully decant the supernatant, add 70% ice-cold ethanol and micrcentrifuge for 3 minutes at 4 ^{O}C. Carefully decant the supernatant and dry the pellet on the bench top under in a vacuum desiccator.

For quantitation of incorporation:

1. Remove 10ul of the mixture prepared in step#4.
2. Add 1ul of [a- ^{32}P] dCTP.
3. Incubate at room temp. For 5 minutes.
4. Incubate at 42 ^{O}C for 1.5 hr.
5. Add 1ul 0.5M EDTA, pH 8.0 and freeze at -20 ^{O}C (if not using immediately)
6. Thaw (if necessary and spot contents onto membrane filter (Total Blot+).
   Wash with ice-cold Trichloroacetic acid and determine bound radioactivity. Use this result, with the activity of the isotope, amount of RNA used and efficiency of b-counter to determine total cDNA synthesized)

II. Conversion of cDNA to double stranded product

1. Re-suspend pellet from first stand synthesis in 284 ul water and add the following: **(Note: order of addition is important!)**

   4ul 5mM dNTPs (50uM final, each)
   80ul 5x buffer (1xfinal – supplierd with enzyme)
   12ul 5mM b-NAD+ (150uM final)
   2ul 10 uCi/ul [a- ^{32}P] dCTP (50uCi/ml final)

2. Vortex and spin briefly to concentrate.
   Next add:
   4ul (4U) RNase H (10u/ml final)
   4ul (20U) E.coli DNA ligase (not T4 DNA ligase) (50 U/ml final)
3. Vortex and spin briefly to concentrate. Incubate 12-16 hrs at 14 ^{O}C.
4. If quantitation of incorporation is desired, remove 10ul to a separate tube and freeze at -20 ^{O}C.
5. Extract the reaction with 400ul buffered phenol. Back extract phenol phase with 200ul TE and combine aqueous phases.
6. Extract with an equal volume of buffered phenol: Chloroform and back extract with 300ul TE, combining aqueous phases as before.
7. Divide the sample into two tubes, and add 150ul 7.5M ammonium acetate and ethanol precipitate and wash as before.

Reference:

1. Ausebel, F., Ed. Current Protocols in Molecular Biology, pp 5.5.2-5.5.5