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Nick translation using DNA polymerase I and α-^{32}P-dCTP

1. Reagents

- Deoxycytidine-5'-triphosphate, α-^{32}P (NEG013H)
- DNA polymerase I (0.6 units/µL)
- DNase I (0.2 units/mL)
- Deoxynucleoside Triphosphate Mixture (100 µM each of dATP, dGTP, dTTP)
- Reaction buffer (200 mM Tris-HCl, 20 mM $MgCl_2$, 20 µg/mL BSA, 50 mM dithiothreitol pH 7.4
- Nick translation stop buffer (250 mM EDTA)
- Purified water
- DNA probe purification column
- Filters, vials, and scintillation cocktail
- Liquid scintillation counter, such as a Tri-Carb

2. Nick translation reaction

One nick-translation reaction – 0.5 µg of DNA/30 µL volume.

Sample DNA Reaction Mix	Volume (µL)
α-^{32}P-dCTP	10 µL
Buffer	5 µL
Cold dNTPs	4 µL
ddH_2O	5 µL
DNA	2 µL (0.5 µg)
DNA polymerase I (0.6 U/µL)	2 µL
DNase I (0.2 U/mL)	2 µL
	30 µL

1. Mix tube gently and centrifuge it briefly
2. Incubate as recommended by the enzyme supplier.
3. Stop the reaction with 5 µL of 250 mM EDTA (40 mM final) or via another method compatible with your purification column.

3. Preparation for analysis

To determine the amount of ^{32}P-dCTP incorporated into the DNA molecule (specific activity or counts per minute per µg), the nick translation reaction is analyzed as follows:

1. Make a 1:10 dilution of the nick translation reaction as follows: 2 µL (0.286 µg of DNA) of the reaction added to 18 µL ddH_2O. Label this vial "1:10C"
2. Make a 1:20 dilution of the nick translation reaction as follows: 5 µL of the 1:10 dilution added to 5 µL ddH_2O. Label this vial "1:20C"
3. Spot 2 µL of each dilution onto 13 mm 0.45 µm filter disks in duplicate (mark which sample is which). Allow these to air dry while you purify the probe. These filters will be used after purification of the probe to determine its specific activity.

4. Probe purification

Purify probe using an appropriate column. The volume used for elution in this protocol was 200 µL.

5. Analysis of probe

1. After probe purification, make a 1:10 dilution of the purified probe (2 µL of purified probe added to 18 µL ddH_2O). Label this "1:10E".
2. Make a 1:20 dilution of the purified probe (5 µL of 1:10 dilution added to 5 µL ddH_2O). Label this "1:20E".
3. Spot 2 µL from each dilution onto 0.45 µm filter disks in duplicate (mark which sample is which). Allow filters to air dry.
4. You should now have the following 8 filter disks:
    a. 2 of the 1:10C filters
    b. 2 of the 1:20C filters
    c. 2 of the 1:10E filters
    d. 2 of the 1:20E filters
5. One filter disk from each duplicate set will be counted directly.
6. One filter disk from each duplicate set will be precipitated in 10% TCA. Label the TCA disks with a T to distinguish them from the direct counts. The filters are run at two dilutions to ensure that the numbers are accurate. One dilution, or the average of two, can be used for computations.
7. TCA precipitation (1 filter from each filter set):
    a. Place one filter disk from each duplicate set into a small beaker of cold 10% TCA which is on ice. Include a blank filter throughout the entire TCA precipitation procedure to be used as an internal control. Allow this to sit on ice for 15 minutes.
    b. Dispose of TCA into a radioactive waste container.
    c. Add more cold 10% TCA. Let sit for 5 minutes on ice.
    d. Dispose of TCA into a radioactive waste container.
    e. Rinse the filters briefly with cold 70% ethanol. Dispose of the ethanol into a radioactive waste container.
    f. Allow filters to dry completely under a lamp.
8. Put all filters, the TCA and non-TCA (direct count) filters in labeled scintillation vials. Add scintillation fluid and count each sample for 1 minute.

Sample counts:	Direct	TCA precipitated
	1:10C- 970088.0	1:10C-T- 715672.0
	1:20C- 456844.0	1:20C-T- 357087.0
	1:10E- 353270.0	1:10E-T- 344641.0
	1:20E- 177078.0	1:20E-T- 172294.0

9. Note: depending upon your scintillation counter, it may be necessary to further dilute these samples in order to count for a full minute.

% incorporation of labeled nucleotides:

TCA precipitation $\frac{\text{1:10C-T}}{\text{1:10C}} = \frac{715672}{970088} \times 100 =$ 73.7% of total isotope added in the reaction was incorporated into the probe

Specific activity:

TCA precipitation $\frac{\text{1:10C-T x 10 (dil. factor)}}{\text{Mass dna/µL}} = \frac{7156720 \text{ cpm/2 µL}}{0.0286 \text{ µg/ 2 µL}} = 2.5 \times 10^8$ cpm/µg

For optimal sensitivity in probe hybridization experiments, all probes should have a specific activity of > or = 1.0 x 10^8 cpm/µg.

Precipitability of Probe:			
1:10E-T	=	344641 =	97.6% of the purified probe is TCA-precipitable, indicating that
1:10E		353270	unincorporated ^{32}P-dCTP has been removed during purification

Probe concentration:		
TCA Precipitate	1:10E-T x 10 (dil factor) =	3 446 410.0 = 1 732 205 cpm/µL
	Volume probe	2 µL

How to use this number. Example: our hybridization protocol asks us to add 10^8 cpm/10 mL of buffer; divide this by 1,723,205 cpm/µL. We need to add 58 µL of the purified probe to the hybridization solution.

6. Appendix

The method for radiolabeling DNA using the nick translation reaction is based on a study reported by Kelly et. al, in 1970, describing replication of DNA by E.coli DNA polymerase I. These investigators report that before chain copying can occur using purified polymerase I, scissions or nicks have to be introduced into a single strand. Pancreatic DNase I is readily used to accomplish nicking *in vitro*. DNA polymerase I requires a terminal nucleotide bearing a 3'-hydroxyl group (primer terminus) from which the DNA chain is extended.

It has been postulated that the mechanism for removing nucleotides in the path of a growing DNA chain is the 5' → 3' exonuclease activity of DNA polymerase I first recognized by Klett et. al, and Deutscher and Kornberg. As nucleotides are removed and added simultaneously, the nick is moved or translated linearly along the DNA chain without net DNA synthesis taking place, the process referred to as nick translation. The overall direction of chain growth is 5' → 3'.

In the presence of labeled deoxynucleoside triphosphates, pre-existing unlabeled nucleotides in the DNA chain are replaced by radioactive duplicates. Specific activity of the DNA product depends on the specific activity of the labeled nucleotide and the extent of nucleotide replacement. The degree of radiolabeling as well as the size of the DNA are critical to the sensitivity of probes used in nucleic acid hybridization experiments.

1. Deutscher, M.P. & Kornberg, A. Enzymatic synthesis of deoxyribonucleic acid. XXIX. Hydrolysis of deoxyribonucleic acid from the 5' terminus by an exonuclease function of deoxyribonucleic acid polymerase. *J. Biol. Chem* **244**, 3029-3037 (1969).
2. Kelly, R.B., Cozzarelli, N.R., Deutscher, M.P., Lehman, I.R. & Kornberg, A. Enzymatic synthesis of deoxyribonucleic acid. XXXII. Replication of duplex deoxyribonucleic acid by polymerase at a single strand break. *J. Biol. Chem* **245**, 39-45 (1970).
3. Klett, R.P., Cerami, A. & Reich, E. Exonuclease VI, a new nuclease activity associated with E. coli DNA polymerase. *Proc. Natl. Acad. Sci. U.S.A* **60**, 943-950 (1968).