PLANT MOLECULAR BIOLOGY REPORTER
Volume 5, Number 4, 1987 Pages 380-386

EXPERIMENTAL PROTOCOLS

A Simplified Method for the Detection of Neomycin Phosphotransferase II Activity in Transformed Plant Tissues

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Introduction

The development of plant cell transformation systems using the Ti plasmid of *Agrobacterium tumefaciens* as a vector has depended upon the expression of chimeric genes carrying dominant selectable markers to identify transformants. One of the widely utilized selectable markers is the bacterial Tn5 neomycin phosphotransferase II (NPT II) enzyme that confers resistance to the antibiotics kanamycin, neomycin, and G-418 (reviewed by Fraley et al., 1986). Initial assays of NPT II activity were performed with bacterial extracts to determine if R-factors conveying resistance to antibiotics were present.

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These assays measured the transfer of radiolabel from a γ^{32}P-ATP cofactor to the antibiotic in a reaction mixture by the binding of the radiolabelled product to phosphocellulose paper (dot blot). Paper blots were counted in a scintillation counter (Davies, 1980; Davies and Smith, 1979; Ozanne et al., 1969).

The first NPT II assays of transformed plant material were done by gel filtration accompanied by paper chromatography (Herrera-Estrella et al., 1983). Reiss et al., (1984) (modified by Schreier et al., 1985) developed a sensitive, quantitative method for the detection of NPT II activity that has since been utilized extensively for the detection of transformed plants (e.g., McCormick et al., 1986). This method employs electrophoretic separation of the enzyme and antibiotic phosphorylation in an overlaid agarose gel, followed by blotting with phosphocellulose paper. With the need to screen greater numbers of transformed plants in recent years, a faster method of screening tissue for NPT II activity using less radiolabel was desirable. The following protocol, based on the original bacterial screening methods, can be used to screen large numbers of transformed plants quickly while reducing the necessary amount of radiolabel. This protocol is simpler than the dot blot protocol described recently by Platt and Yang (1987) which entails using a nitrocellulose "prefilter" to block phosphoprotein adsorption to the phosphocellulose paper and uses a large amount of radiolabel.

In our experience, dilution of γ^{32}P-ATP with "cold" nucleotide increases the amount of radioactive neomycin phosphate formed while reducing non-specific background signals. These effects are likely achieved in several ways. Dilution of the radioactive ATP with the cold nucleotide reduces the amount of isotope available for non-specific protein kinase side reactions, which lowers the background signal often seen in gel assays. Under the same conditions, NPT II appears to be limited only by the amount of ATP available; hence, adding more nucleotide leads to proportionately more product and no loss of signal. The inclusion of higher levels of cold ATP in combination with flouride (inhibitor of phosphatases) helps prevent hydrolysis of neomycin phosphate and ATP by phosphatases. This helps suppress non-specific adsorption to phosphocellulose.

Procedure

1. Grind callus or leaf tissue, frozen in liquid nitrogen, with a mortar and pestle. Add 150 μl to 500 μl of 1× extraction buffer (dependent upon amount of tissue available, ≅1 ml buffer/g tissue) and continue grinding.

2. Transfer the ground sample into an Eppendorf tube and vortex for 45-60 seconds. Maintain sample tubes on ice. Centrifuge 5 minutes at 4°C in a

microcentrifuge and transfer the supernatant to a new tube. Run Bradford protein assays on this material (Bradford, 1976) to determine protein concentrations for enzyme activity quantification.

3. Mix in an Eppendorf tube 15 µl of sample extract and 15 µl of the assay mixture and mix well. Incubate the mixture for 30 minutes at 37°C.

4. Cut an appropriate size piece of Whatman™ P81 (cellulose phosphate) paper for the number of samples to be spotted. Soak the paper in a solution of 20 mM ATP, 100 mM pyrophosphate and dry. This eliminates non-specific binding to the paper and background in the autoradiogram (Ozanne et al., 1969).

5. After incubation is completed, centrifuge the tubes for 5 minutes at 10,000 rpm. This step is especially necessary with plant extract samples that have been frozen for storage and then rethawed. These samples may be gelatinous and label can stick non-specifically to the paper causing false positives.

6. Spot 20 µl of each sample supernatant onto the dried P81 paper allowing sufficient room between samples for spreading (2 cm /spot). Usually non-transformed plant tissue and bacterial extract (extract of *E. coli* with pKC7, Rao and Rogers, 1979) controls are spotted on the paper.

7. After the spots have dried, wash the paper for 2 minutes in 10 mM sodium phosphate buffer, pH 7.5 at 80°C.

8. Wash the blot in the 10 mM phosphate buffer at room temperature for 10 minutes (phosphate buffer helps eliminate background counts). Repeat this procedure 3-5 times. Dispose of all washes as radioactive waste.

9. Dry the blot and expose to x-ray film 16 hrs. to 48 hrs. The positives will show up on the film as dark spots and can be identified by placing the film over the original blot in the correct orientation. For precise quantification, the spots can be cut out of the blot (or duplicate blots can be made in #6 above) and counted on a scintillation counter.

Buffer solutions

Extraction Buffers (can use either A or B, same for gel assays)

A. 2× Stock (Reiss et al., 1984)

		Final Conc.
Glycerol	2 ml	20%
0.5M Tris-HCl, pH 6.8	2.5 ml	125 mM
β-mercaptoethanol	1 ml	10%
SDS	20 mg	0.2%
Final volume in H_2O	10 ml	

B. 2× Stock

		Final Conc.
Glycerol	2 ml	20%
0.5 Tris-HCl, pH 6.8	2.5 ml	
DTT	154 mg	100 mM
10% Na-deoxycholate	2 ml	2%
Final volume in H_2O	10 ml	

Reaction Buffer (Reiss et al., 1984) 5× Stock

		Final Conc.
Tris	20.183 g	335 mM
$MgCl_2$	21.348 g	210 mM
NH_4Cl	53.490 g	2 M

Dissolve in 300 ml H_2O.
Titrate to pH7.1 with 1 M
Maleic Acid (use pure crystalline, need 100-200 ml).
Raise volume to 500 ml with H_2O.

Assay Mixture

Reaction Buffer (above) 1×	4.936 ml
ATP (10 mM stock)	5 µl
γ^{32}P-ATP (10 µCi/µl stock)	1.5 µl
Neomycin (22mM stock)	7 µl
NaF or KF (1M stock)	50 µl
Total Volume	5 ml

Comments

The use of the dot blot assay has made the detection of NPT II activity in transformed plant tissues quicker and easier with much less exposure to radiation. The dot blot assay can be done on prepared extracts in approximately 2-3 hours followed by an overnight exposure of the autoradiogram. In contrast, the gel assay can take 2-3 days to complete. In the gel assay, the number of samples that can be loaded per gel is a limiting factor to total number of samples assayed. Large numbers of samples require the running of more gels. This is not a limiting factor in the dot blot assay. Figure 1 shows that the sensitivity of the dot blot assay is comparable to that of the gel assay. Figure 2 shows that the dot blot assay can be used with a variety of plant species and

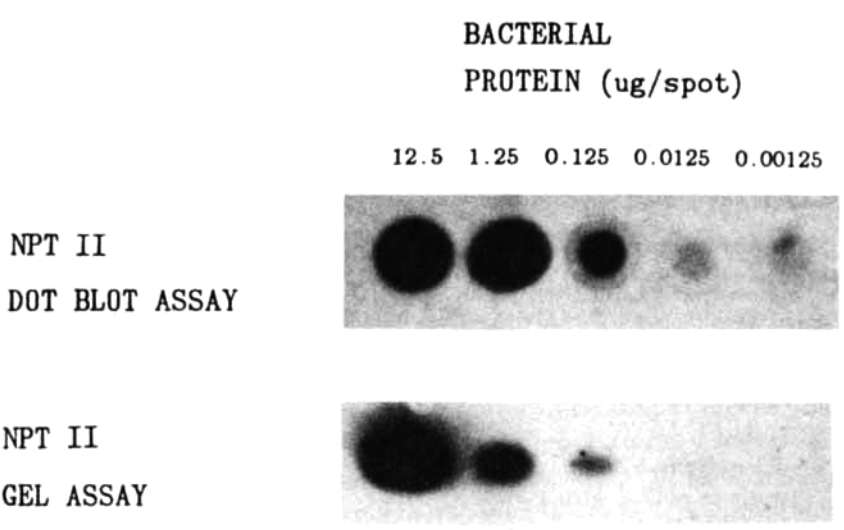

Figure 1. A comparison of the NPT II dot blot assay to the original NPT II gel assay for minimum detectability of the enzyme. Using an extract of *E. coli* cells containing the pKC7 plasmid (Rao and Rogers, 1979), the amount of protein was determined by a Bradford assay (Bradford, 1976) and titrated for use in both NPT II assays. The results show the detectability to be similar in both protocols and there is no detectability lost with the dot blot method. The dot blot autoradiogram exposure was 39 hours and the gel assay autoradiogram exposure was 1.5 hours.

tissue types with the same reliable results of the gel assay. The original NPT II gel assay used aproximately 45 μCi of ^{32}P on a per sample basis (450 μCi of ^{32}P per overlay gel with an average of 10 samples per acrylamide gel). The dot blot assay uses approximately 0.045 μCi per sample. This radiation safety factor is an important benefit.

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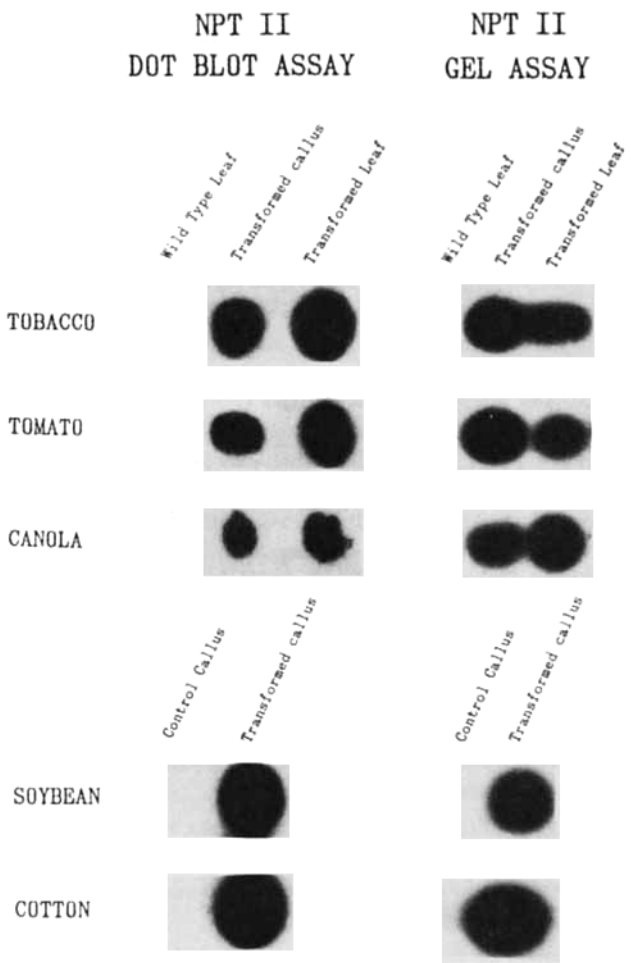

Figure 2. A comparison of the NPT II dot blot assay and the NPT II gel assay in the detection of transformed calli and plants in different plant species. These included tissue samples from tobacco (*Nicotiana tabacum*), tomato (*Lycopersicon esculentum*), canola (*Brassica napus*), soybean (*Glycine max*), and cotton (*Gossypium hirsutum*). The transgenic tissues were transformed with pMON 200 type Ti plasmids in *Agrobacterium tumefaciens* (Fraley et al., 1985). Each tissue sample was extracted and the protein concentration was determined by the Bradford assay (Bradford, 1976). Comparable samples in each of the assays were prepared from the same stock extract. All samples were normalized to obtain 12.5 μg per spot. Autoradiogram exposures for the dot blot assay and gel assay were 16 hours and 40 minutes, respectively.

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