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A Technique for Radiolabeling DNA Restriction Endonuclease Fragments to High Specific Activity[1]

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A technique for conveniently radiolabeling DNA restriction endonuclease fragments to high specific activity is described. DNA fragments are purified from agarose gels directly by ethanol precipitation and are then denatured and labeled with the large fragment of DNA polymerase I, using random oligonucleotides as primers. Over 70% of the precursor triphosphate is routinely incorporated into complementary DNA, and specific activities of over 10^9 dpm/μg of DNA can be obtained using relatively small amounts of precursor. These "oligolabeled" DNA fragments serve as efficient probes in filter hybridization experiments.

KEY WORDS: radiolabeling; DNA fragments; hybridization; Southern transfers; agarose gels; cloned DNA.

Many techniques in molecular biology require the use of DNA probes labeled to high specific activity. Such techniques include Southern (1) and Northern (2) blot hybridizations, colony screening (3), plaque hybridization (4), and *in situ* hybridization (5). Although an entire plasmid or phage genome containing a desired insert can be radiolabeled and used as a probe in such experiments, the signal to noise ratio is often suboptimal under these conditions, especially when the insert is small compared to the size of the vector. The signal to noise ratio is particularly important when detecting rare sequences, such as nonrepeated genes in the DNA of higher eucaryotes.

There are two general methods available to obtain probes with the high signal to noise ratio and specific activity required for such experiments. With M13-derived vectors, systems are available to allow the production of single-strand-specific probes which limit background hybridization (6,7). With plasmid or lambda phage vectors, a desired restriction endonuclease fragment containing the insert can be recovered from an agarose gel (by electroelution, binding to hydroxyapatite or glass, or other methods [reviewed in Refs. (8,9)]) and subsequently radiolabeled by nick translation (10). Radiolabeling only the insert, rather than the entire vector, considerably increases the signal to noise ratio of the resultant probe. This method has proven extremely useful over the past several years, but the techniques for recovering the DNA fragments can be time consuming, and contaminants from the agarose gel sometimes inhibit nick translation (Refs. (11,12) and our unpublished results).

The technique reported here incorporates the advantages of a technique for rapidly recovering DNA fragments from agarose gels together with a reliable way to radiolabel the fragments to very high specific activity ("oligolabeling"). The "oligo-labeling" reaction can also be used to radiolabel DNA fragments eluted from gels by other techniques. The resultant probes are efficient tools for filter hybridizations.

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MATERIALS AND METHODS

Materials

NaI (S-8379), salmon sperm DNA (D-1626), polyvinylpyrrolidone (PVP-10), bovine serum albumin (A-4503), Ficoll-70 (F-2878), Ficoll-400 (F-4375), and agarose (A-6013) were purchased from Sigma Chemical Company, St. Louis, Missouri. Sarkosyl (NL-30) was from ICN Pharmaceuticals, Plainview, New York. Bovine serum albumin (nucleic acid enzyme grade) was purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Maryland. Restriction enzymes and the large fragment of DNA polymerase I were obtained from Bethesda Research Laboratories, Inc., or from New England Biolabs, Inc., Beverly, Massachusetts. [^{32}P]dCTP (PB.10205) and [^{32}P]dATP (PB.10204) were purchased from Amersham Corporation, Arlington Heights, Illinois, and nitrocellulose paper was from Schleicher and Schuell, Keene, New Hampshire. Deoxyribonucleoside triphosphates (2050, 2070, 2080) and oligodeoxyribonucleotides (hexamers, Catalog No. 2166) were purchased from P-L Biochemicals, Inc., Milwaukee, Wisconsin.

The recombinant plasmids chGH800/pBR322 (containing a cDNA insert of human growth hormone (13)), JW101 and JW151 (containing cDNA inserts of alpha-globin and gamma-globin, respectively (14)), and p0vE12 (containing a cDNA insert of chicken ovalbumin (15)) were grown in *Escherichia coli* strain HB101 and the plasmid DNA was purified by standard methods (16,17). Experiments involving recombinant DNA were performed in accordance with the appropriate NIH guidelines.

Solutions Required

Recovery of DNA fragments. Saturated NaI: NaI (900 g) was dissolved in 490 ml of water at 70–80°C. The solution was filtered and stored at room temperature until crystals appeared (usually 12–48 h). The refractive index of the final solution was 1.5000–1.5010. If the solution turned yellow (through oxidation), enough solid sodium sulfite was added to make the solution colorless. The sodium sulfite treatment could be repeated when required (over several months) without harmful effects; TNE: 100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA, pH 7.5. W1: NaI/ethanol/TNE (4/3/2), made fresh on the day of use. W2: Ethanol/TNE (7/3). W3: Ethanol/water (19/1). E: 3.0 mM Tris, 0.01 mM EDTA, pH 7.5.

Labeling of DNA fragments. TM: 250 mM Tris–HCl, 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol, pH 8.0. DTM: 100 uM each of dATP, dGTP, dTTP in TM. OL: 1 mM Tris, 1 mM EDTA, pH 7.5, to which 90 optical density units per milliliter of oligodeoxyribonucleotides was added. LS: 1 M Hepes[2] (pH 6.6)/solution DTM/solution OL (25/25/7), stored at −20°C.

General Methods

Electrophoresis. Plasmid DNA was cleaved with an appropriate restriction endonuclease and the digest was electrophoresed through an agarose slab gel (14.3 cm × 0.3 cm) in a Tris–acetate buffer as described by Sugden *et al.* (18). Electrophoresis was at 50–70 V for 2–4 h. The gel was stained for 15–60 min in 250 ml ethidium bromide (2 μg/ml) in water. The restriction fragment of interest was cut from the gel under ultraviolet illumination (UV Products, San Gabriel, Calif.). Care was taken to excise the fragment cleanly, thereby minimizing the volume of agarose. The excised fragment was sliced into pieces 0.5–1.0 cm long.

Blot hybridization. Nitrocellulose filters containing transferred DNA were prepared by the modification of Southern's (1) procedure described by Wahl *et al.* (19). The prehybridization, hybridization, and washing were done at 60°C according to the protocol described in Ref. (20), except that prehybridization and

[2] Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; Pol I, DNA polymerase I; bp, base pair.

hybridization were done in Seal-N-Save bags (Sears) using 10 ml of hybridization solution. The inclusion of denatured salmon sperm DNA (at a final concentration of 200 μg/ml) was crucial to limit background hybridization. After labeling, probes were centrifuged through Sephadex G-75 (21) in 20 mM NaCl, 20 mM Tris, 2 mM EDTA, 0.2% SDS, pH 7.5, then boiled for 5 min and cooled on ice. To 10 ml of the hybridization buffer was added 2–4 × 10^6 cpm (Cerenkov).

Measurement of incorporation of radioactivity into probes. Incorporation of radioactive precursors was monitored by acid precipitation. Aliquots of the reaction were diluted in 20 mM EDTA, pH 7.5, and a solution of 1 mg/ml bovine serum albumin (Sigma A-4503) in 80% trichloroacetic acid was added, for a final concentration of 12% trichloroacetic acid. After incubation on ice for 15 min, precipitates were collected on fiberglass filters and counted using Cerenkov radiation. The efficiency of Cerenkov radiation counting of ^{32}P-labeled DNA samples acid precipitated in this way was 75% of that obtained by Cerenkov counting of the same samples eluted from the filter into aqueous solution. Incorporated percentages given under Results have been corrected for this reduced efficiency of Cerenkov counting of acid precipitated DNA samples.

Recovery and Labeling of DNA Fragments

Step 1. Solubilization of gel. The gel fragments excised from an agarose gel were placed in a preweighed dust-free 15-ml Corex centrifuge tube. The net weight (in grams) of the gel fragment is called "*W*" in the following recipe: 1 M Tris–HCl, pH 7.5 (0.05 ml × *W*), and saturated NaI (2.1 ml × *W*) were added to the tube, which was covered with Parafilm (American Can Co., Greenwich, Conn.) and turned end over end on a Labquake shaker (Labindustries, Berkeley, Calif.) for 30 min. The tube was vigorously Vortexed for 0.5–1 min and then placed on the shaker for an additional 10–20 min. At this point there should be no visible gel fragments remaining.

Step 2. Recovery of DNA fragments from solubilized gel. Ethanol, 1.575 ml × *W*, was added, and, after mixing, the solution was incubated at room temperature for 1 h. It was then centrifuged at 15°C for 2 h at 12,750 rpm in the SS-34 rotor of a Sorvall centrifuge. The supernatant was decanted and the pellet washed, once with solution W1, twice with W2, and once with W3, as follows: the tube was gently filled all the way to the top (approximately 18 ml) and allowed to stand at room temperature for 0.5–1 min; the liquid was then decanted and the tube lip wiped with a tissue while inverted. All washes were at room temperature. After the W3 wash, the tube was dried for a few minutes under vacuum. One milliliter of solution E was added, and the tube was covered with Handiwrap with the aid of a rubberband. The tube was placed in a water bath at 95–100°C for 2.5 min, Vortexed for 10 s (while hot), and returned to the 95–100°C bath for another 2.5 min. The tube was centrifuged at 1000*g* for 2 min at room temperature to get all the liquid to the bottom of the tube. The eluate was transferred to a cuvette and the optical density measured with a Corey spectrophotometer. DNA yield in micrograms (*Y*) was determined using the formula $Y = (A_{260} - A_{300}) \times 43$. (The "yield" from a mock precipitation, containing 3 g of 1% agarose but no DNA, was less than 0.1 μg when measured in this way.) The eluate was then transferred to a 1.5-ml polypropylene microfuge tube, frozen, and lyophilized. The lyophilate was dissolved in water at 25 μg/ml and stored at −20°C.

Step 3. Labeling of DNA fragments ("oligo-labeling"). A 25-μl reaction mixture was prepared as follows: 11.4 μl of solution LS, 1 μl of nucleic acid enzyme grade BSA (10 mg/ml), and the appropriate volume of water were placed in a 1.5-ml polypropylene microfuge tube on ice. An aliquot of the solution containing the recovered DNA fragment (1–5 μl) was heated to 95–100°C for 2 min in a sealed capillary tube, immediately cooled to 0°C in an ice bath, and added to the reaction mixture.

[^{32}P]dCTP (5–10 μl) and 2.5 units of the large fragment of DNA polymerase I were then added. The components were mixed by gently tapping the tube. The tube was then centrifuged at room temperature in a microfuge for two seconds to get all the liquid to the bottom of the tube, which was then incubated at room temperature.

For preparing probes for filter hybridizations, we routinely used 0.062 μg DNA (2.5 μl) and 50 μCi dCTP (5 μl; 3000 Ci/mmol). The reaction was allowed to proceed for a few hours or overnight (whichever was more convenient).

RESULTS AND DISCUSSION

As described under Materials and Methods, the procedure involves three simple steps: (1) solubilization in sodium iodide of an agarose gel slice containing the desired restriction fragment; (2) selective ethanol precipitation of the fragment from a NaI–agarose solution; and (3) labeling of the fragment using the large fragment of DNA polymerase I with random oligodeoxyribonucleotides as primers.

Steps 1 and 2

Solubilization of agarose gels with chaotropic salts has been a widely used starting point for recovering DNA by several methods (reviewed in Refs. (8,9)). The method used here is a modification of a method described earlier (22). Although the original technique described used a large amount of carrier DNA together with acetone precipitation, we have found that small amounts of DNA can be effectively precipitated without carrier DNA, using ethanol. As little as 0.02 μg/ml of DNA could be effectively precipitated using this technique. The percentage of DNA precipitated seemed to be constant (40–60%) in the range 0.02–0.5 μg/ml. Gels ranging from 0.6 to 1.5% agarose gave equivalent results; higher percentage gels should be diluted with water to an agarose concentration of 1.5% or less. DNA fragments as small as 300 bp were effectively precipitated (smaller fragments were not tested). Although the DNA was not quantitatively precipitated, a large enough proportion was obtained to provide for numerous separate labelings. A numerical example follows: Starting with 50 μg of plasmid DNA, of which the fragment of interest comprised 20% of the total plasmid, the yield of fragment we routinely obtained at the end of the entire procedure was approximately 3 μg. (Total theoretical yield of the fragment is 10 μg; 50% was lost in the ethanol precipitation steps; another 20% was lost during gel electrophoresis.

TABLE 1

EFFECT OF VARIOUS REACTION CONDITIONS ON THE INCORPORATION OF [^{32}P]dCTP INTO DNA USING OLIGO-LABELING[a]

Expt	Conditions	Label incorporated[b] (%)
1	Standard reaction[c]	85
2	Holoenyzme substituted for large fragment of Pol I	22
3	Minus template DNA	1
4	Minus oligodeoxyribonucleotide primers	8
5	Minus Hepes, so that pH of the reaction was 8.0	42
6	Reaction mixture titrated to pH 6.2	18

[a] Oligo-labeling reactions were performed as described under Materials and Methods, using the human gamma-globin cDNA insert from plasmid JW151.

[b] Determined by trichloracetic acid precipitation, as described under Materials and Methods, after a 3-h reaction time.

[c] As described under Materials and Methods, using 0.062 μg DNA and 50 μCi dCTP (3000 Ci/mmol).

subsequent handling of the sample, transfer from one tube to another, etc.) Since each radiolabeling was routinely performed using 0.062 μg of DNA template, the amount of DNA obtained was sufficient for 48 labeling reactions.

Step 3

It was shown over 10 years ago that oligonucleotides can serve as primers for copying single stranded templates by a variety of DNA polymerases (23–27). We tested several parameters in an effort to optimize the labeling obtained with denatured DNA fragments and *E. coli* polymerase I for use as hybridization probes.

The choice of the enzyme was crucial (Table 1). With Pol I holoenzyme, labeling was rapid, but the absolute percentage incorporated was less than with the large fragment, and the product was unstable. This difference probably arose from the presence in the holoenzyme of the 5′–3′ exonuclease activity (28,29). Since the primers used were short (6 bases), the 5′–3′ exonuclease could rapidly degrade the primer and then begin to degrade

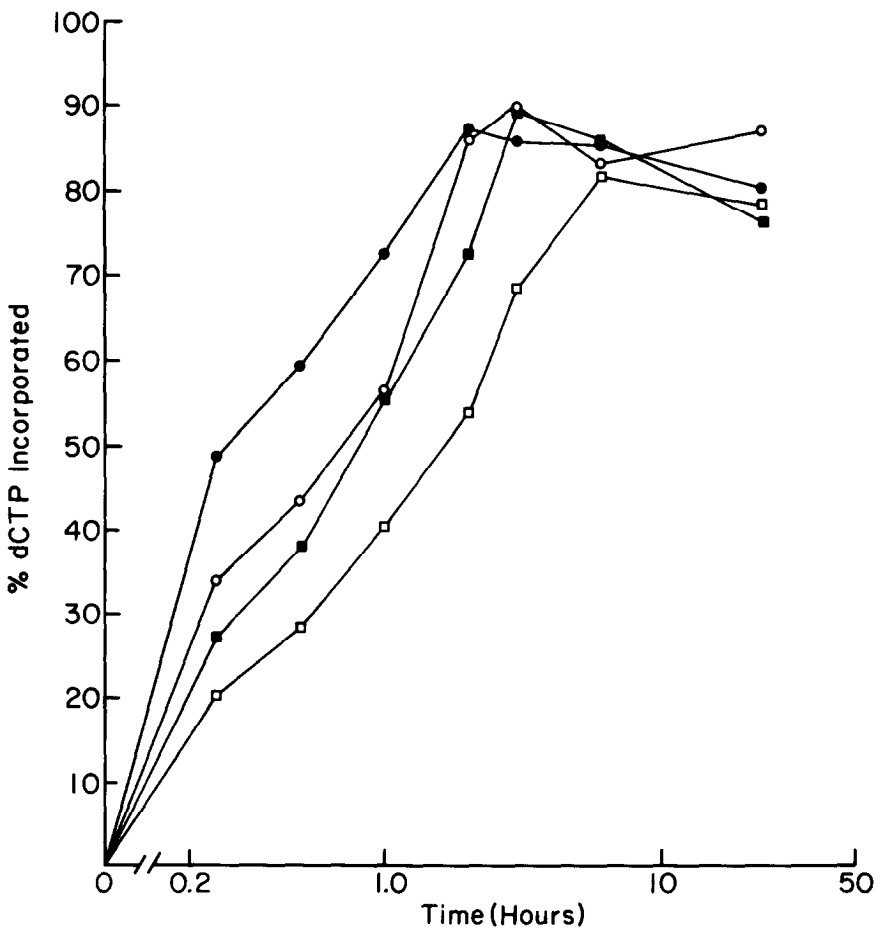

FIG. 1. Effect of precursor concentration on kinetics of [^{32}P]dCTP incorporation into DNA. A 1200-bp DNA restriction fragment containing the gamma-globin cDNA insert of plasmid JW151 was recovered from an agarose gel as described under Materials and Methods. 0.062 μg of this DNA was then "oligo-labeled" as described under Materials and Methods using 0.062 μg DNA template, and [^{32}P]dCTP (3000 Ci/mmol) in the following amounts: (●) 50 μCi; (○) 100 μCi; (■) 150 μCi; (□) 200 μCi.

TABLE 2

SPECIFIC ACTIVITIES OF PROBES OBTAINED USING VARIOUS AMOUNTS OF PRECURSORS AND TEMPLATES

Labeling protocol	Amount of DNA template (μg)	Labeled precursor[b] (μCi)	Label incorporated (% at plateau)[c]	Specific activity of probe (dpm/μg)
A	0.062	dCTP (50)	87	1.2×10^9
B	0.062	dCTP (100)	90	1.9×10^9
C	0.062	dCTP (150)	89	2.4×10^9
D	0.062	dCTP (200)	82	2.7×10^9
E	0.062	dCTP (100) + dATP (100)	84	3.7×10^9
F	0.125	dCTP (50)	88	0.7×10^9
G	0.031	dCTP (50)	74	1.7×10^9

[a] Oligo-labeling reactions were performed as described under Materials and Methods using the human gamma globin cDNA insert from plasmid JW151.

[b] Specific activity of precursor was 3000 Ci/mmol.

[c] Plateau was reached in 3 h or less in protocols A, B, C, E, and F (see Fig. 1). Plateau was reached in 6–12 h with protocols D and G.

the labeled product. An important practical point is that some commercially available "large fragments" are heavily contaminated with the small fragment. Obviously, use of such enzymes will result in reduced levels of incorporation similar to those seen with the holoenzyme.

No label was incorporated into acid insoluble products without template DNA (Table 1). Without added oligonucleotide primers, there was a small amount of incorporation, probably resulting from partial renaturation of the DNA fragments taking place during the incubation, or internal "foldback" sequences in the template serving as primers for the polymerase.

Control of the pH was also important to obtain a stable product during labeling. The reaction performed at pH 6.6 was not as rapid as that performed at pH 8.0, but the extent of incorporation was greater and the product was more stable (Table 1). To our knowledge, the only enzymatic difference between the two pH's is the much reduced 3′–5′ nuclease activity at the lower pH (30). At pH 6.2, the labeling proceeded at a much slower rate than at pH 6.6 (Table 1).

We chose as primers hexamers obtained after DNase I digestion of calf thymus DNA, because they are commercially available and have given reproducible labeling results with a wide variety of template DNA fragments. We have also used unfractionated DNase I digests of calf thymus DNA and salmon sperm DNA with success. However, it was somewhat difficult to standardize the products obtained after DNase I digestion without subsequent fractionation, so we feel that the hexamers are a more reliable source of primer. Although hexamers were smaller than the size of oligomer which provides the highest rate of priming by Pol I (31), hexamers adequately primed the reaction. The size of the probes produced from a 1200-bp restriction fragment template had a number average size of 800 bases.

The reaction rate depended upon both DNA template and radioactive precursor concentrations. A 80–90% incorporation of isotope was obtained after 3 h using 50 μCi of [^{32}P]dCTP (3000 Ci/mmol) and as little as 0.062 μg DNA template (Fig. 1). Longer reaction times were required to achieve maximal specific activity of the product using larger concentrations of precursors (Fig. 1). However, the reaction products were remarkably stable (Fig. 1) and reactions could be done overnight if desired. Because of this stability, there is no need to frequently monitor the course of the reaction. We generally prepare probes using 0.062 μg DNA and 50 to 100

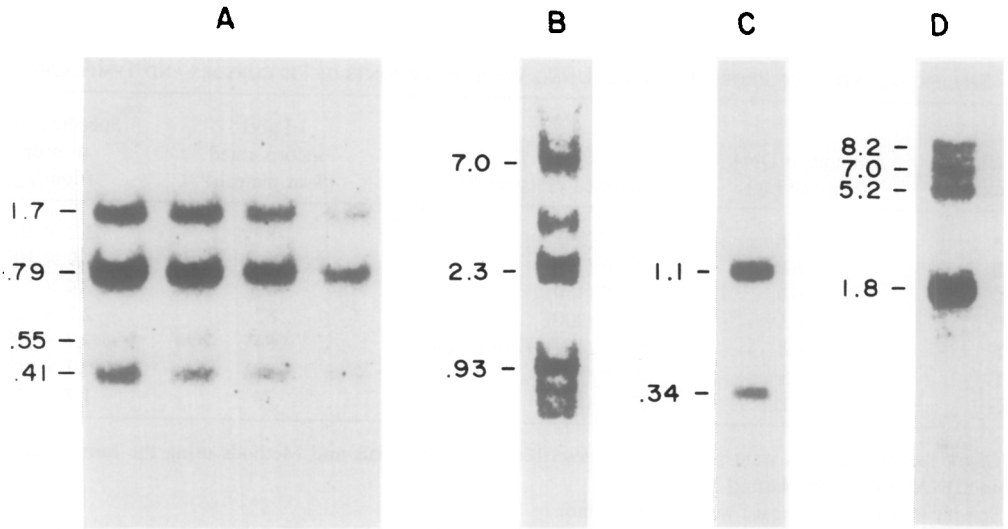

FIG. 2. Autoradiographs of Southern blots hybridized with oligo-labeled DNA fragment probes. The plasmids were cleaved with an appropriate restriction endonuclease, and the fragments containing the cDNA inserts were recovered from agarose gels and oligo-labeled as described under Materials and Methods. The labeled DNAs were then used as hybridization probes against Southern blots: (A) *Hin*fI digests of chicken DNA (2, 4, 6, 8 μg from right to left) using ovalbumin probe; (B) *Hha*I digest of human DNA (5 μg), using human growth hormone probe; (C) *Msp*I digest of human DNA (5 μg), using alpha-globin probe; (D) *Hpa*II digest of human DNA (5 μg), using gamma-globin probe. DNA fragments were labeled using labeling protocol A of Table 2; exposure time was 24–48 h. Autoradiographic exposures were made with Kodak XAR-5 film using Dupont Lightning-Plus intensifying screens at −70°C (32,33). The sizes in kilobase pairs of the major fragments are indicated to the left of each blot.

μCi of [^{32}P]dCTP (3000 Ci/mmol), using a several-hour (or overnight) incubation.

DNA fragments could be labeled to high specific activity with this method for two reasons. First, the majority of the precursor could be incorporated into a DNA product (Fig. 1); second, very small amounts of DNA could serve efficiently as templates (Table 2). Specific activites of the probes ranged from 0.7 $\times 10^9$ to 3.7 $\times 10^9$ dpm/μg, depending upon the amounts of precursor triphosphate and template used (Table 2). The labeled DNA functioned efficiently as probe for filter hybridizations, as seen in the examples in Fig. 2. A 24- to 48-h exposure was adequate to visualize the bands corresponding to single copy genes in complex DNA.

The "oligo-labeling" technique could also be used to label restriction fragments eluted from gels by techniques other than the ethanol precipitation technique described here (data not shown). The Pol I large fragment does not appear to be inhibited by agarose contaminants. These contaminants can inhibit the nick translation reaction, probably by interfering with the DNase I nicking step (Refs. (11,12) and our unpublished results).

In summary, we have described a technique for recovering and radiolabeling restriction endonuclease fragments of DNA. The technique is efficient in terms of time, and has the advantage that a stable labeling product, of very high specific activity, can be produced using small amounts of DNA template and radioactive precursor. We have already used this technique for labeling over a dozen different cloned eucaryotic sequences, including those from insect, avian, rodent, and human cells, and have found it to be both simple and reliable.

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