A Simple Method to Make Better Probes from Short DNA Fragments

*Tamás Kapros, Anthony J. Robertson, and Jakob H. Waterborg**

Abstract

A detailed method is presented for the creation of head-to-tail multimers of short blunt restriction fragments, ligated into a plasmid vector in a single-tube reaction. Random priming of the concatemer insert readily yields hybridization probes of high specificity, unattainable from the short monomer fragments.

Index Entries: Hybridization; probe; directed ligation; digoxigenin; histone; alfalfa.

Study of closely related genes or individual members of gene families and their expression by hybridization is limited by the often short blocks of sequences that are distinct and characteristic. Preparation of effective hybridization probes from short sequence templates, such as the 125-bp 3' UTR of one of the replication-dependent histone H3.1 genes in alfalfa *(1)*, by random priming is difficult. The small size of the template makes repetitive preparation from a plasmid digest inconvenient, the labeling efficiency with digoxigenin-dUTP is low, and heterogeneity in probe length results in reduced sequence specificity and signal strength. We have developed a simple method that, in a one-tube reaction, allows us to ligate small amounts (ng) of short sequences into tandemly arranged multimers and to insert these into a suitable vector. The resulting concatemer insert is readily prepared and yields probes of high specificity and effectiveness.

One can prepare directed head-to-tail sequence multimers by T4 DNA ligation if restriction enzymes in the reaction cut all head-to-head or tail-to-tail ligation products. This prevents creation of sequences with a high potential for secondary structures, which have been shown to be unstable in *E. coli (2)*. The method described here extends this approach based on a combination of distinct restriction sites with complementary termini *(3)* to restriction enzymes that produce blunt termini. This allows for a much

*Address to which all correspondence and reprint requests should be sent: Division of Cell Biology and Biophysics, University of Missouri, Kansas City, MO 64110.

wider combination of possible restriction enzyme pairs. We show an example of how to create readily appropriate fragment ends. Application of the method is described for a very small amount of a DNA fragment obtained by restriction digestion of a plasmid miniprep. We describe the optimized conditions for inclusion of a third blunt-cutter restriction enzyme in the same one-tube reaction. This will prevent vector self-ligation, promote insertion, and obviates the need to purify ligated multimer. Polyethylene glycol at 7% favored all concurrent reactions.

Digestion of plasmid pH3c-1 by restriction enzymes *Hinf*I and *Cla*I produces a 123-bp 3' UTR fragment *(1)* specific for one member of the alfalfa histone H3.1 gene family (*4,5*; Robertson and Waterborg, unpublished observations). Its start at 8bp beyond the stop codon excludes protein coding sequence homology with other histone H3 genes. It ends 2 bp beyond the polyadenylation signal sequence and thus excludes polyA tracts as well as the polyG tail, a result of the original cDNA cloning procedure *(1)*. pBluescript II SK vector (Stratagene, polycloning site: *Sma*I·*Pst*I·*Eco*RI·*Eco*RV·*Hind*III·*Cla*I·*Hinc*II) was chosen to create suitable and distinct blunt restriction termini by insertion at the *Pst*I and *Cla*I sites. The insert was prepared from the *Pst*I delimited insert of pH3c-1 *(1)* by digestion with *Hinf*I (all enzymes: Promega), fill-in of termini with Klenow polymerase, heat inactivation, digestion with *Cla*I and isolation from low-melting agarose *(6)*. The vector was prepared similarly by digestion with *Pst*I, trimming back with T4 DNA polymerase, heat treatment and digestion with *Cla*I *(7)*. Digestion of the ligation product by *Sma*I and *Hinc*II yields the 123-bp 3' UTR extended by only a few base pairs and terminated by two distinct blunt termini.

Direct repeating multimers of this UTR fragment were produced by incubating 100 ng fragment, eluted from agarose *(6)*, in a total volume of 15 µL for 3h at 25°C with 1.5 U T4 DNA ligase, 3 U *Sma*I, 3 U *Hinc*II in 0.5X Promega T4 DNA ligase buffer (15 m*M* Tris-HCl, pH 7.8, 5 m*M* $MgCl_2$, 5 m*M* DTT, and 0.5 m*M* ATP), 0.5X Stratagene Universal buffer (50 m*M* K-acetate, 12.5 m*M* Tris-acetate, pH 7.6, 5 m*M* Mg-acetate, 0.25 m*M* β-mercaptoethanol, 5 µg/mL BSA), and 7 % (w/v) polyethylene glycol 8000 (Sigma). In a parallel reaction, 1 µg pcDNA II (Invitrogen, polycloning site: ··*Xho*I·*Bst*XI·*Eco*RV·*Pst*I·*Eco*RI·*Bst*XI··) was digested in 10 µL for 2h at 37°C with 4 U *Eco*RV in 1X Stratagene Universal buffer. Subsequently, 5 µL of this digest (500 ng pcDNA II), 4 U *Eco*RV, 3 U *Sma*I, 3 U *Hinc*II, and 3 U T4 DNA ligase in 25 µL 0.5X Promega T4 DNA ligase buffer and 0.5X Stratagene Universal buffer was added to the ligation reac-

tion and incubated for a further 2 h at 25°C, 30 min at 37°C, and 10 min at 70°C to allow insert ligation, digest all remaining or created restriction sites, and inactivate all enzymes, respectively.

E. coli DH5α (Gibco-BRL) was transformed with 5 µL reaction mixture, plated on LB agar with carbenicillin (0.1 mg/mL), and supplemented with 0.1 mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 20 mg/mL) and IPTG (isopropyl-thio-β-D-galactoside, 20 mg/mL) for blue-white colony selection *(7)*. Miniprep DNA from white colonies was digested with *Bst*XI, and insert size was assessed by gel electrophoresis. The expected direct repeat organization of inserts with multiple UTR fragments was confirmed by restriction analysis.

UTR multimer insertion products created under identical conditions except for the addition of *Sma*I and *Hinc*II gave very different results. The only UTR multiples obtained, at a very low frequency and as dimers, proved to be direct repeats, suggesting that head-to-head and tail-to-tail ligation products were not stable and maintained. In addition, monomer inserts and empty vectors were seen. No such products were observed with appropriate excess of all restriction enzymes in the reaction.

One clone containing a direct tetramer repeat was used to prepare digoxigenin labeled probes. The complete insert (0.5 kb) was released by *Bst*XI digestion, purified from low melting agarose with Geneclean II (Bio101) and incubated with digoxigenin-dUTP, random hexanucleotide primers, dNTPs, and DNA polymerase as described for the Genius system (Boehringer). Southern analysis with this probe of pH3c-1 DNA, digested with *Pst*I and *Eco*RV, and of plasmid pH3c-11, a cDNA clone for alfalfa histone H3.2, digested with *Pst*I and *Pvu*II *(1,4)*, demonstrated its specificity for the 3' UTR of the histone H3.1 cDNA with a twofold increase in signal strength relative to probe made from the 220-bp 3' UTR containing *Eco*RV-*Pst*I pH3c-1 fragment used before *(4)*. Crosshybridization with homologous and heterologous coding sequences or with vector or polyA fragments was not detected.

The activity of enzymes and thus their relative amounts to be used were assessed in test reactions under various conditions. In establishing optimal enzyme concentrations Promega and Stratagene technical instructions were also used. We found that at 25°C each of the restriction enzymes as well as T4 DNA ligase work efficiently in the buffer combination described above. Optimal enhancement of blunt end ligation by polyethylene glycol under our conditions occurred at 7%. Yield of longer multimers was enhanced by larger excess of vector sequences.

The vector chosen to receive the directed multimer insert should not contain a site for any of the delimiting restriction enzymes. *Eco*RV vector digestion for *Sma*I-*Hinc*II fragments represents one possible combination. Alternatives could be *Sma*I-*Eco*RV delimited multimer fragments inserted in the presence of all three enzymes into *Hinc*II digested pGEM9Zf or pGEM11Zf (Promega) or *Hinc*II-*Eco*RV fragments inserted into *Sma*I linearized pTRXN (USB) or pGEM7Zf (Promega). Other combinations of restriction enzymes that support directed blunt ligation and that do not cut most or all currently used plasmid vectors could be selected from enzymes like *Bal*I, *Hpa*I, *Nru*I, *Pme*I, *Pml*I, *Sna*BI, *Srf*I, *Stu*I, and *Swa*I and their isoschizomers. In case the vector linearizing enzyme could cut the insert, purified linearized and dephosphorylated vector DNA should be added to the multimer ligation reaction in the absence of linearizing enzyme.

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