

Sequence-Independent Method for *in Vitro* Generation of Nested Deletions for Sequencing Large DNA Fragments

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Recently, two PCR-based methods for *in vitro* preparation of templates for sequencing large DNA fragments have been published (1, 2). According to these techniques, the long fragment of interest is digested by several restriction endonucleases in separate reactions. After that, either vectorette PCR (1) or suppression PCR (2) (originally described in Refs. 3 and 4, respectively) is used for the amplification of only one fragment from each restriction. As a result, a set of nested DNA fragments suitable for partial sequencing is generated. Thus, these methods make it possible to avoid time-consuming *in vivo* subcloning stages. However, their weak points are the use of many restriction enzymes and possible absence of recognition sites in some regions of DNA.

Here, we propose a novel application of the recently developed "*in vitro* cloning" technique (5) for preparing suitable templates for all the regions of a long DNA fragment to be sequenced (*in vitro* subcloning). Since our first report on *in vitro* cloning we have introduced two major improvements to this strategy. First, we employed a method of PCR in 96-well plates in the presence of ethidium bromide (EtBr²). After amplification, the wells containing PCR products are colored by EtBr, while the empty wells remain black. This eliminates the need to analyze all 96 wells by gel electrophoresis. It is possible to yield 10–20 individual *in vitro* clones per plate. The second improvement consists of the use of suppression PCR adaptors with identical outer parts. Amplification of individual molecules is then carried out using a single PCR primer. Such amplification is known to be much less prone to nonspecific amplification background problems than the amplification with two primers (6, 7). We have found that the use

of a single primer makes it possible to amplify an individual molecule in a single PCR of 40–45 thermal cycles without any detectable background. This allowed us to exclude the stage of nested PCR from the *in vitro* cloning protocol.

The schematic representation of our method is shown in Fig. 1. In our model experiment, the 5680-bp vector pSELECT-1 (Promega) without insert was used. Six hundred nanograms of this plasmid was incubated with 0.1 ng DNase I (Sigma) in a 20- μ l volume containing freshly prepared 1 $\times$ buffer (50 mM Tris-HCl, pH 7.5, 10 mM MnCl₂, 0.1 mg/ml BSA) at 16°C for various periods (from 1 to 60 min). The reactions were stopped by adding an equal volume of 100 mM EDTA and the DNA was analyzed by agarose gel electrophoresis (Fig. 2A). The sample showing the least smearing and visible remains of supercoiled plasmid (Fig. 2A, lane 2, 1 min of digestion) was taken for further manipulations.

The chosen DNase I-digested DNA sample was phenol-chloroform extracted and ethanol precipitated. Then 100 ng DNA was treated with 5 U Klenow fragment (Amersham) in a 10- μ l volume containing 1 $\times$ ligase buffer (New England BioLabs) and 100 μ M each dNTP. After heat inactivation of Klenow enzyme (70°C for 10 min) the DNA was ligated to a pseudo-double-stranded suppression adaptor (5) (mixture of two oligos: 5'-GTAATACGACTCACTA-TAGGGCTCGAGCGGCCGCCCGGGCAGGT and 5'-ACCTGCCC) bearing the T7 promoter sequence (underlined) on the 5' end of the long oligo. Ligation was carried out at room temperature for 2 h in a 20- μ l volume containing 1 $\times$ ligase buffer, 400 U ligase (New England BioLabs), and 2 μ M adaptor.

The next step is to determine the titer of target molecules in the ligation mixture by means of the amplification of its sequential dilutions (Fig. 2B). A series of sequential 10-fold dilutions of the ligation mixture was prepared in buffer containing 10 mM Hepes (pH 7.5) and 50 mM NaCl. Forty PCR cycles were performed starting with 1 μ l of each dilution. Because of the suppressive PCR effect (4, 5), only fragments flanked at one end by vector's T7 promoter are amplified. The length of each fragment corresponds to the distance between vector's T7 promoter and particular cleavage site. In 10³- to 10⁶-fold dilutions amplified DNA looks like a smear, while in 10⁷- to 10⁸-fold dilutions DNAs differ from each other (sets of bands or a single band), and the last dilution (10⁹-fold) is empty. Each band in 10⁸-fold dilution corresponds to a single DNA molecule taken at the start of PCR. Thus, according to our estimation 10⁸-fold dilution (Fig. 2B) contains approximately 2–3 molecules/ μ l.

To obtain each fragment in a separate tube, 10 μ l of this dilution (20–30 target molecules) was added

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² Abbreviations used: EtBr, ethidium bromide; BSA, bovine serum albumin.

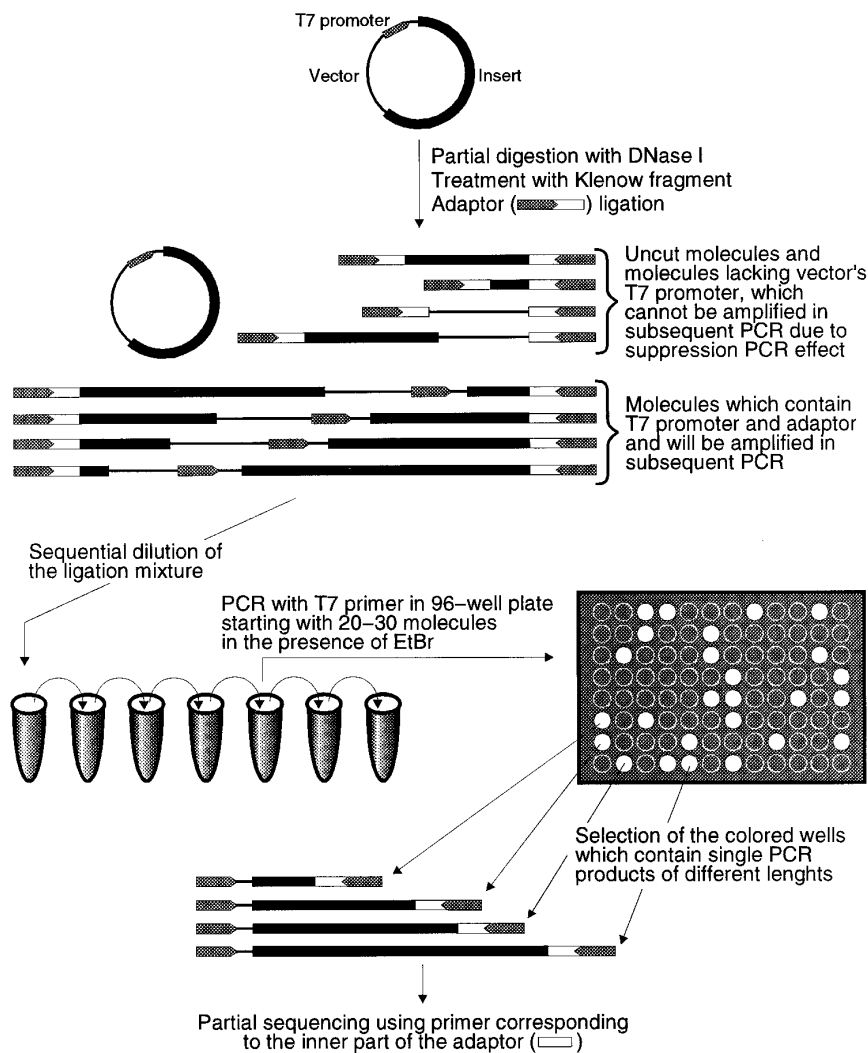


FIG. 1. Scheme of the suggested method. Shaded box represents the T7 promoter (arrow shows the direction of RNA synthesis); open box, the inner part of the suppression adaptor; black box, insert DNA; thin line, vector DNA.

to 1 ml of a PCR mixture containing 2 $\mu\text{g/ml}$ ethidium bromide, which was divided between 96 wells (10 μl per well). Amplification was conducted without mineral oil using an M.J. Research PTC-100 thermal cycler with a heated lid. All PCRs performed contained 40 mM tricine-KOH (pH 9.2 at 22°C), 3.5 mM $\text{Mg}(\text{OAc})_2$, 10 mM KOAc, 75 $\mu\text{g/ml}$ BSA, 200 μM of each dNTP, and 0.5 μM T7 primer (5'-GTAATAC-GACTCACTATAGGGC). Advantage KlenTaq polymerase mix (CLONTECH) was used. PCR cycling parameters were as follows (calculated temperature): 94°C for 10 s, 66°C for 30 s, 72°C for 5 min; 45 cycles. As a result, the 96-well plate containing 24 brightly colored wells was obtained (Fig. 3A), 19 of which contained unique PCR products (*in vitro* clones). We have selected 10 of them whose length ranged from 0.5 to 5 kb with approximately 500-bp intervals (Fig. 3B), representing a set of nested de-

letions. Each PCR product can be partially sequenced using a primer corresponding to the inner part of the adaptor. Thus, this set is sufficient to obtain the sequence of the whole pSELECT-1 by any method capable of determining about 500 bases per single reading.

It should be emphasized that *in vitro* clones are to be sequenced directly by any method suitable for PCR products to avoid misreadings arising from PCR replication errors. Under direct sequencing, even replication errors introduced during the first PCR cycle will not result in erroneous reading—in this case one would observe two bands or peaks of half intensity corresponding to the same nucleotide position. Due to the high fidelity of modern “long and accurate” PCR enzyme systems (11), one is likely to encounter this problem once per each 10 kb sequenced with the help of our method. In such a case the situation can be clarified by

sequencing another *in vitro* clone of similar length. If the replication error occurs at the second cycle or later, the correct band will exceed the erroneous one at least threefold in intensity, so that would not pose a problem for correct base identification.

It may be suggested that the presence of ethidium bromide in PCR might result in greater misreading probability. This possibility may be excluded by adding ethidium bromide to the reactions after PCR is performed.

The length of inserts which may be sequenced using our method is limited only by the ability of the chosen PCR system to amplify long products. Our experiments, with the enzyme system and amplificator used, showed that it is quite easy to obtain the complete collection of subclones covering a 5-kb range with 500-bp intervals, though clones less than 3 kb long were about twice more abundant than the longer ones due to slight DNase I overdigestion. Obtaining the longest subclones may pose a problem if initial DNase I digestion was too extensive. Ideally, the extent of DNase I digestion should be less than one cut per molecule on average.

In contrast to previously described analogous techniques (1, 2), our method does not depend on the distribution of restriction sites in the DNA fragment of interest. Furthermore, the single digestion reaction is needed instead of a treatment with dozen(s) of restriction endonucleases. The proposed method resembles the classical method for generation of nested deletions

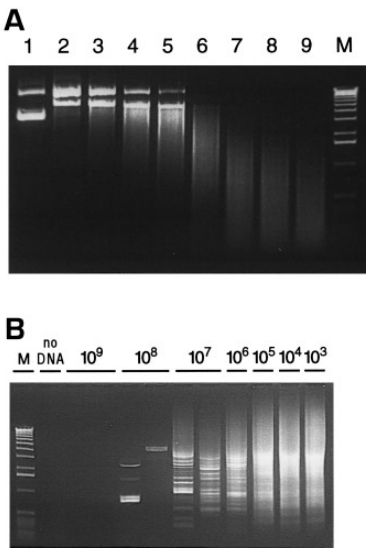


FIG. 2. (A) Selection of the DNA digestion conditions. Lane 1, untreated vector pSELECT-1. Lanes 2–9, incubation of DNase I for 1, 2.5, 5, 10, 20, 30, 40, and 60 min, respectively. Lane M, 1-kb ladder (Gibco BRL). (B) Results of PCR starting from sequential dilutions of adaptor-ligated DNA. The dilution extent of the ligation mixture is indicated above the lanes. Lane M, 1-kb ladder (Gibco BRL).

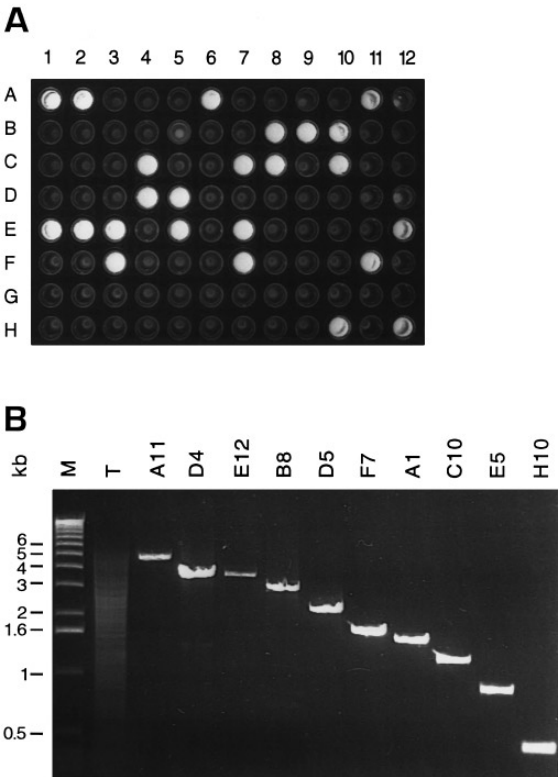


FIG. 3. Results of *in vitro* subcloning. (A) Plate under UV illumination after *in vitro* cloning. (B) Agarose gel electrophoresis of PCR products (0.5 μ l per lane) from some colored wells (numbers of the wells are shown above the lanes). Lane T, the product of amplification of undiluted ligation mixture (15 PCR cycles). Lane M, 1-kb ladder (Gibco BRL).

based on exonuclease III treatment (8), but it relies on *in vitro* procedures only.

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Transfection Assay for Dual Determination of Toxicity and Gene Expression

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Delivery of DNA into eukaryotic cells has become an important technique in molecular biology. Several methods to this effect have been described, such as virus-, polymer-, cationic lipid-, or calcium phosphate-mediated transfer as well as physical techniques. In order to determine the efficiency of transfection or the promoter specificity and strength, reporter genes such as those for β -galactosidase or luciferase are used. Determination of the β -galactosidase expression by colorimetric assays using *o*-nitrophenyl- β -D-galactopyranoside (ONPG,² 1) or chlorophenol red galactopyranoside (CPRG, 2) as substrates is common. Most techniques for the introduction of genes into living cells are accompanied by toxicity as a consequence of disturbance of cellular functions. In order to characterize cell damage or cell proliferation, different colorimetric assays have been described previously (3–10).

All methods used to date measure either toxicity or gene expression after the gene delivery. In the present study we describe a colorimetric assay for the simultaneous determination of the acidic phosphatase activity as a marker of *in vitro* toxicity and the β -galactosidase activity as a marker of transfection efficiency, both carried out in the same plate. This combined assay

allows the measurement of a large number of samples in a 96-well plate within a short time.

To that aim we have developed the following protocol for assaying cells on a 96-well plate: 1 to 3 days after transfection of cells with the lacZ reporter gene, the medium was removed and the cells were washed once with phosphate-buffered saline, pH 7.4. To each well 80 μ l acidic phosphatase assay buffer, containing 10 mM *p*-nitrophenyl phosphate (pNPP), 0.02 M 2-[*N*-morpholino]ethanesulfonic acid (pH 5.5), and 0.1% Triton X-100, was added. The plates were placed in an incubator at 37°C, 5% CO₂ for 15 to 30 min. Thereafter the assay was completed by addition of 20 μ l 0.5 M Tris-HCl (pH 8.0). The optical density was measured at 405 nm (reference filter 690 nm). After calculation of the cell numbers using the *p*-nitrophenol concentration measurements, 150 μ l of β -galactosidase substrate solution (1 mg/ml CPRG, Boehringer Mannheim, Germany, in Hanks' balanced salt solution, HBSS) was added to the same plate and the optical density was measured again at various times at 540 nm (reference 690 nm).

In order to combine the acidic phosphatase assay with the β -galactosidase reporter gene assay, the absorption spectra of the products resulting from the enzyme activity should not overlap. The substrate for the acidic phosphatase assay, i.e., pNPP, does not absorb in the visible range; the reaction product *p*-nitrophenol has an absorption maximum at about 400 nm but does not absorb light at wavelengths higher than 500 nm. The product of the β -galactosidase reaction—chlorophenol red—has a strong absorption band between 540 and 590 nm with a maximum at 580 nm. Therefore the substrate and the reaction product of the acidic phosphatase assay will not interfere with the ensuing β -galactosidase determination. Since the CPRG substrate absorbs at 405 nm and thus would interfere with the acidic phosphatase assay, the latter must be carried out first.

In contrast to other acidic phosphatase assays described in the literature (5, 9, 10), here a buffer with lower ionic strength was used. This does not influence the sensitivity of the method but thereafter allows conveniently a change of the pH value to about 7.8 by addition of stop reagent (0.5 M Tris-HCl, pH 8.0), which is suitable for the ensuing measurement of the β -galactosidase activity.

Similarly to the widely used 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (3), the acidic phosphatase assay in a 96-well plate showed a linear response to cell numbers between 1000 and 30,000 per well of different adherent tumor cell lines (F98 rat glioblastoma; N64 human glioblastoma; MCF-7 and MaTu human mamma carcinoma; CC531 rat colon carcinoma); however, we found that standard deviations from six samples were significantly lower

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² Abbreviations used: ONPG, *o*-nitrophenyl- β -D-galactopyranoside; CPRG, chlorophenol red galactopyranoside; pNPP, *p*-nitrophenyl phosphate; HBSS, Hanks' balanced salt solution; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DOCSPEP, 1,3-dioleoyloxy-2-[*N*-5-carbamoyl-spermine]-propane.