Single-Stranded DNA Production from Phagemids Containing GC-Rich DNA Fragments

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Single-stranded (ss)DNA is a preferred template for dideoxy DNA sequencing. It is most commonly obtained by cloning the studied sequences into an M13 vector (1); however, large inserts tend to be unstable. Plasmid-phage chimeric vectors (phagemids) offer an alternative (7). They replicate as normal plasmids in *E. coli*, but upon infection with a helper phage (5,7) they produce phagemid ssDNA and package it into phage-like particles. ssDNA can be easily recovered from the culture supernatant by a standard procedure (6).

Some helper phage constructs like M13K07 and its derivatives such as VCSM13 were designed to ensure that phagemid ssDNA is packaged and exported preferentially over phage ssDNA (5,7). However, helper phage ssDNA can contaminate the phagemid ssDNA to various extents. This causes ambiguities in sequencing reactions, because some primers (e.g., the commonly used T7 or T3 primers) show weak selective binding to helper phage ssDNA (data not shown). Also, yields of phagemid ssDNA vary considerably.

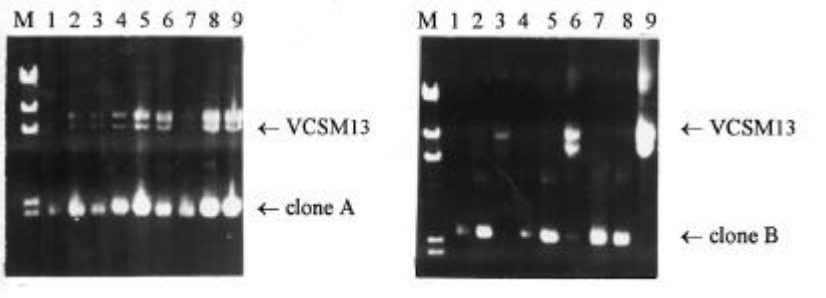
In our work, additional problems were encountered in sequencing *Streptomyces* spp. DNA [GC content of 70%–74% (2)]. Double-strand sequencing of *Streptomyces coelicolor* A3(2) DNA gave poor results. Sequencing of a single-stranded template obtained after VCSM13 helper phage infection was more successful, but the use of recommended methods of phagemid ssDNA production resulted in a variable amount of DNA rescue, often with an abundance of a helper phage ssDNA.

We examined the following factors influencing phagemid ssDNA production: *(i)* initial density of cell cultures, *(ii)* multiplicity of infection (MOI) and *(iii)* insert length of a recombinant.

The procedure was applied to phagemids (clone A and clone B) in which pBluescript® SK(+) vector (Stratagene, La Jolla, CA, USA) contained 0.3- and 1.3-kb inserts, respectively, of *S. coelicolor* A3(2) DNA. The host strain was XL1-Blue *E. coli* (Stratagene) (*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F′*proAB, lacI*^{q}*ZDM15*, Tn*10(tet*^{r}*)*]). Cultures grown 3–4 h were used as a source of exponentially growing cells. Cell concentrations were estimated from the optical density $(OD)_{600}$ of cultures, assuming an OD_{600} of 1.0 approximately equal to 8×10^8 cells/mL (6). The quantity of ssDNA was measured by densitometry using the CS-1 Image Documentation System Version 1.20 (Cybertech, Berlin, Germany) and single-stranded M13mp18 DNA as a standard. All measurements were based on 1.5-mL cultures (a standard centrifuge tube volume for DNA minipreparations).

Contrary to reports in previously discussed protocols (3,6,7), we found exponential growth of the inoculum culture to be a prerequisite. When overnight culture or glycerol stock cultures were used as inoculum, we often observed no growth. The yield of phagemid ssDNA was often very low, and large quantities of helper phage ssDNA were produced.

The most surprising observation was that for phagemid ssDNA production, MOI was much less important than initial cell density at the time of infection. This can be seen in Figure 1, lanes 1, 5 and 9, where samples of the same MOI (1.67 plaque-forming units [pfu]/cell), but different initial cell densities are compared. In lane 1 (density 6×10^5 cells/mL), recombinant ssDNA, although obtained at low efficiency, was



Lane	Initial helper phage titer (pfu/ml)	Initial concentration of bacterial cells (cell/ml)	Clone A			Clone B		
			Quantity of ssDNA (μg/sample)		Ratio*	Quantity of ssDNA (μg/sample)		Ratio*
			phagemid	helper phage		phagemid	helper phage	
1	$1x10^6$	$6x10^5$	0.8	0.0	>>1	0.5	0.0	>>1
2	$1x10^6$	$6x10^6$	2.6	0.6	4.3	1.3	0.3	4.3
3	$1x10^6$	$6x10^7$	1.1	0.6	1.8	0.0	0.6	0.0
4	$1x10^7$	$6x10^5$	2.2	0.9	2.4	0.4	0.2	2.0
5	$1x10^7$	$6x10^6$	4.0	2.0	2.0	1.4	0.3	4.7
6	$1x10^7$	$6x10^7$	1.9	1.6	1.2	0.2	2.5	0.1
7	$1x10^8$	$6x10^5$	1.9	0.5	3.8	1.8	0.4	4.5
8	$1x10^8$	$6x10^6$	3.8	2.9	1.3	1.5	0.3	5.0
9	$1x10^8$	$6x10^7$	3.0	2.3	1.3	0.0	3.8	0.0

*Ratio - ratio of recombinant phagemid ssDNA to helper phage VCSM13 DNA

Figure 1. Production of ssDNA from phagemids containing different fragments of *S. coelicolor* A3(2) DNA relative to production of VCSM13 helper phage ssDNA. Left panel: clone A, 0.3-kb insert; right panel: clone B, 1.3-kb insert. Lanes marked M: *Hin*dIII digest of phage λDNA. Lanes 1–9 are described in the table. Cultures of individual transformants were grown overnight at 37°C and 180 rpm on a shaking platform in 2× TY medium containing 100 mg/L ampicillin and 12.5 mg/L tetracycline. The overnight culture was used to inoculate 2× TY medium containing ampicillin (100 mg/L). After 4 h of growth, 2, 20 or 200 μL of this culture (approximate OD_{600} = 0.570) were used to inoculate 1.5 mL 2× TY medium containing 75 mg/L ampicillin, giving initial densities of 6×10^5, 6×10^6 and 6×10^7 cells/mL, respectively. VCSM13 phage (from a titrated stock) was added at 10^6, 10^7 or 10^8 pfu/mL. After 1 h of incubation at 37°C, kanamycin was added at a final concentration of 75 mg/L, and incubation with vigorous agitation was continued at 37°C for an additional 19 h. Phage particles were precipitated from the culture supernatant, and ssDNA was purified according to a standard procedure (6).

free of helper phage ssDNA. In lane 5 (density 6×10^6 cells/mL), recombinant ssDNA was present in a larger amount but with helper phage ssDNA comprising up to a half of phagemid ssDNA of clone A. As the initial cell density increased to 6×10^7 cells/mL (lane 9), phagemid ssDNA decreased and was negligible in clone B. At the same time, the quantity of helper phage ssDNA increased.

In the recombinant with the longer insert, the proportion of phagemid to helper phage ssDNA changed more drastically with the change of initial cell density, and the quantity of DNA was generally lower.

Dilution of overnight cultures used as inoculum to obtain corresponding cell concentrations reduced the phagemid ssDNA production by 50% or more, while the proportion of helper phage ssDNA remained similar (data not shown).

A similar dependence of phagemid ssDNA rescue on initial cell density was observed with pBluescript SK(+) phagemid vector lacking an insert. However, faster growth of bacteria with the vector alone produced high cell densities and correspondingly larger quantities of both types of DNA.

For comparison, ssDNA production from pBluescript SK(+) containing the 1.1-kb insert of average GC content (52.7%) [human carcinoembryonic antigen cDNA fragment (4)] was examined under the same conditions (data not shown). The yield of phagemid ssDNA decreased with the increase of initial cell concentration. However, no significant contamination with helper phage was found, irrespective of the amount and the age of the inoculum. Therefore, the phagemid ssDNA was of sufficient quality for sequencing even if obtained with low yield.

In summary, examining conditions for ssDNA production from phagemid recombinants with inserts of high GC content yielded DNA templates of reasonable quality for dideoxy DNA sequencing. Only cultures in exponential growth should be used to inoculate cultures infected with helper phage. Initial cell density is the most important factor influencing phagemid ssDNA production and should be relatively low (10^5–10^6 cells/mL). This differs from protocols in procedures previously recommended. Phage MOIs can be varied without strongly influencing phagemid ssDNA production.

REFERENCES

1. **Bankier, A.T. and B.G. Barrell.** 1989. Sequencing single-stranded DNA using the chain-termination method, p. 37-78. *In* C.J. Howe and E.S. Ward (Eds.), Nucleic Acids Sequencing—A Practical Approach. IRL Press, Oxford.
2. **Hutter, R. and T. Eckhardt.** 1988. Genetic manipulation, p. 89-184. *In* M. Goodfellow, S.T. Williams and M. Mordarski (Eds.), Actinomycetes in Biotechnology. Academic Press, London.
3. **Karger, B.D. and J. Jessee.** 1990. Preparation of single-stranded DNA from phagemids. Focus *12*:28-29.
4. **Oikawa, S., H. Nakazato and G. Kosaki.** 1987. Primary structure of human carcinoembryonic antigen (CEA) deduced from cDNA sequence. Biochem. Biophys. Res. Commun. *142*:511-518.
5. **Russel, M., S. Kidd and M. Kelley.** 1986. An improved filamentous phage for generating single-stranded plasmid DNA. Gene *45*:333-338.
6. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
7. **Vieira, J. and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. *153*:3-11.

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