Chimera-Free Cloning of Single DNA Inserts Using “GC Cloning”

David Mead, Rebecca Hochstein, Keynttisha Jefferson, Ronald Godiska, Spencer Hermanson
Lucigen Corporation, Middleton, WI 53562

Abstract

The efficiency of shotgun DNA sequencing depends to a great extent on the quality of the random libraries used. It is important to minimize chimeric inserts to facilitate accurate sequence assembly. Single-insert clones are usually ensured by ligating asymmetric linkers, typically with a BstXI recognition site, to the insert DNA. Subsequently, the excess linkers must be completely removed from the insert DNA before ligation to a vector containing complementary BstXI ends. We have developed a novel “linker free” cloning strategy to eliminate the need for linker addition and removal in constructing high-quality shotgun libraries. It is based on a new GC cloning technology and a unique DNA end blocking chemistry developed at Lucigen. The pSMARTGC vector contains a single 3’-C overhang, which is compatible with the single 3’-G overhang added to blunt ended DNA using PyroPhage DNA polymerase or many other non-proofreading polymerases. The unique combination of a C tailed vector and G tailed insert blocks the ligation of multiple fragments. This protocol is robust and showed five to ten-fold higher yields of clones compared to previous protocols, and is significantly faster than TA cloning. The level of chimerism is ~ 1% in the library, and the background of clones without an insert was <1%. Another important benefit is the ability to construct complex libraries using 10-100 ng insert without compromising the sequence coverage of level of empty background. The procedure is very rapid, as libraries were completely processed in a day. High copy, low copy, single copy and linear vector versions of the GC cloning vectors with chimera free capabilities have been constructed.

Introduction

A common method for cloning PCR products is TA cloning. This method is based upon a 3´A residue being added to the ends of the PCR products by the thermostable DNA polymerase. The 3´A tails allow direct ligation to a vector with 3´T tails.

However, current TA cloning kits have several drawbacks. Available vectors often do not stably maintain fragments that are AT-rich, large (e.g., >8 kb), or contain toxic ORFs; cloning efficiency is low; the background of empty vector clones is problematic; and the kits are relatively expensive.

Importantly, addition of a single 3´ base can also be used to prevent formation of chimeras in random shotgun libraries, replacing the troublesome step of linker ligation. We show here that addition of a 3´G nucleotide to blunt fragments is highly effective both for construction of chimera-free libraries and for direct cloning of PCR products.

Efficient G-tailing of blunt DNA using Taq DNA polymerase.

Taq DNAP was used to add a single 3´nucleotide extension to blunt DNA fragments. The fragments were treated with Taq polymerase and a single dNTP for 30’ at 70º C. They were then self-ligated with T4 DNA ligase (Figure 1, Lanes A, T, C, or G). As controls, the blunt DNAs were not ligated (Lane 0), or they were ligated without treatment (Lane “Blunt”).

The A and G reaction products showed no observable mobility shift, indicating that A- and G-tailing did take place; moreover, it blocked self-ligation of the fragments. The T and C reactions permitted moderate and significant self-ligation, respectively, demonstrating minimal T or C tailing by Taq DNAP. The addition of G tails was unexpected, as previous reports provided no evidence of G tailing in standard PCR conditions.

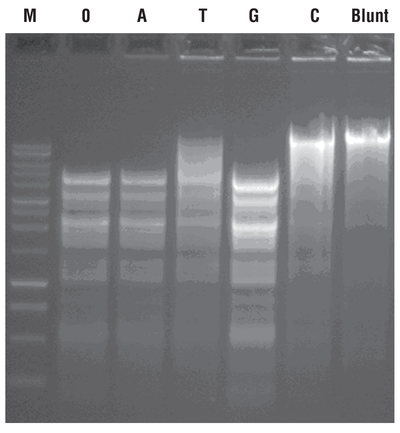

Figure 1. Protection of blunt DNA from self-ligation by A or G tailing using Taq DNA polymerase. Lane M) 1 kb ladder. Lane 0) lambda/*Hinc* II DNA, no treatment. Lanes A-C) lambda/*Hinc* II DNA treated with Taq DNA polymerase in the presence of dATP (A), dTTP (T), dGTP (G), or dCTP (C).After treatment with Taq polymerase, the DNA was ligated by T4 DNA ligase. Lane Blunt) lambda/*Hinc* II DNA, self-ligated.

GC Cloning Technology

GC cloning is analogous to TA or TOPO TA cloning, except that a 3’G tailed product from a non-proofreading polymerase is ligated to a C-tailed vector (Figures 2 and 3). The pSMART® GC vectors provide a single 3´C overhang. The 3´G tails on the inserts are added during PCR or by direct tailing of blunt fragments.

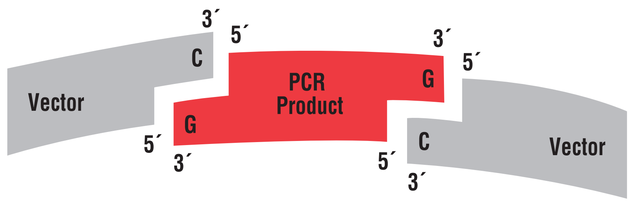

Figure 2. GC Cloning Concept. PCR performed using EconoTaq™ (Lucigen) or any other non-proofreading DNA polymerase adds a single G overhang to the PCR products. Alternately, incubation of blunt-ended DNA with EconoTaq DNA Polymerase adds the 3´-G overhang. Ligation to the complementary C-overhang of pSMARTGC or pGC Blue vectors is fast and highly efficient.

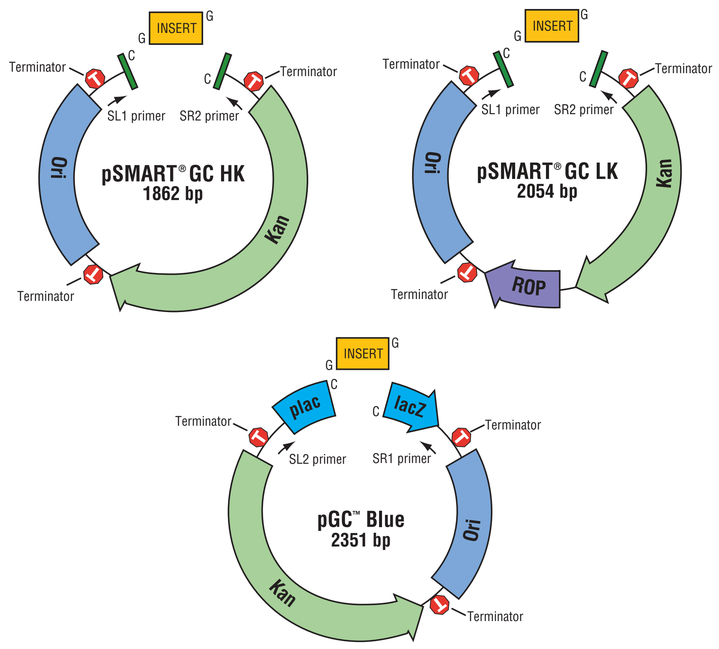

Figure 3. The GC cloning vectors. pSMARTGC HK is high copy number; pSMARTGC LK is low copy number; pGC Blue is high copy. Ori, origin of replication; Kan, Kanamycin resistance gene; plac, lac promoter; *lacZ*, *lacZ* alpha peptide gene; (T), transcriptional terminators.

GC Cloning versus TOPO TA Cloning

A PCR fragment encoding chloramphenicol resistance was used to compare GC Cloning to TOPO TA Cloning. The PCR product was directly ligated into pSMARTGC HK, pGC Blue, and the TOPO TA vector pCR®II-TOPO.

Transformants were selected on kanamycin plates to determine cloning efficiency (Total colony forming units [CFUs], Figure 4A). Colonies were subsequently patched to chloramphenicol plates to determine the number of clones with full-length inserts encoding chloramphenicol resistance (Cm^R, Figure 4B).

GC cloning proved more successful than TOPO TA cloning: the pSMARTGC HK reaction yielded 8-fold more clones with the correct insert, and pGC BLUE reaction gave 3-fold more clones with the correct insert (Fig. 4B).

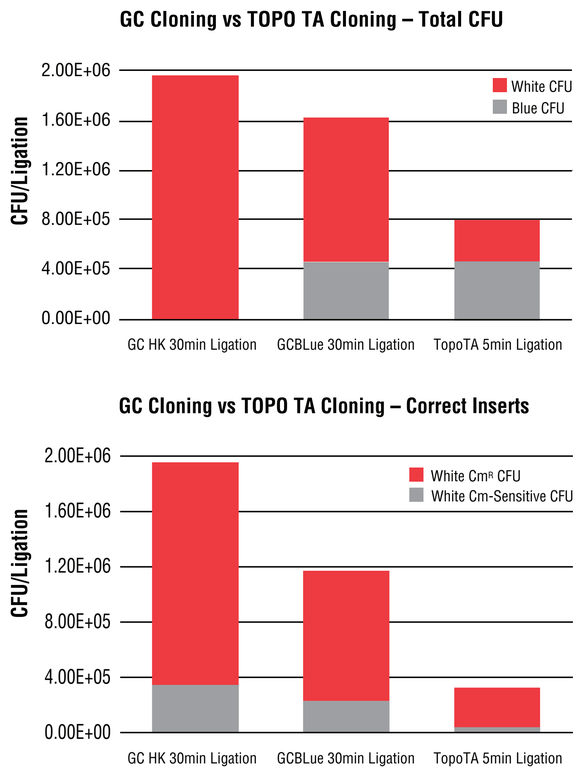

Figure 4. GC cloning vs. TOPO TA cloning.

Panel A) Total CFUs per ligation. Each vector was ligated to a chloramphenicol resistant expression cassette directly from a PCR reaction using manufactures’ protocols. All reactions were transformed into Lucigen’s E. cloni 10G electrocompetent cells, then grown on plates containing kanamycin, IPTG and XGAL. Blue and white CFUs were counted directly.

Panel B) Correct Inserts per ligation. White colonies from the kanamycin plates were patched onto chloramphenicol plates to determine the number of clones with the expected Cm^R inserts. The GC Cloing vectors pSMARTGC HK and pGC BLUE gave 8X and 3X more Cm^R clones, respectively, than the pCRII TOPO TA vector.

Chimera-free Cloning

In random library construction, BstXI linkers are typically ligated to the ends of the insert DNA fragments to prevent formation of concatamers. The linker-ligation process is very time-consuming, requiring overnight ligation and multiple rounds of agarose gel fractionation and column purification. The resulting DNA yields are very low.

In contrast, GC cloning provides a quick and easy alternative for construction of chimera-free libraries, termed the CloneSmart Chimera-free system (patent pending). The kit contains the C-tailed pSMARTGC or pGC Blue vectors, along with PyroPhage™ thermostable phage DNA polymerase to add G tails to target DNAs.

Linker-Ligation Method	GC Cloning Method
• Shear	• Shear
• End-repair • Purify DNA • Ligate BstXI linkers O/N • Remove linkers by column, gel, etc.	• End-repair/block
• Size fractionate • Purify and quantitate gel slice • Ligate to vector • Transform and plate • Blue/white colony screen	• Size fractionate • Purify and quantitate gel slice • Ligate to pSMART vector • Transform and plate • Pick all colonies

Chimera Free Cloning Results

Lambda phage genomic DNA was hydrodynamically sheared to 1-3 kb, end-repaired using the DNA Terminator Kit (Lucigen), and gel purified to select for 2 kb inserts. Half the sample was cloned into the blunt pSMART LCKan vector. The remainder was tailed using Chimera Free Tailing Buffer and Pyrophage Tailing Enzyme and cloned into the pSMARTGC LK vector.

Plasmid DNA from recombinant clones was linearized and run on a gel to determine the size of inserts (Figure 5). Chimeric clones containing multiple inserts were easily recognized by their increased size. The G-tailed reaction produced only 1/102 (1.0%) chimeric clones and 1/102 self-ligated clones. Blunt-ended insert DNA yielded 12/102 chimeric inserts (12%) and 0/102 self-ligated clones.

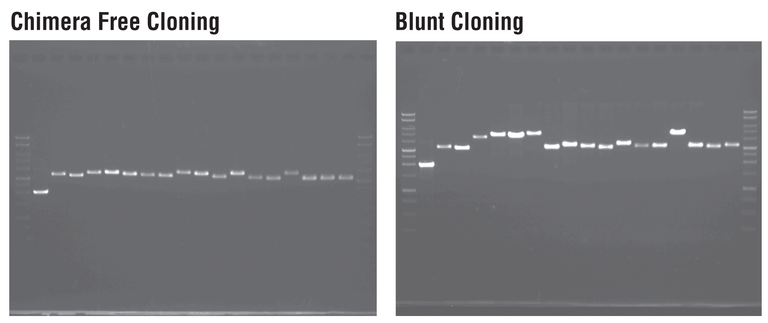

Figure 5. Gel analysis of clones from Chimera-free cloning vs blunt cloning. DNA minipreps from 102 colonies from each transformation were linearized with SmaI to determine the size of inserts.

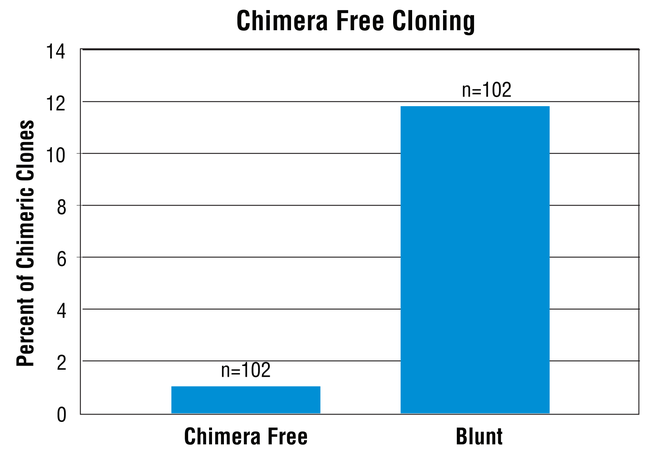

Figure 6. Twenty micrograms of lambda phage genomic DNA was physically sheared to between 1 and 3 kb using a Hydroshear Device. The sheared DNA was ethanol precipitated, resuspended in water, and end-repaired using a DNA Terminator reaction (Lucigen) to generate blunt ends. Half of the sample was set aside for blunt cloning, the remainder was tailed using Chimera Free Tailing Buffer and Pyrophage Tailing Enzyme. The prepared inserts were cloned into Lucigen’s pSMART LCKan vector for blunt cloning and pSMART-GC LCKan vector for Chimera Free cloning. Ligations were transformed, plated and 102 colonies each were grown in overnight cultures for minipreps. All clones were digested with SmaI and electorphoresis was used to determine the size of inserts.

Summary

- **GC cloning provides a fast and efficient method of cloning PCR products or G-tailed fragments.** The cloning efficiency is higher with GC-compatible ends compared to TA ends.
- **GC cloning vectors are based on the powerful, unbiased CloneSmart system.** The CloneSmart vectors lack an indicator gene and possess transcriptional terminators, which provides much greater insert stability in cloning otherwise deliterious sequences.
- **No screening needed.** GC Cloning Vectors are qualified to produce >99% recombinant clones in a typical experiment. In contrast, TA or TOPO TA cloning is much less efficienct, resulting in a large precentage of empty vector that requires additional time and labor for analysis.
- **Chimera-free cloning is faster, easier and more efficient than linker based methods.** No vector preparation or linker purification is needed to assure single-insert cloning, saving considerable time and labor.
- **Beta Testing at two genome centers shows the level of chimerism is 1% using bacterial and BAC shotgun libraries.**

Lucigen® Advanced Products for Molecular Biology

Lucigen Corporation 2120 W. Greenview Dr., Ste 9 Middleton, WI 53562 www.lucigen.com