

Efficient isolation of novel microsatellite loci using the StabyCloning™ System

Delphi Genetics Inc.
Sharpened Tools for Lifescience Discoveries



Technical Note

Technical notes provide customers with innovative applications and clear protocols specifically designed for Delphi Genetics products.

INTRODUCTION



Some molecular genetics applications require the cloning of small inserts and the selection/screening of recombinants. Traditionally, such selection/detection relies on the disruption of a marker gene (like *ccdB* or *lacZ*). Unfortunately, these markers are badly inactivated by small inserts (for example 300 bp-long or less). Moreover, several research groups have pointed out that microsatellite libraries built with topoisomerase I functionalized pCR2.1 plasmids (Invitrogen, Carlsbad, CA, USA) yield a high proportion (up to 90%) of clones generating double sequences (1). This result seems to be due to unwanted cleavages by the *Vaccinia* topoisomerase I. This cloning anomaly can therefore dramatically decrease the experiment efficiency and increase costs (as many sequencing products are useless). The probability of unwanted cleavages depends on the length and sequence of the insert as well as on the vector sequences flanking the insert.

Such corruption of genomic libraries is widespread, probably because of this type of irregular enzymatic activity. One solution to avoid this kind of pitfall is the use of alternative enzymes for promoting the cloning reaction. Clearly, the good old T4 DNA ligase is a perfect alternative as it is not associated to cloning artifacts. When using the StabyCloning™ Kits, blunt-ended DNA fragments are cloned in a positive selection vector using ligase. The whole process (ligation + transformation + plating) takes one hour, i.e., as quick as when using TOPO® cloning. All colonies are independent clones and the insert is correctly oriented. StabyCloning™ is very effective for a large range of insert sizes: from 1 base pair to more than 14000bp.

The proprietary StabyCloning™ technology has been extensively tested and validated in Michel C. Milinkovitch's Lab at the Université Libre de Bruxelles (2,3) where StabyCloning™ has consistently outperformed PCR Cloning Kits from other leading suppliers for the cloning of small DNA fragments such as microsatellite loci.

In the following section, we provide a protocol that has been in use for several years in the aforementioned Lab. The main focus of this protocol is the *de novo* isolation of microsatellite loci that can subsequently be used for pedigree analyses and population/conservation genetic studies. The protocol below is modified from reference (4).

PROTOCOL

- 1) Genomic DNA is first digested with *AluI* and then ligated to a blunt-end adaptor (oligo A: 5'-CTCTTGCTTACGCGTGGACTA-3', oligo B: 5'-PO₄- TAGTCCACGCGTAAGCAAGAGCACA-3') using DNA ligase.
- 2) Fragments ranging from 400 to 1000 bp are

extracted from an agarose gel after electrophoresis.

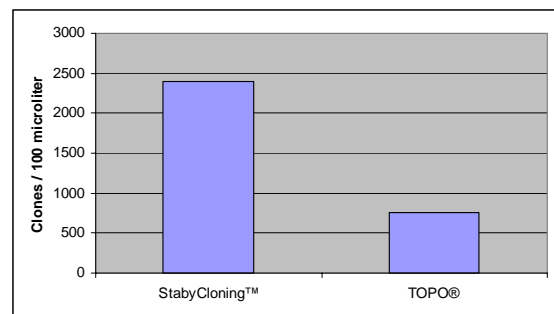
- 3) These fragments are subsequently purified, denatured, and hybridized with a mixture of biotinylated oligonucleotide probes containing microsatellite repeats.
- 4) Fragments hybridizing with the probes are recovered with streptavidin-coated beads.
- 5) Those fragments are amplified using a modified version of OligoB (incorporating the *StabyCloning* 14 bp tail CCT-TCG-CCG-ACT-GA in 5')
- 6) The PCR products are ligated to Delphi Genetics pSTC1.3 no-background vector using T4 DNA Ligase included in the *StabyCloning*[™] Kit, then transformed into competent cells (CYS21, Delphi Genetics) and plated.
- 7) Recombinant molecules are isolated from clones using standard plasmid extraction methods.
- 8) Sequences of inserted genomic fragments are obtained by cycle sequencing followed by electrophoresis (for example, on an ABI 3730 sequencer; Applied Biosystems), following the manufacturer's instructions.
- 9) Sequences are fed into sequence analysis programs (e.g., ref. 5) that identify microsatellite repeats and design optimal primers flanking each repeated sequence.

BENEFITS OF THE *StabyCloning*[™] KIT FOR THE ISOLATION OF MICROSATTELITES

- **Efficient cloning of small inserts such as microsatellite loci:** the positive selection process is based on gene activation rather than gene disruption. Hence, only clones bearing the desired construct are selected. It is well known that the selection of recombinants with small cloned inserts can be troublesome because disruption of a selection gene is not always efficient with small inserts. This is the case for positive selection using *ccdB* only (e.g. "Zero Background" vectors from Invitrogen) or the Blue/White selection system based on the insertional disruption of *lacZ*. Therefore the proportion of clones bearing the correct insert is increased when using the *StabyCloning*[™] technology.
- **No deletions:** The use of DNA Ligase in

the *StabyCloning*[™] kit avoids any topoisomerase-associated cloning anomalies. Sequence reads are therefore consistently long and void of double sequences.

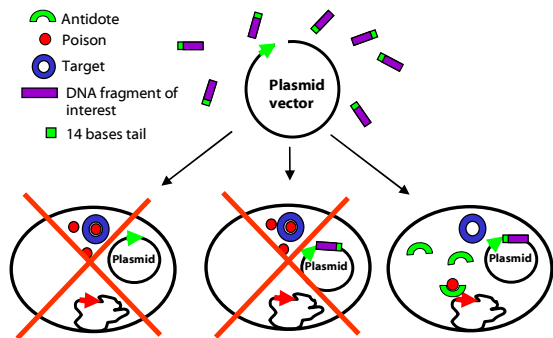
- **Speed of the reaction:** The whole procedure (ligation + transformation + plating) takes 1 hour. No antibiotic resistance gene expression is required, therefore the reaction can be directly plated and all the colonies on the plate are independent clones.
- **Higher number of clones** (see figure below)



100 µl transformed bacteria have been plated in each case. The insert size was 500 bp. Using *StabyCloning*[™], 2400 independent clones are obtained, i.e., three times more than when using TOPO[®] cloning (Invitrogen).

ABOUT THE *StabyCloning*[™] SYSTEM

The *StabyCloning*[™] Kit is designed for the rapid, precise, and efficient cloning of PCR products generated using proofreading polymerases. The system uses a proprietary positive selection strategy based on the plasmid toxin/antitoxin module *ccd*. The complete cloning procedure is performed within one hour (including plating). *StabyCloning*[™] relies on the activation of an antitoxin gene rather than on the disruption of a toxin gene. DelphiGenetics' new selection scheme exhibits therefore unique advantages over other selection approaches. When a sequence of 14 base pairs (encoding the last 4 codons and the stop codon of the antidote gene) is added to the 5'-end of the DNA fragment to be cloned, the fusion of this sequence with the truncated antitoxin gene restores an active gene encoding a protein (CcdA) able to counteract the action of the toxin located in the chromosome of the host bacteria. The 14-bp sequence is incorporated into the target DNA fragment by using a modified PCR primer (the appropriate 14-base tail is added at the 5'-end of one of the two PCR primers).



As illustrated in the figure, this process allows (i) the selection of recombinant plasmids that incorporate the fragment of interest (non-recombinant plasmids contain an inactive, truncated, *ccdA** gene, hence the host bacteria cell dies), and (ii) orientation of the fragment of interest (only one of the two possible orientations will restore an active, non-truncated, *ccdA* gene). Moreover, restoration of the *ccdA* gene stabilizes the plasmid into the bacterial population without the need for antibiotics (see www.delphigenetics.com for additional information on the advantages of this stabilization system).

KIT COMPONENTS

- pSTC1.3 blunt ended vector
- CYS21 bacteria
- T4 DNA ligase and reaction buffer
- Regeneration medium
- Forward and Reverse sequencing primers
- Primer Control mix

StabyCloning™ Products are available with either electro- or chemically- competent cells.

ORDER REFERENCES

- STC1-10: electro-competent cells, 10 reactions
- STC1-12: chemically-competent cells, 10 reactions
- STC1-20: electro-competent cells, 20 reactions
- STC1-22: chemically-competent cells, 20 reactions

RELATED PRODUCTS

Please visit www.delphigenetics.com for additional information on our DNA engineering and protein expression products

REFERENCES

- (1) Forbes A. et al.; Resolving a DNA sequence artefact associated with topoisomerase I generated clones in the plasmid pCR 2.1. *BioTechniques* 42:458-462 (2007).
- (2) Tzika A. C. et al.; Molecular Genetic Analysis of a Captive-Breeding Program: The Vulnerable Endemic Jamaican Yellow Boa (submitted to *Conservation Genetics*) 2008.
- (3) Tzika A. C. et al. Population structure of an endemic vulnerable species, the Jamaican boa (*Epicrates subflavus*). *Molecular Ecology* 17, 533-544 (2008).
- (4) Bloor et al.; Microsatellite Libraries by Enrichment. University of Liverpool.
<http://www.genomics.liv.ac.uk/animal/MICROSAT.PDF>
- (5) <http://ueg.ulb.ac.be/oligofactory/index.jsp>

LEGAL

StabyCloning™ is a trademark of Delphi Genetics.

TOPO® is a registered Trademark of Invitrogen Corporation.

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