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Blocking oligonucleotides improve sequencing through inverted repeats

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Gateway™ cloning is a popular method for genome analysis and protein expression (for a compilation of publications using Gateway, see <http://www.ncifcrf.gov/rtp/pel/pubs.asp>). In Gateway cloning, bacteriophage λ recombination sites, called *att* sites, are first added to the DNA sequence of

interest, typically during PCR. The presence of these flanking *att* sites allows the DNA of interest to be cloned and subsequently subcloned into expression vectors entirely by in vitro site-specific recombination reactions (1). The recombination reactions are efficient compared to those based on ligases, and powerful

Figure 1. Sequencing through inverted repeats. Gateway entry clones contain the gene (or DNA of interest) between inverted repeats of 100-bp *attL* sites (95 bp inverted repeats, 3 mismatches). (A) During sequencing, the duplex DNA is denatured to single strands (bottom strand shown from 3' to 5'). (B) As the size of the gene decreases to less than 1 kb, the inverted repeats on each strand pair to each other more efficiently. (C) Subsequent annealing of the sequencing oligonucleotide (F, forward) results in the extension of the sequencing reaction being stopped at the double-stranded region of the hairpin. (D) Formation of the hairpin can be inhibited by the addition of a blocking oligonucleotide that is complementary to the distal repeat *attL2*, which allows (E) the annealing and extension of the sequencing primer to proceed. Note that because *attL1* and *attL2* are almost identical, the blocking oligonucleotide also anneals to *attL1*, thus the same blocking oligonucleotide facilitates sequencing from both forward and reverse primers.

Table 1. Sequences of attL1 and attL2 in Entry Clones Derived from Recombination Between pDonr223 and PCR Products Containing attB1.1 and 2.1

Name	Sequence
attL1	5'-AGCCTGCTTTtTTGTACAAAGTTGGCATTATAaAAAGCATTGCTcATCAATTTGTTGCAAC- GAACAGGTCACTATCAGTCAAATAAAATCATTATTTG-3'
attL2	5'-ACCCAGCTTTcTTGTACAAAGTTGGCATTATAAgAAAGCATTGCTtATCAATTTGTTGCAAC- GAACAGGTCACTATCAGTCAAATAAAATCATTATTTG-3'
Blocking oligonucleotide 521 (40-mer)	5'-GTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCreVA-5'
Blocking oligonucleotide 522 (40-mer)	5'-CGAACAGGTCACTATCAGTCAAATAAAATCATTATTTGrevA-5'
Blocking oligonucleotide 264 (60-mer)	5'-GGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAreVA-5'

The inverted repeats are underlined, and the mismatches between the inverted repeats are bold and lowercase.

selections and uniform conditions allow for highly efficient and automated cloning. For example, 12,000 predicted open reading frames of *Caenorhabditis elegans* have been amplified, and the re-

sulting PCR products have been cloned by this method (2). Gateway clones of PCR products are called entry clones, and the inserts in these clones are typically sequenced

to detect and discard plasmids that have mutations that resulted from primer synthesis or PCR errors. In the process of sequencing several hundred inserts in entry clones, we have noticed that sequence read lengths drop as the sizes of the genes decrease. With inserts smaller than approximately 500 bp, the quality of sequence data obtained under standard sequencing conditions is poor. This is presumably due to the presence of inverted repeats of 100-bp attL recombination sites that flank DNA segments in entry clones. Because each sequencing cycle produces single-stranded template DNA, the nearly identical attLs can hybridize to form a 15-bp hairpin. If the hairpin forms before the sequencing primer can be extended through the attL, then sequence is not obtained for that cycle (Figure 1). Here we describe a simple way to minimize this difficulty.

pDonr223 is identical to pDonr221 (Invitrogen, Carlsbad, CA, USA), except that it confers spectinomycin resistance instead of kanamycin resistance. Blocking, sequencing, and PCR oligonucleotides were purchased from Qiagen (Valencia, CA, USA) and desalted but not otherwise purified. The blocking oligonucleotides contained their 3'-terminal base in reverse orientation (3'→3' linkage) so that both ends of the blocking oligonucleotides were 5' OH. Modified attB sequences were used for improved cloning efficiency (3): attB1.1 was added to 5' primers 5'-GGGGCAACTTTGTACAAAAAGTTG-3', and attB2.1 was added to 3' primers 5'-GGGGA-CAACTTTGTACAAGAAAGTTG-3'.

Figure 2. Improved sequencing of a 390-bp insert entry clone by the addition of blocking oligonucleotides. Scans A–E were obtained using the forward sequencing primer, and scans F–G were obtained using the reverse sequencing primer. The black bar shows the locations of (scans A–E) attL1 or (scans F–G) attL2. The blocking oligonucleotides hybridize to the distal attL site on each strand (attL2 on the bottom strand using the forward sequencing primer, and attL1 on the top strand using the reverse sequencing primer). Fwd, forward; Rev, reverse.

Table 2. Effect of Blocking Oligonucleotide 264 (60-mer) on Phred Scores, Forward and Reverse Primers, and with Clones of Various Sizes

Insert Size (bp)	Sequencing Primer	Blocking Oligonucleotide	Phred Scores ^a
		264 Concentration (μM)	
390	Forward	0	5
		1	201
		5	316
		10	351
		20	484
	Reverse	0	152
425	Forward	25	460
		0	69
	Reverse	25	378
		0	94
600	Forward	25	458
		0	120
		5	404
		10	630
		25	663
	Reverse	50	664
		0	0
		5	89
		10	152
		25	155
638	Forward	50	460
		0	66
	Reverse	25	656
		0	237
		25	587
719	Forward	0	319
		n.d.	n.d.
	Reverse	0	167
		25	518
887	Forward	0	266
		25	642
	Reverse	0	56
		n.d.	n.d.
		n.d., not determined	
^a Sequence read lengths (8).			

Sequencing primers were forward, 5'-CCCAGTCACGACGTTGTA-AAACG-3', and reverse, 5'-GTAACAT-CAGAGATTTTGAGACAC-3'. Table 1 shows the sequences of *attL1* and *attL2* in entry clones derived from recombination between pDonr223 and PCR products containing *attB1.1* and 2.1.

The *attB1.1* and *attB2.1* sequences were added to the 5' ends of amplification primers in the appropriate reading

frame (1). PCR was performed by standard techniques. Amplification products were purified with QIAquickTM (Qiagen) PCR kits and then cloned into pDonr223 with Gateway BP ClonaseTM, according to the manufacturer's instructions (Invitrogen). *Escherichia coli* (DH5α) colonies were cultured overnight in CIRCLE-GROW[®] (Qbiogene, Carlsbad, CA, USA), and sequencing template plasmid was purified from 1 mL of cells by manual alkaline sodium dodecyl sulfate (SDS) extraction and dissolved to 150–200 ng/μL in TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) containing 50 μg/mL RNase A. Sequencing reactions comprising 6 μL mini-prep plasmid DNA, 3.2 pmol sequencing primer, and 1 μL blocking oligonucleotide (for concentrations, see below) were brought to a final volume of 14 μL. An aliquot (3.85 μL) of this mixture was combined with 2.2 μL ABI PRISM[®] BigDyeTM terminators (version 1; Applied Biosystems, Foster City, CA, USA), 1.1 μL BigDye v1.1 5× dilution buffer (Applied Biosystems), and 3.85 μL water, cycled

25 times at 96° for 10 s, 50° for 5 s, and 60°C for 4 min, and applied to an ABI PRISM 3700 sequencer (Applied Biosystems). Note that higher concentrations of blocking oligonucleotides might be required when using more dilute BigDye terminator mixture.

Figure 1 shows the mechanism by which the inverted *attL* sites of Gateway entry clones are presumed to inhibit the extension of sequencing

primers. As the size of the cloned DNA segment decreases, the rate at which the complementary *attL* sites find each other and hybridize to form a hairpin increases. Because each sequencing cycle begins with a denaturation step that produces a single-stranded template, if the hairpin forms before the sequencing primer is extended through the proximal *attL*, then the extension of the sequencing primer in each cycle will be impeded.

We expected to diminish this undesirable process by making the distal *attL* site double-stranded with complementary oligonucleotides before it could hybridize to the proximal *attL* site. We did this by adding oligonucleotides that were blocked at their 3' termini and complementary to the distal *attL* site to each sequencing reaction. We tested several candidate oligonucleotides for their effect on the sequencing reactions of a template with an insert of 390 bp cloned between the *attL1* and *attL2* inverted repeats (Figure 2). Without any blocking oligonucleotide (Figure 2A), the extension of the forward primer was stopped approximately 20 bases into the stem of the hairpin, which is AT-rich. The addition of blocking oligonucleotide 521 (Figure 2B), which hybridizes toward the top of the stem of the hairpin, actually decreased the read length to 17 bases into the stem. Oligonucleotide 522, which hybridizes at the bottom of the stem, gave very good sequence over its length of 39 bases (Figure 2C), at which point the signal strength dropped dramatically. The addition of both 521 and 522 (Figure 2D) allowed for sequence to be read through the hairpin, but better signal strength was seen with oligonucleotide 264, which is a single, longer oligonucleotide (Figure 2E) (60-mer).

The sequence quality with the reverse primer was not as severely affected by the hairpin formation (Figure 2F), and the sequence quality did not improve as dramatically with blocking oligonucleotide 264 (Figure 2G). Table 2 summarizes the results of several experiments with blocking oligonucleotide 264 at several oligonucleotide concentrations, forward and reverse sequencing primers, and cloned genes of various sizes. We then added 1 μL 25 μM blocking oligonucleotide 264 to our entry clone sequencing reactions.

Inverted repeats represent difficult

regions in genome sequencing projects (4–6). The method described here has limited applicability to these situations because one must know the sequence of the inverted repeats to design a blocking oligonucleotide. In such circumstances, it would be preferable to separate the inverted repeats by cloning them separately or to increase the distance between the inverted repeats by inserting a transposon (of known sequence) into the loop of the hairpin (7). Alternatively, one could design sequencing oligonucleotides with higher melting temperatures and attempt to minimize hairpin formation by raising the annealing temperature in each sequencing cycle. For sequencing Gateway entry clones using standard forward and reverse primers, the blocking oligonucleotide method described here is a less expensive and easier way to acquire long sequence reads.

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DNase I treatment of cDNA first strands prevents RT-PCR amplification of contaminating DNA sequences

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Retropseudogenes arise in evolution by reverse transcription of processed mRNAs and incorporation of the resulting cDNAs back into the genome (1). They are intronless DNA sequences that share a high degree of homology with the cDNA of their corresponding active genes. The human genome is estimated to contain approximately 23,000 to 33,000 retropseudogenes (2). An example of a gene family exhibiting retropseudogenes is the high mobility group protein family of which the *HMGA1* (HMGA protein family) and *HMGB1* (HMGB protein family) genes have been shown to contain many retropseudogenes (3–5). Several retropseudogenes have also been described for the housekeeping genes *GAPDH* and β -actin (*ACTB*) (6,7). In a lot of cases, the sequence homology of the retropseudogenes compared with the functional mRNA can be so high that retropseudogenes may serve as an appropriate template in a PCR using genomic DNA and a primer set detecting a cDNA sequence of the active gene (4,8).

Unfortunately, PCR products amplified from retropseudogenes cannot be distinguished in size from those amplified from cDNA, as retropseudogenes lack introns. A similar problem exists for specific amplification of intronless genes because in a RT-PCR, discrimination of cDNA and DNA amplification products by differing sizes of PCR products is not possible. Although the proportion of intronless human genes is generally thought to be no higher than 5% of all genes, there do exist some families of intronless genes like the histone genes and the G-protein-coupled receptor (GPCR) gene family (9).

Thus, genomic DNA (gDNA) contamination of cDNAs affects specific amplification of both housekeeping or target genes exhibiting at least one retropseudogene and intronless genes. Accordingly, a gDNA-free cDNA is needed to perform reliable RT-PCR analyses. Herein we describe an improvement of the standard method to eliminate detectable gDNA contamination in cDNA preparations.