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A Solution for the Split Peak and $n-10$ Artifacts at the vWA Locus in PowerPlex® 16 and PowerPlex® ES Systems

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INTRODUCTION

In recent years we have received reports of a split or $n-1$ peak at the vWA locus from PowerPlex® 16 and PowerPlex® ES^(a-c,j) users. In addition, a vWA artifact has been observed that runs at approximately the $n-10$ position (approximately 10 bases smaller than the main peak, although the size can vary significantly from injection to injection) on the multicapillary ABI PRISM® 3100 and 3100-Avant and Applied Biosystems 3130 and 3130xl Genetic Analyzers and at approximately the $n-18$ position (18 bases smaller than the main peak) on the single-capillary ABI PRISM® 310 Genetic Analyzer (1). The PowerPlex® 16 and PowerPlex® ES Systems use the same primer pair to amplify the vWA locus and therefore generate identical products. Recently we determined the root cause of both of these artifacts and devised a novel solution to prevent their occurrence on these capillary electrophoresis platforms. This article is intended as a brief summary of this work. A more detailed report was published recently for those readers desiring a more in-depth analysis (2).

THE vWA SPLIT PEAK AND ITS SOLUTION

The vWA split peak appears as two forms in electropherograms. In the most extreme cases it manifests as two distinct peaks, with the second peak migrating at the $n-1$ position relative to the main peak. In other cases the split peak presents as a shoulder on the left (i.e., smaller) side of the main peak (Figure 1). This significant variation in the size of the split peak relative to the main peak ruled out the simple explanation that this peak was due to incomplete adenylation. Further evidence to substantiate this interpretation was the fact that a sample showing a split on an Applied Biosystems 3130 or 3130xl Genetic Analyzer would not show a split when analyzed on an ABI PRISM® 310 Genetic Analyzer. These data suggested that the vWA split peak was caused by secondary structure rather than by a distinct DNA fragment. From the results of several experiments and observations that we recently published (2), we determined that the split peak is due to free unlabeled vWA primer annealing to its complementary sequence at the 3' end of the denatured TMR-labeled strand after injection into the capillary array. This happens despite the fact that the POP-4™ polymer should maintain the denatured state of the DNA. The partially double-stranded (PDS) nature of this species allows it to migrate faster than the single TMR-labeled strand. Annealing occurs during electrophoresis through the exposed portion of the capillary outside of the oven. As soon as the PDS species enters the oven or the environment in the capillary becomes sufficiently denaturing, the primer melts off the TMR-labeled strand, and the two species (i.e., the single TMR-labeled strand and previously PDS species) continue migration through the rest of the capillary, separated by the distance created while in the PDS state. This distance depends on when melting occurs. If it occurs later, two distinct peaks are seen, as the PDS species migrates farther away from the single-stranded species. Conversely, if melting occurs sooner a shoulder is observed.

Post-amplification addition of three oligonucleotides prevents the reannealing of complementary sequences to the labeled vWA strand during capillary electrophoresis, eliminating the split peak and $n-10$ artifacts at the vWA locus.

To prevent this annealing we devised a simple competitor oligonucleotide or sacrificial hybridization sequence (SHS), which is complementary to the unlabeled vWA primer. This SHS oligonucleotide is present in the new Internal Lane Standard 600 (ILS 600) and, thus, is added post-amplification to the amplified product. Following electrokinetic injection, the SHS oligonucleotide anneals to the unlabeled vWA primer, thereby preventing formation of the PDS species and consequently the split peak.

THE vWA n-10 ARTIFACT AND ITS SOLUTION

If the vWA split peak were due to a PDS species, then it was possible that the n-10 artifact also was due to double-stranded DNA (dsDNA). Specifically, it could represent double-stranded amplicon formed by annealing of the complementary unlabeled and TMR-labeled amplified strands. Several lines of evidence corroborated this hypothesis. First, two n-10 peaks often are seen with a heterozygote but only one with a homozygote. In the heterozygote the n-10 peaks appear to be separated from one another by the same distance as the main peaks. Second,

there is some variation in migration such that in certain cases the n-10 peak may migrate "on-ladder" and be designated as an allele, whereas in other cases it may migrate "off-ladder" (Figure 1). Such variation in sizing is consistent with a peak caused by secondary structure rather than a discrete DNA fragment that is smaller than the main peak. Third, we noticed that the n-10 artifact was reduced in intensity relative to the main peak when the SHS oligonucleotide was titrated into the loading cocktail. One would expect an oligonucleotide that is complementary to the unlabeled vWA primer (and also the 5' end of the unlabeled amplified strand; Figure 2) to interfere with annealing of the two amplified strands. Fourth, this artifact migrates at the n-18 location on the ABI PRISM® 310 Genetic Analyzer. Differential migration on two instrument platforms suggests that this peak is due to secondary structure rather than a discrete single-stranded DNA species.

Because the SHS oligonucleotide was capable of reducing the intensity of the n-10 peak, we reasoned that adding additional sequences that were complementary to the 3' end of the unlabeled strand would effectively prevent the unlabeled strand from annealing to the TMR-labeled strand. We chose to use two 30mer oligonucleotides, which we named complementary oligonucleotide target 1

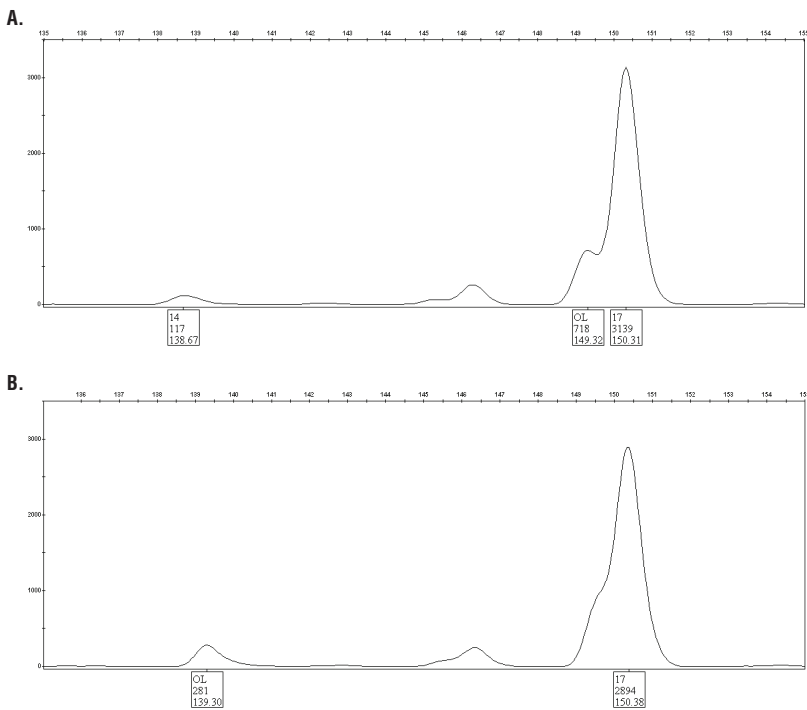


Figure 1. Artifacts at the vWA locus in the PowerPlex® 16 System. We amplified 1 ng of human genomic DNA using the PowerPlex® 16 System and GeneAmp® PCR System 9700 for 32 cycles, then analyzed amplified products using an Applied Biosystems 3130 Genetic Analyzer. **Panel A.** The vWA locus shows a defined split peak in roughly the n-1 position relative to the main peak and an n-10 peak, which migrates 11.64 bases shorter than the main peak at the 14 allele position. **Panel B.** The split peak manifests as a shoulder. Note that the n-10 peak now migrates 11.08 bases shorter than the main peak and migrates "off-ladder". Peak labels are (from top to bottom) allele designation, peak height and size.

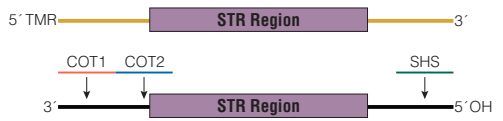


Figure 2. Schematic diagram of COT1, COT2 and SHS oligonucleotide hybridization to the vWA amplicon. The yellow line represents the TMR-labeled strand of the vWA amplicon. The black line represents the complementary unlabeled strand. The grey box denotes the location of short tandem repeats. The sites where the COT1, COT2 and SHS oligonucleotides anneal to the unlabeled strand are indicated.

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and 2 (COT1 and COT2; Figure 2). Two oligonucleotides were chosen instead of one 60mer because of the higher efficiency with which two full-length 30mers can be made compared to one 60mer. The presence of these three oligonucleotides (SHS, COT1 and COT2) in the new ILS 600 effectively eliminated both the split peak and n-10 artifacts (Figure 3). This also resolved the n-18 artifact seen on the ABI PRISM® 310 Genetic Analyzer (2). Testing of the new ILS 600 by laboratories outside of Promega has confirmed this result.

EFFECT OF NEW ILS 600 ON SIZING AND PEAK HEIGHTS

We conducted a concordance study of 118 samples amplified with the PowerPlex® 16 System and analyzed with the old ILS 600 (Cat.# DG2611) or new ILS 600 containing SHS, COT1 and COT2 oligonucleotides (Cat.# DG1071). All samples produced concordant genotypes with the old and new ILS 600. In addition, sizing of alleles observed in these samples and allelic ladders when run with the old ILS 600 were indistinguishable from those with the new ILS 600 (2). Finally, we compared the sizing of alleles in PowerPlex® ES, PowerPlex® Y, AmpFISTR® Profiler Plus™ ID, Cofiler® and SGM Plus® kits. In all cases, the presence of the SHS, COT1 and COT2 oligonucleotides had no effect on sizing of alleles in these STR systems. We also examined the effect of these additional oligonucleotides on peak heights. We observed no significant difference in peak heights between amplified samples or allelic ladders run with the old and new ILS 600.

CONCLUSION

We determined that the split peak and n-10 artifacts at the vWA locus in the PowerPlex® 16 and PowerPlex® ES Systems are due to partially and fully

double-stranded DNA molecules, respectively. These form after electrokinetic injection by annealing of complementary DNA sequences in the portion of the POP-4™ polymer-filled capillary array that resides on the cathode side of the oven on the ABI PRISM® 3100 and 3100-Avant and Applied Biosystems 3130 and 3130xI Genetic Analyzers (2). To ensure that such reannealing does not occur, we added three competitor oligonucleotides (SHS, COT1 and COT2) that anneal to the unlabeled vWA primer and unlabeled vWA amplicon strand to the ILS 600. This prevented the TMR-labeled vWA amplicon strand from annealing to

these unlabeled single-stranded DNAs and, thus, eliminated the two artifacts. The presence of these oligonucleotides in the new ILS 600 has no adverse effect on sizing, genotyping or peak heights. As a result, the new ILS 600 allows easier data interpretation at the vWA locus in the PowerPlex® 16 and PowerPlex® ES Systems by eliminating two problematic artifacts.

REFERENCES

1. PowerPlex® 16 System Technical Manual #TMD012, Promega Corporation.
2. McLaren, R. et al. (2008) Post-injection hybridization of complementary DNA strands on capillary electrophoresis platforms: A novel solution for dsDNA artifacts. *Forensic Sci. Int. Genetics* **2**, 257–73.

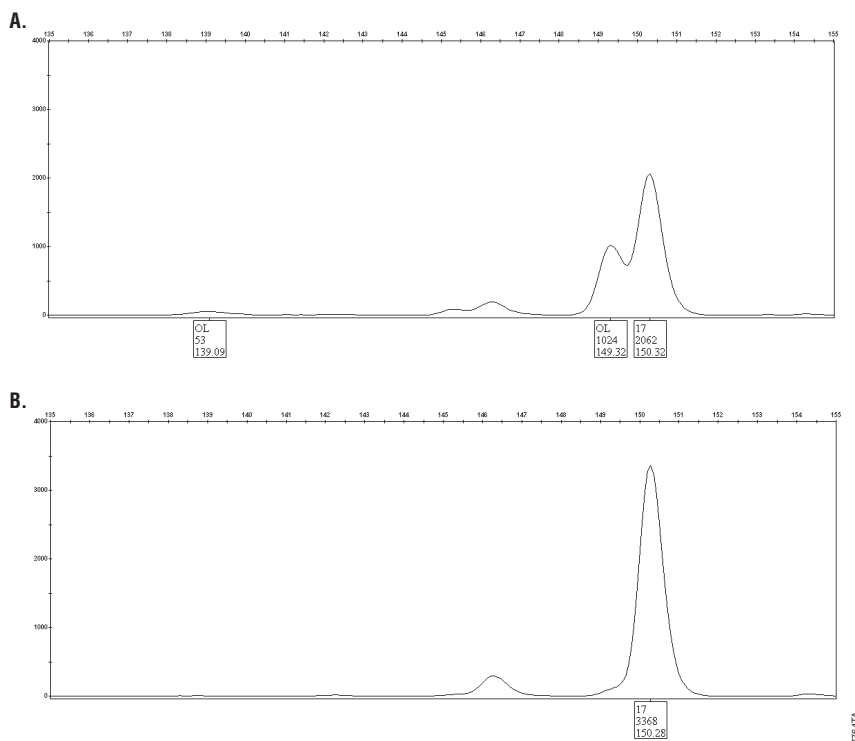


Figure 3. The new ILS 600 eliminates the split peak and n-10 artifacts. We analyzed a PowerPlex® ES-amplified sample using an Applied Biosystems 3130 Genetic Analyzer and old ILS 600 (Panel A) or new ILS 600 containing SHS, COT1 and COT2 oligonucleotides (Panel B). The presence of the SHS, COT1 and COT2 oligonucleotides in the new ILS 600 eliminates the split peak/shouldering of the main peak and n-10 artifact. Note similar results are observed with PowerPlex® 16. Peak labels are (from top to bottom) allele designation, peak height and size.