Evaluating Double-Peak Melt Curves for the LinkSeq™ HLA-B*27 Assay

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Aim

A "double-peak" melt curve can be problematic when assessing LinkSeq™ HLA-B*27 results. This study is intended to provide additional insight into the cause and potential remedies.

Methods

HLA typings were performed using the LinkSeq™ HLA-B*27 (One Lambda, Inc, West Hill, CA) LinkSeq™ 384 (One Lambda, Inc, West Hill, CA) , and QTYPE 384 kits (CareDx. Inc, Brisbane, CA) on a QuantStudio™ 6 Pro (Thermo Fisher Scientific), with analysis performed by Suretyper™, Score6™, and Design and Analysis Software (DA) (Thermo Fisher Scientific).

Results

LinkSeq™ HLA-B*27 typing results of one routine sample were inconclusive based on the presence of above threshold melt curve peaks in both the control and target range. A complete typing was performed by LinkSeq™ 384 identifying a homozygous HLA-B*40:01 (Figure 1).

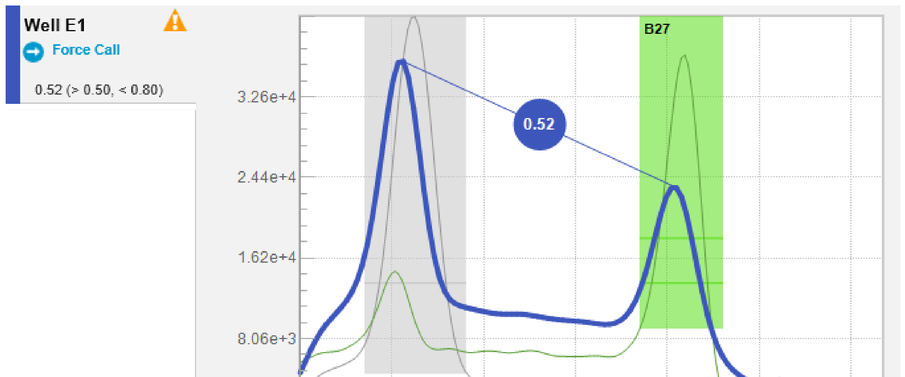

Figure 1: Double peak false positive results on LinkSeq™ HLA-B27 in the presence of homozygous HLA-B*40:01

Primer targets of the forward 5' (32L) and reverse 3'(44R) were compared via sequence alignment with a reference of HLA-B*27:05 against all common HLA-B alleles. Several alleles, including HLA-B*40, -B*41, -B*44, -B*49 and -B*50, exhibit no difference in sequence to HLA-B*27:05 across the forward primer binding site. Bases at 44R are mismatched to HLA-B*27:05 for these alleles (Figure 2).

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AA Codon                    5                  10                  15                  20                  25
B*27:05:02:01      GC TCC CAC TCC ATG AGG TAT TTC CAC ACC TCC GTG TCC CGG CCC GGC CGC GGG GAG CCC CGC TTC ATC ACC GTG
B*40:01:02:01      -- --- --- --- --- --- --- --- --- --- G-- A-- --- --- --- --- --- --- --- --- --- --- --- ---
B*40:02:01:01      -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
B*41:01:01:01      -- --- --- --- --- --- --- --- --- --- G-- A-- --- --- --- --- --- --- --- --- --- --- --- ---
B*44:02:01:01      -- --- --- --- --- --- --- --- T-- --- G-- A-- --- --- --- --- --- --- --- --- --- --- --- ---
B*49:01:01:01      -- --- --- --- --- --- --- --- --- --- G-- A-- --- --- --- --- --- --- --- --- --- --- --- ---
B*50:02:01:01      -- --- --- --- --- --- --- --- --- --- G-- A-- --- --- --- --- --- --- --- --- --- --- --- ---

AA Codon                   30      32L         35                  40          44R     45                  50
B*27:05:02:01      GGC TAC GTG GAC GAC ACG CTG TTC GTG AGG TTC GAC AGC GAC GCC GCG AGT CCG AGA GAG GAG CCG CGG GCG CCG
B*40:01:02:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --G A-- --- --- --- --A
B*40:02:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --G A-- --- --- --- --A
B*41:01:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --G A-- --- --- --- --A
B*44:02:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --G A-- --- --- --- --A
B*49:01:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --G A-- --- --- --- --A
B*50:02:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- A-- --- --- --G A-- --- --- --- --A

AA Codon                   55                  60                  65                  70                  75
B*27:05:02:01      TGG ATA GAG CAG GAG GGG CCG GAG TAT TGG GAC CGG GAG ACA CAG ATC TGC AAG GCC AAG GCA CAG ACT GAC CGA
B*40:01:02:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- A-- --C A-- --- --- T-- ---
B*40:02:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- A-- --C A-- --- --- T-- ---
B*41:01:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- A-- --C A-- --- --- T-- ---
B*44:02:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- A-- --C A-- --- --- T-- ---
B*49:01:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- A-- --C A-- --- --- T-- ---
B*50:02:01:01      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- --- A-- --C A-- --- --- T-- ---
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Figure 2: Sequence alignment with HLA-B*27:05 and common HLA-B alleles found to have no difference at the 3' end of the forward primer (32L).

Primer stringency was evaluated through changes in annealing temperature. Deviations from the recommended 64°C did not significantly reduce the double-peak effect or did not produce viable target amplicons. A homozygous HLA-B*44 sample was used in this testing (Figure 3).

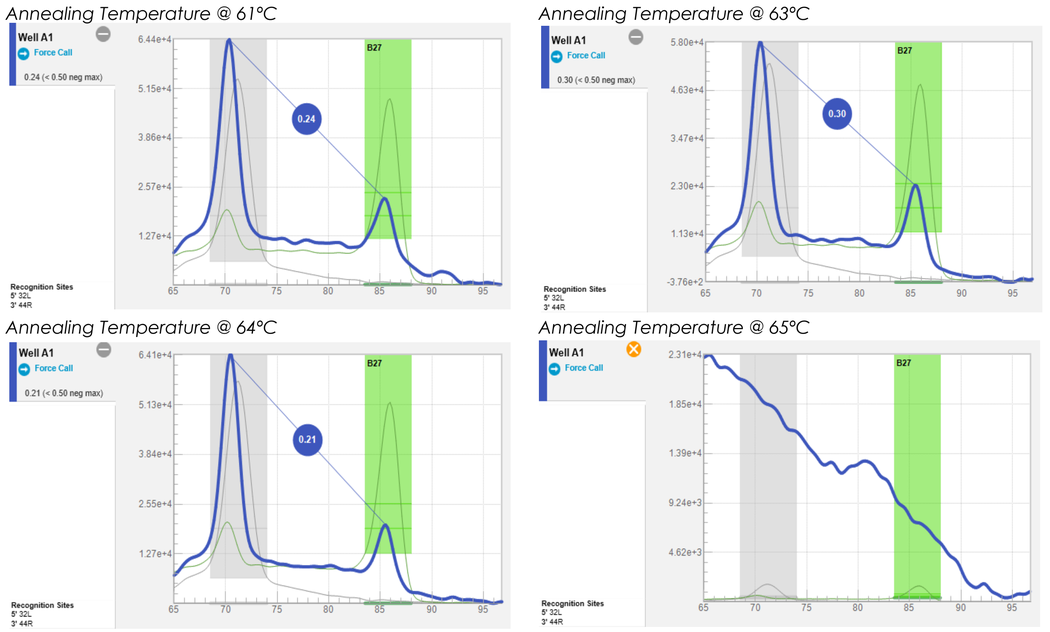

Figure 3: Runs with a homozygous HLA-B*44 were performed with annealing temperatures at 61°C, 63°C, 64°C (recommended), and 65°C. A "positive" peak is present near threshold at annealing temperatures up to 64°C, but target amplicons are unidentifiable at 65°C.

HLA-B*27 amplicons had a consistently higher melting temperature (Tm) (≥86.0°C) which could be distinguished from those of the double-peak "positive" when overlaid (Figure 4).

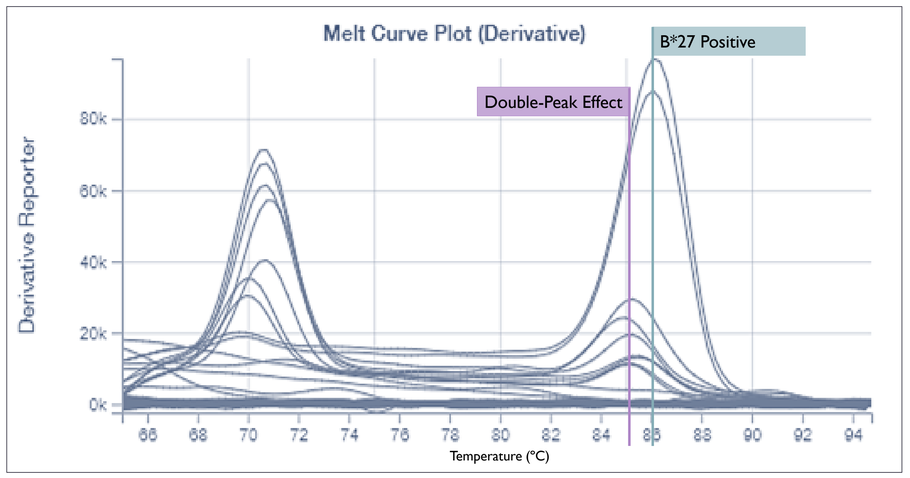

Figure 4: An overlay of HLA-B*27 positive, HLA-B*27 negative, and HLA-B*44 homozygous melt curve peaks. HLA-B*27 peaks were consistently higher in temperature than those of HLA-B*44.

Conclusion

LinkSeq™ typing for HLA-B*27 may occasionally produce flagged or questionable negative results in the presence of several alleles, especially if the alleles are homozygous. Such a result is likely due to sequence homology with the forward primer, and enough similarity with the reverse to initiate and sustain some amplicon production. There are other possible variables at play, such as primer annealing temperature or concentration, but simple changes to the run profile did not alter the effect.

Slight differences in base composition can be used to further differentiate between true and false positive amplicons, with true positives having a higher Tm peak in this study. Tools available in the DA software may assist in evaluating run data. No double-peak results were confirmed to be true positive for HLA-B*27. Furthermore, all known HLA-B*27 typings resulted in a clear positive peak.

References

1. Robinson J, Barker DJ, Georgiou X, Cooper MA, Flicek P, and Marsh SGE IPD-IMGT/HLA Database. Nucleic Acids Research (2020), 48:D948-55

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