P11.090

Evaluation of Multiple High Resolution Melting (HRM) Techniques Conducted on Three Commercially Available HRM Platforms

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INTRODUCTION

HRM is a genetic screening tool that allows investigators to rapidly detect, identify and focus on samples with relevant mutations before they proceed to expensive and time consuming traditional or next generation sequencing. This study used diverse HRM methods to compare three commercially available HRM capable platforms: the LightScanner® (Idaho Technology, Inc.) / Eppendorf MasterCycler®Realplex²(Eppendorf),theLightCycler®480(Roche),and the StepOnePlus™ (Applied Biosystems). These instruments use 96 and or 384 well plates. HRM instruments generate sufficient data density to detect subtle temperature and fluorescence differences caused by differences within the target sequence. The quality of HRM data is dependent on many variables including assay design, target characteristics, chemistry, expertise and instrumentation.

METHODS

A suite of assays was developed to highlight some common HRM techniques: mutation scanning, small amplicon genotyping (SAG) and unlabeled probe genotyping (See Figure 1 and 2.). Scanning assays require only two oligo nucleotides, the forward and reverse primers that encompass the region of interest. Small amplicon genotyping assays still require only two primers but the primers are designed to flank the mutation so that the SNP is the only variation that is being interrogated. LunaProbes™ or unlabeled probe assays use three oligonucleotides, the forward and reverse primers and the probe. The forward and reverse primers are usually designed to produce an amplicon of 150-300 base pairs in size. LunaProbe assays require asymmetric PCR in which the forward to reverse primers are present in unequal concentrations. In most cases a ratio between 1:5 or 1:10 works well. The asymmetric primer concentrations produce an excess of one of the strands allowing the unlabeled probe to hybridize to its target. When the lunaprobe assay is melted the unlabeled probe melts first and creates a probe signal at a lower Tm, then the amplicon melts at the higher Tm creating an amplicon signal. The unlabeled probe is blocked on the 3' end to prevent it from amplifying. The LunaProbe assay can genotype samples using the probe melt signal and scan the samples using the amplicon melt signal. Each assay was optimized on the three HRM platforms. The assays used LightScanner® Master Mix and the LightScanner® High Sensitivity Master Mix that include the double stranded DNA dye LCGreen®Plus (Idaho Technology, Inc.). The High Sensitivity Master Mix has internal standards added that the LightScanner software can use to calibrate the HRM data of individual samples within the plate to the calibrator peaks allowing more accurate Tm data and reduce the variation between samples, producing tighter grouping and better differentiation of the different homozygous (homozygous wild type and Homozygous mutant) homoduplexes formed during PCR.

Figure 1: Assay design strategies for scanning, small amplification genotyping and LunaProbes and high resolution melting techniques.

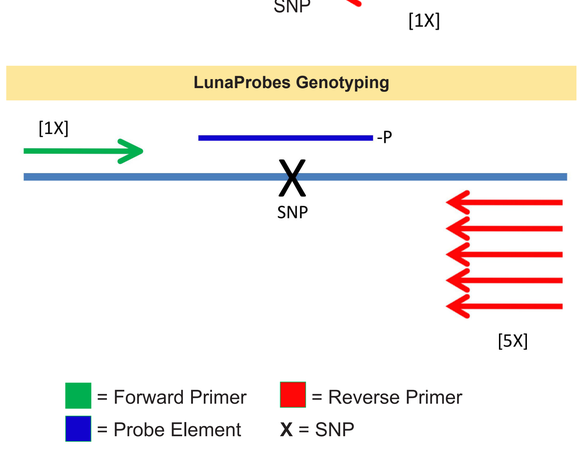
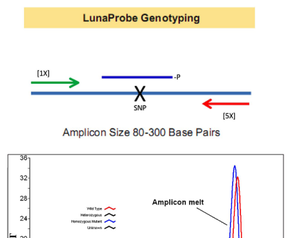


Figure 2: LunaProbes advantages and considerations.

– Advantages:
- Amplicon size ranges from 75-300bp
- Gives a probe peak (for genotyping) and an amplicon peak (for scanning)
- Can detect mutant allele fractions down to 5%

– Disadvantages:
- Requires Asymmetric PCR
- Requires 3oligos (2 primers and a probe)
- The probe must be blocked (costs more to synthsize)



RESULTS

The LightScanner/Eppendorf MasterCycler Realplex², LC480 and the StepOnePlus platforms were able to differentiate the seven different genotype groups in the LIPC gene scanning assay (See Figure 3.). All three platforms were able to distinguish between heterozygous A:T genotype from the homozygous genotype samples in the CPS1 (SAG) class 4 SNP assay. However only the LightScanner was able to differentiate the heterozygous A:T and the two homozygous A:A and T:T genotyped samples using the LightScanner High Sensitivity Master Mix (See Figure 4.). The OTC (LunaProbe) assay targets a class 4 SNP. The LightScanner and the StepOnePlus instruments correctly grouped all three genotypes of the OTC class 4 SNP (LunaProbe) assay (See figure 5.). All three instruments detect allele fractions of 10% in the ADH4 (LunaProbe) assay (See Figure 6.). The Human THO1 (SAG) assay contains a tetra nucleotide repeat region. All three instruments were able to differentiate the 8 genotyped samples (See Figure 7.). The LightScanner was able to separate all 5 genotyped groups. The LighCycler480 could not differentiate between the H63D WT and C282Y mutant samples. The StepOnePlus could not differentiate between the S65C mutant and the C282T WT samples. (See Figure 8.).

RESULT FIGURES

Figure 3: LIPC -gene scanning assay- 301 base pairs, 48.8% GC, with 4 known SNPs. The use of the LightScanner Master Mix allows 100% sensitivity on the Lightscanner instrument and StepOnePlus instrument. The Lightcycler 480 could not differentiate between the homozygous variant (magenta) and the homozygous WT (gray) samples for one of the SNP positions.

Figure 4: CPS1 gene -small amplicon genotyping assay- 51 base pairs, 39.1% GC, A>T SNP with nearest neighbor base symmetry. All three instruments could differentiate between the homozygous and heterozygous samples. The LightScanner was able to differentiate all three genotypes (heterozygous, homozygous WT and homozygous mutant) samples. The LightScanner software has the ability to correct for very small intersample temperature variations between the samples using the internal temperature controls included in the LightScanner High Sensitivity Master Mix allowing the LightScanner software correctly genotype the samples in this difficult small amplicon genotyping assay.

Figure 5: OTC gene -LunaProbes assay- 188 base pairs, 34.6% GC, A>T SNP with nearest neighbor base symmetry. This data shows the probe generated signal only. All three systems were able to differentiate the three alleles.

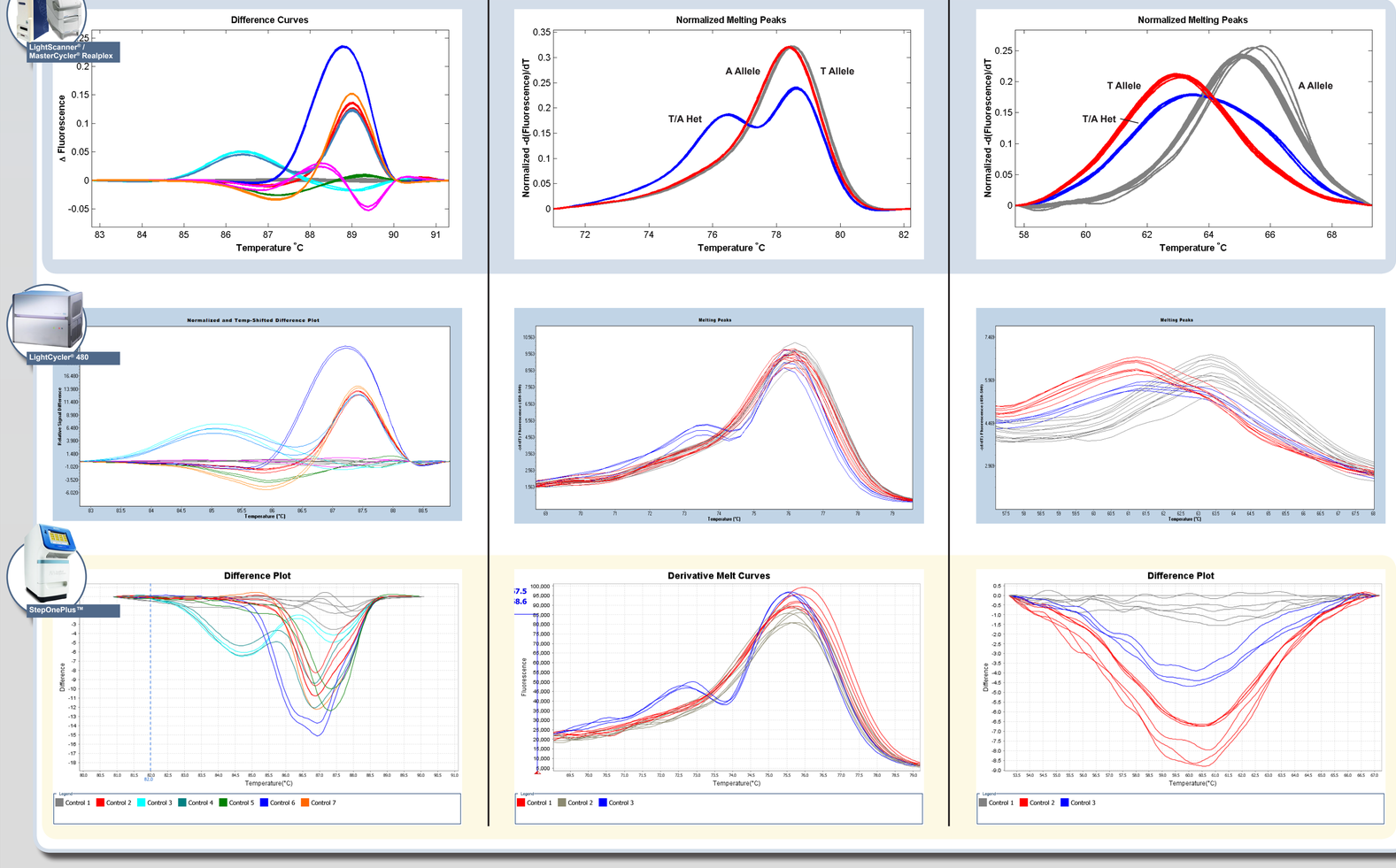
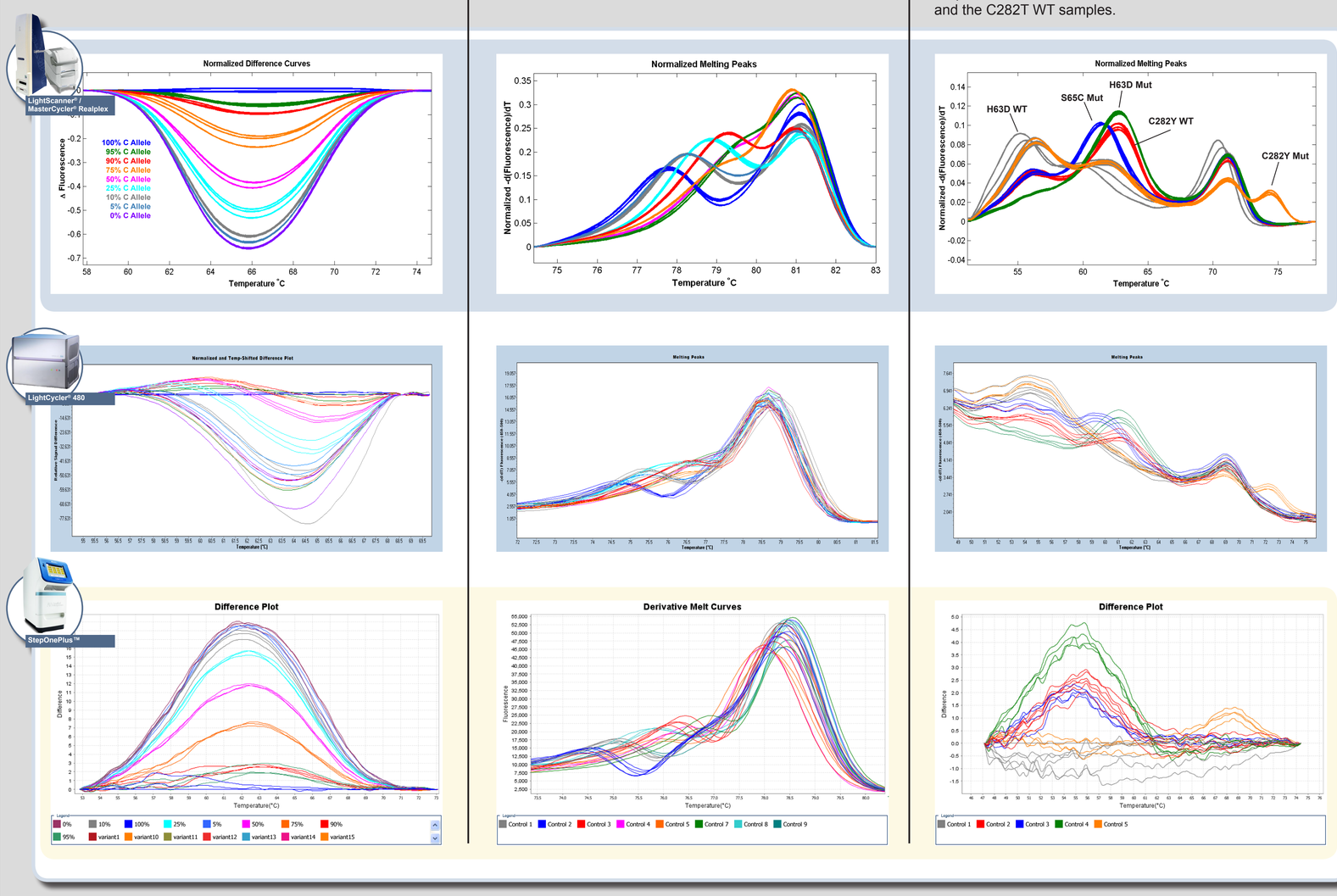


Figure 6: ADH4 gene, rs3762894 –LunaProbes assay- 108 base pairs, 35.2% GC, G>C SNP. All three instruments can detect an allele fraction of 10% for this target. The LightScanner can detect down to the 5% allele fraction.

Figure 7: Human THO1 gene, -small amplicon genotyping assay- 78 base pairs + (n) repeats, a common tetra-nucleotide repeat. All three instruments were able to differentiate the eight samples that contained different numbers of the tetra nucleotide repeat region.

Figure 8: HFE gene, -multiplexed LunaProbes assay- HFE C282Y: 87 base pairs, 59.8% GC and HFE H63D: 111 base pairs, 50.5% GC. The LightScanner was able to separate all 5 genotyped groups. The LighCycler480 could not differentiate between the H63D WT and C282Y mutant samples. The StepOnePlus could not differentiate between the S65C mutant and the C282T WT samples.



CONCLUSIONS

The LightScanner/Eppendorf MasterCycler Realplex², the LightCycler 480 and the StepOnePlus systems performed high resolution melting with the different scanning and genotyping methods. The LightScanner/ MasterCycler Realplex² platform provided more precise mutation discovery, differentiation and genotyping of difficult target regions including class 4 SNPs. Possible reasons for this are as follows; higher data acquisition rate, better signal-to-noise ratio, and more sophisticated software algorithms. In addition, the LightScanner system is able to perform melts in substantially less time. A typical HRM range of 20°C takes less than 10 minutes total time vs. 45 minutes plus on the other systems. This allows for tremendous throughput for end-point genotyping and mutation scanning experiments. Although the LightScanner system is not "walk-away" from amplification to melting, there are advantages to uncoupling the two functions from a systems standpoint. For example, one LightScanner 96 system can melt and analyze plates amplified from multiple PCR only instruments or instruments without HRM capabilities.

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Presented at European Society of Human Genetics Conference (ESHG) May 2011

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