Sensitivity and Specificity of High Resolution Melting for Detection of Heterozygous and Homozygous Variants Across Nine Instruments

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Mei Li, Luming Zhou, Ying Wang and Carl T. Wittwer

Department of Pathology, University of Utah Medical School

Background

Molecular diagnostics plays a crucial role in differential diagnosis and personal therapy. Genotyping is a major part of molecular diagnosis. Recently, high resolution melting has become a popular method for genotyping due to its speed, sensitivity, low cost and reduced contamination risk. However, it is not clear if all instruments that claim high resolution melting have comparable sensitivity and specificity. Therefore, we evaluated genotyping by high resolution melting in various instruments with different fragments.

Materials and Methods

The single nucleotide polymorphism (c. 3405-29A>T) of the CPS1 gene was amplified by PCR in the presence of LCGreen Plus in four fragment lengths (51bp, 100bp, 272bp, 547bp). This variant is nearest-neighbor symmetric and the wild type (A/A) and homozygous variant (T/T) are predicted to have identical melting temperatures. Amplicons were pooled for the three genotypes and used for blinded genotyping on all nine instruments, including HR-1™ (Biofire Diagnositics), LS32 (LightScanner® 32, Biofire Diagnositics), LS96, Rotor36/72 (Rotor-Gene® Q, 36 and 72 sample carousels, Qiagen), LC480 (LightCycler®480, Roche), CFX96 (CFX96TM, Bio-Rad), StepOne+ (StepOne Plus™, Life Technology), Eco™ (Illumina), Piko (PikoReal™ 96, Thermo Scientific). Homozygous variant and heterozygous variant (A/T) were detected under recommended high resolution melting conditions in all instruments. Data were analyzed with identical custom software.

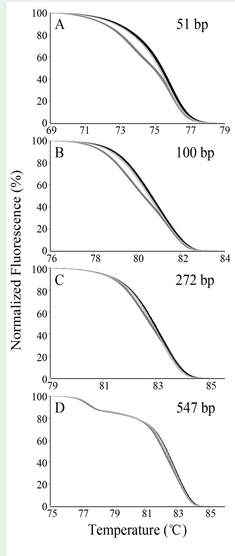

Figure 1. Normalized melting curves of the three genotypes within four amplicons of different sizes.

Table 1. Instrument characteristics

Instruments	Volume (µL)	Format	Channel		Detection mode[a]	Acquasition mode	Ramp rate[b] (°C/s)	Data points/°C	Time to single sample (min)	Batch size	Throughput (samples/hour)
			Excitation (nm)	Emission (nm)							
HR-1	10	cappilary	455	>490	single sample (PD)	continuous	0.3	66	1.5	1	40
LS32	10	cappilary	445	495	single sample (PD)	continuous	0.3	67	2.5	32	24
LS96	10	plate	470	>510	full plate (CCD)	continuous	0.1	10	5	96	1152
Eco	10	plate	470	525	full plate (CCD)	continuous	0.08	10	7	48	411
LC480	20	plate	450	500	full plate (CCD)	continuous	0.04[c]	18-25[h]	9	96	640
Piko	10	plate	488	535	full plate (CCD)	step	0.01[d]	25	40	96	144
Rotor36/72	10/15	tube	470	510	single sample (PMT)	step	0.01[e]	10	40	36/72	54/108
CFX96	20	plate	470	520	single sample (PD)	step	0.01[f]	10	50	96	115
StepOne+	20	plate	480	520	single sample (PD)	step	0.005[g]	26	95	96	61

[a] PD, photodiode; CCD, charge-coupled device; PMT, photomultiplier tube.
[b] The actual ramp rate messured by a micro-thermocouple or by a temperature-sensitive fluorescence method.
[c] Programmed ramp rate was 25 readings/°C, with dynamic integration time mode.
[d] Programmed ramp was defined as an increment of 0.04 °C with hold of 1 s.
[e] Programmed ramp was defined as a ramp of 0.1 °C with holds of 2 s.
[f] Programmed ramp was defined as an increment of 0.2 °C/step with holds of 10 s.
[g] Programmed ramp was defined as a ramp 0.3%.

Results

The sensitivity and specificity of heterozygous variant detection of four fragments were all 100% in nine instruments. However, for homozygous variants, there were wide differences in the sensitivity and specificity among the various instruments and among the four different fragments. Both the sensitivity and specificity were above 90% in homozygous variant detection for 51bp fragment by Rotor36 and CFX96; for 100bp fragment by HR1, Rotor36/72, CFX96, LS96, LC480, Eco; for 272bp fragment by none of the instruments and for 547bp fragment by HR1 and LS96. The index (average Tm SD/ΔTm) was a good predictor of the instrument ability to distinguish homozygotes (AUC=0.899, p<0.0005).

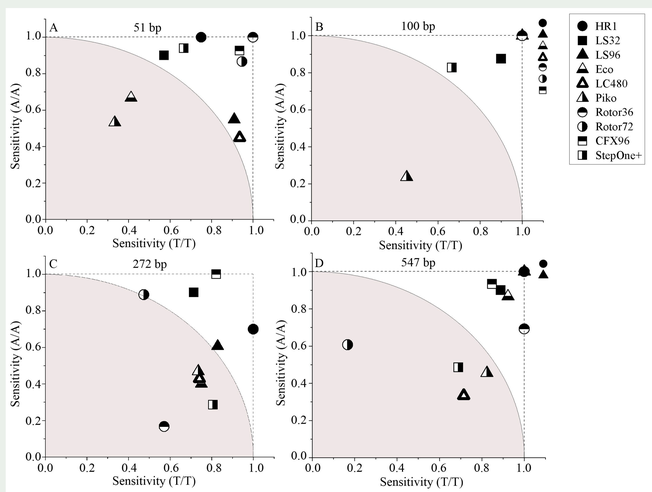

Figure 2. Sensitivity of homozygote detection across nine instruments and four amplicon sizes.

Conclusions

High resolution melting analysis detects heterozygotes with perfect performance, but homozygotes detection is highly dependent on amplicon size and instrumentation. Adjusting the fragment length may increase the resolution of homozygous difference for some single nucleotide variants. The index (average Tm SD/ΔTm) may be used as a metric to predict the performance of high resolution melting assays on different instruments.

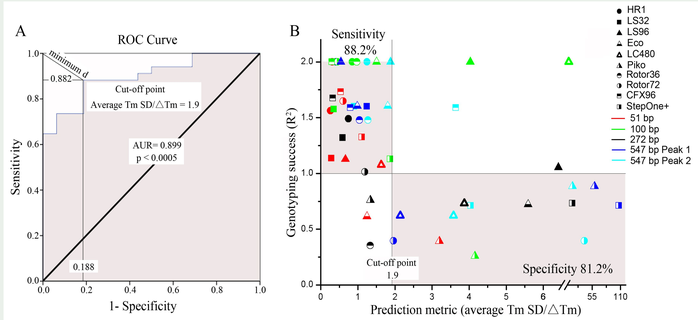

Figure 3. Predicting successful genotyping ($R^2 > 1$) by the metric: average Tm SD/ ΔTm.

References

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. Clin Chem 2003;49:853-60.

Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. Clin Chem 2006;52:494-503.

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A revised abstract is included.