Melting curve analysis Melting temperature o Each double strand DNA (ds DNA) has its own specific melting temperature (Tm), which is defined as the temperature at which 50% of the DNA becomes single stranded.o The temperature at which 50% of strands are hybridized is Tm (melting temperature), which is specific for each sequence, in this case is 81.5?C Melting curve analysis (cont.) o After mathematical processing of such data (arising from fluorescence changes vs. derivative of the temperature, dF/dT), we obtain the specific fluorescence data, Rn (left axis).Calculate Tm o We use the following modified Marmur Doty formula: o Tm = 2(A + T) + 4(C + G) − 7 o Where: Tm = melting temperature in ?C A = number of adenosine nucleotides in the sequence T = number of thymidine nucleotides in the sequence C = number of cytidine nucleotides in the sequence G = number of guanosine nucleotides in the sequence −7 = correction factor accounting for in solution o Marmur Doty Example Calculation.This example will demonstrate the manual calculation of the Tm for the following sequence: o 5'−ACGTCCGGACTT−3' o Step one: plug values into Marmur Doty formula to calculate melting temperature o Tm = 2(A + T) + 4(C + G) − 7 o Tm = 2(2 + 3) + 4(4 + 3) − 7 o Tm = 31.0 ?C o Where: Tm = melting temperature in ?C ?H = enthalpy change in kcal mol−1 (accounts for the energy change during annealing / melting) A = constant of −0.0108 kcal K−1 ?mol−1 (constant that scales energy to temperature) C = oligonucleotide concentration in M or mol L−1 (we use 0.0000005, i.e. 0.5 uM) −273.15 = conversion factor to change the expected temperature in Kelvins to ?C [Na+] = sodium ion concentration in M or mol L−1 (we use 0.05, i.e. 50 mM) One peak = One amplicon, or does it?o Researchers often use melting curve analysis to determine the specificity of the qPCR assay, o One of the other advantage of using the melt curve analysis method is that to find the Single nucleotide Polymerphisms (SNPs).Melting protocols (cont.) o As the temperature increases, dsDNA denatures becoming singlestranded, and the dye dissociates, resulting in decreasing fluorescence (Figure).Melting protocols o Typically, the thermal cycler being used to run the qPCR is programmed to produce the melt curve after the amplification cycles are completed.SNP detection by melting curve analysis o Melting curve analysis exploits the fact that even a single mismatch between the labeled probe and the amplicon will significantly reduce the melting temperature.o Thus, probe/amplicon heteroduplexes containing mismatches, such as the SNP, melt off at lower temperatures than probes bound to a perfectly matched target DNA (i.e., wild type).o Immediately after the last PCR cycle, the samples are momentarily denatured (90 ?C to 94 ?C), the thermal cycler starts at a preset temperature (usually above the primer T m ; e.g., 65?C) and measures the amount of fluorescence.o Melt curve analysis method can be used to check for any primer dimer formation and other anomalies in the qPCR assay developed.Melting curve analysis o The dotted line shows the fluorescence during the heating process; at low temperatures DNA is in double strand form and it has a 100% fluorescence (right axis).o Thus, there are two peaks, the lower peak at left, 72?C, corresponding to the dissociation curve of primer dimers that could be formed during the reaction. degree of GC content (Tm is higher in GC−rich fragments due to the presence of 3 hydrogen bonds) o The reason for this is due to the presence of 3 hydrogen bonds in the GC Pairing as compared to two hydrogen bonds in the AT pairing.mol−1 (accounts for helix initiation during annealing / melting) ?S = entropy change in kcal K−1 ?o The peaks on the right at 81.5?C which show higher intensity, corresponding to the dissociation curve of two specific amplification products

obtained.o The temperature of the sample is then increased incrementally as the instrument (temp rate of 0.1 ?C to 0.3 ?C/Second) while continues to measure fluorescence.o The change in slope of this curve is then plotted as a function of temperature to obtain the melt curve for CFTR exon 17b (Figure 1A).mol−1 (accounts for energy unable to do work, i.e. disorder) R = gas constant of 0.00199 kcal K−1 ?o A well−optimized probe design will provide a Tm difference of 8 ?C to −10 ?C for a single base mismatch under the probe.o These melting temperatures are primarily determined by ?o As they heat, the denatured strands produce fewer signal.dsDNA length, ?and degree of complementarity between strands.o So more energy is to break the GC pair.?