



Jason McKinney<sup>1</sup>, Mikeal Wall<sup>1</sup>, Lindsey Cutler<sup>1</sup>, David Ruddy<sup>2</sup>, Bella Gorbetcheva<sup>2</sup>, John Monahan<sup>2</sup>, David Teng<sup>1</sup>  
 1 - Idaho Technology, Inc. Genomics Research and Development, Salt Lake City, UT, USA  
 2 - Novartis Institute of Biological Research, Oncology Division, Boston, MA, USA

**CONTACT INFORMATION**  
 Jason McKinney  
 jasonm@idahotech.com  
 801-736-6354



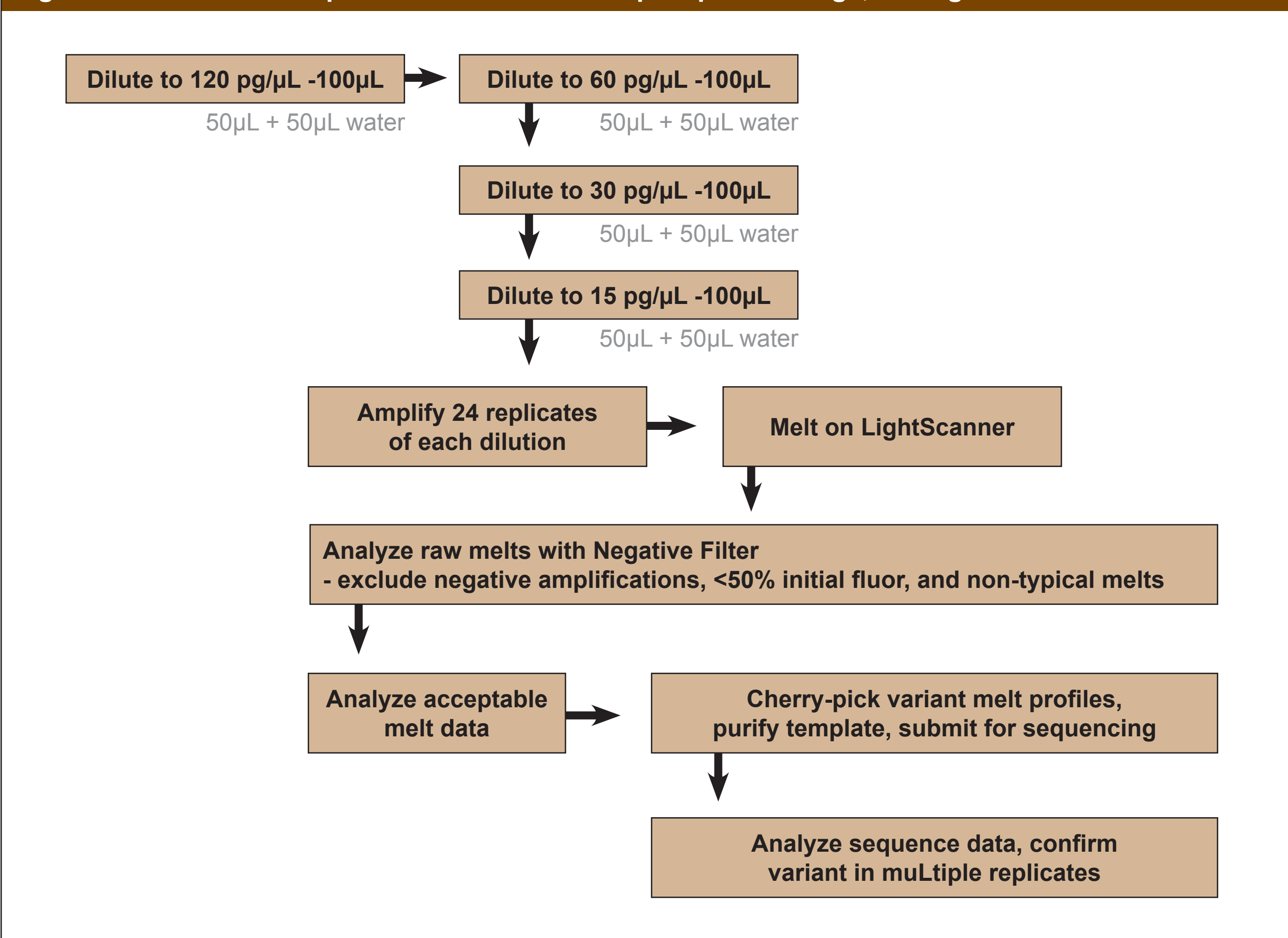
## INTRODUCTION

Vogelstein and Kinzler (PNAS, 1999) first described the concept of Digital PCR (dPCR) as a means to identify mutations in a minor cell population fraction (i.e. primary tumor tissue). By diluting samples down to a single molecule of DNA, it is possible to transform the exponential analog nature of PCR into a digital signal. When PCR is successful on these single molecules, products can be analyzed by sequencing with the expectation that somatic mutations will be observed as a homozygous change rather than a potentially low fraction peak that may be hidden in the common noise from a typical sequencing reaction. By amplifying a sufficient number of replicates of a single sample, the minor cell fraction can be estimated based on the number of mutants observed relative to the total number of replicates which amplified successfully.

## METHODS

We sought to apply High Resolution Melting (HRM) to a modified dPCR approach for defining low fraction variants indicated by an abnormal HRM profile in the primary tumor sample. Digital PCR requires that a single copy of DNA is used as starting template for amplification, thus endpoint detection becomes a digital readout rather than an analog admixture of the DNA. In order to apply HRM, a more robust amplification is desirable across replicates to obtain more reliable HRM profile grouping, thus we chose a target dilution of 5 copies. This dilution target is based on downstream sequencing being approximately 20% sensitive to detect low fraction variants. HRM has demonstrated sensitivity down to 2-5% mutant allele fraction; therefore a single copy of the mutant allele should be sufficient to detect a difference in the melting profile. A total of 17 samples representing suspected low fraction somatic mutations (12) or known variants (5) were assayed in 14 different cancer gene targets (Table 1). For samples with known variants, low fraction mixtures were created containing 5%, 2.5%, and 1.25% of the minor allele. All samples were diluted to target concentrations of 5, 10, 20, and 40 copies and amplified in replicates of 24. Replicates at each dilution that did not amplify robustly were excluded from the high resolution melting analysis. Replicates at each dilution that displayed a suspected variant profile were selected for sequencing confirmation. Figure 1 illustrates the workflow of applying HRM to this dPCR approach.

Figure 1: Determine sample concentration - multiple spec readings, average

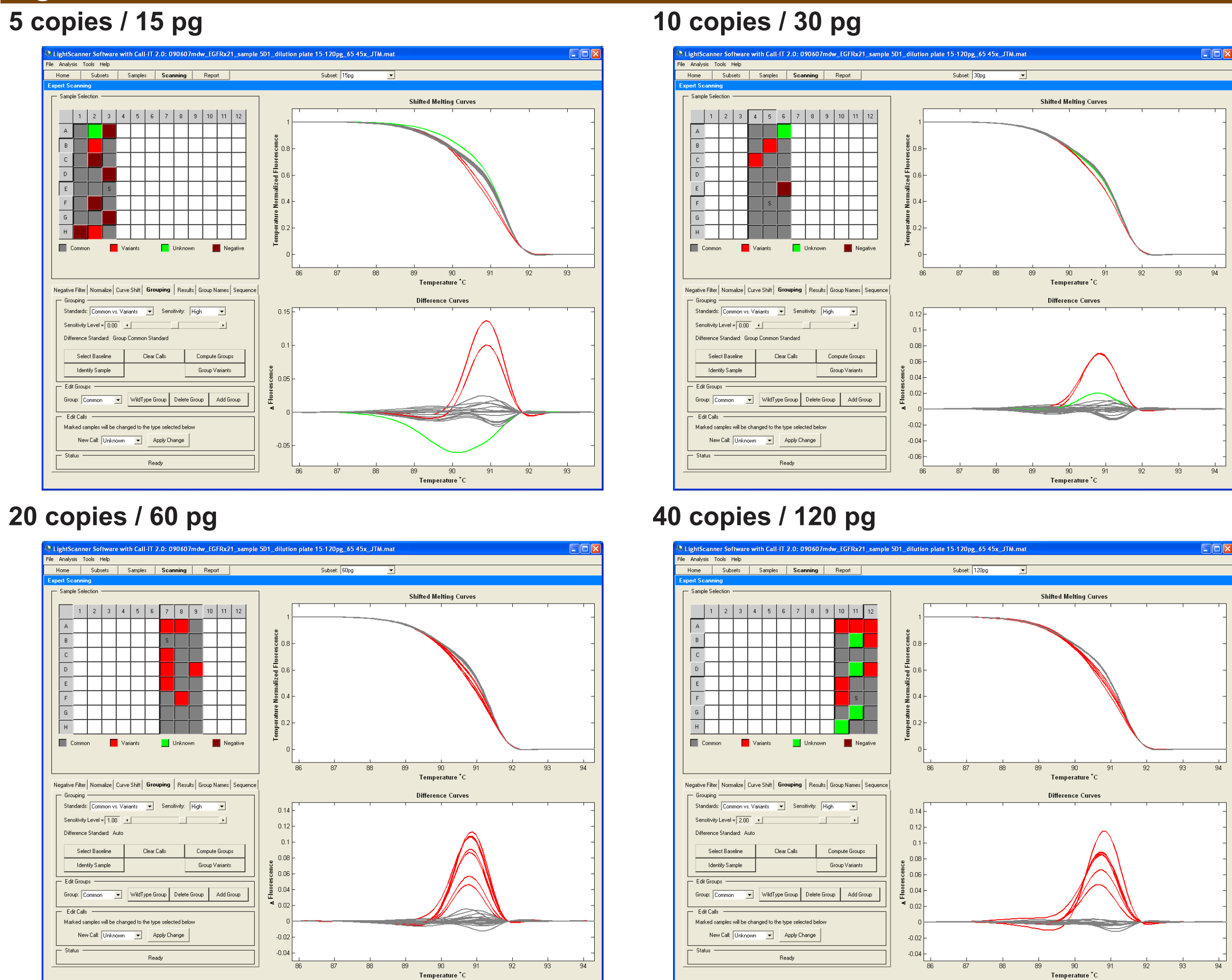


Gene Target	Amplicon Size (bp)	No. of Samples
BRAF exon 15	246	1
EGFR exon 19	148	1
EGFR exon 20	248	1
EGFR exon 21	213	1
JAK2 exon 14	173	1
JAK2 exon 22	236	1
KIT exon 8	210	1
KIT exon 17	180	1
MET exon 14	259	1
PDPK1 exon 5	215	1
PDPK1 exon 10	252	2
PIK3CA exon 3	308	2
PIK3CA exon 10	258	1

## RESULTS

Low fraction somatic mutations were identified in 9 of 12 tumor samples, whereas all 5 samples with known variants were successfully identified down to the 1.25% dilution mixture. Figure 2 illustrates how the “effective” concentration can be estimated by applying the probabilities in Table 2. The number of negative amplifications (6) at the 5 copy (15 pg) dilution suggests the effective concentration of this dilution is closer to 1 - 1.5 copies (3 - 4.5pg). Figures 3 and 4 are examples of how running multiple dilutions on the same plate can be effective at identifying the ideal dilution to resolve low fraction variants. Figures 5a and 5b show the identified variant in BRAF x15 at the 20 and 40 copy dilutions respectively. Allelic fraction is represented in the sequencing traces and appropriately corresponds to the dilutions.

Figure 2



# Copies/Well	% Wells w/ 0 Copies	# Empty Wells/96	# Empty Wells/48	# Empty Wells/24
0.25	77.9%	75	37	18.7
0.5	60.7%	58	29	14.6
0.75	47.2%	45	23	11.3
1	36.8%	35	18	8.8
1.5	22.3%	21	11	5.4
2	13.5%	13	6	3.2
2.5	8.2%	8	4	2.0
3	5.0%	5	2	1.2
3.5	3.0%	3	1	0.7
4	1.8%	2	1	0.4
4.5	1.1%	1	1	0.3
5	0.7%	1	0	0.2
6	0.2%	0	0	0.0
7	0.1%	0	0	0.0
8	0.0%	0	0	0.0
9	0.0%	0	0	0.0
10	0.0%	0	0	0.0

## CONCLUSIONS

These results show how using HRM as a pre-screen to a modified dPCR approach can significantly reduce the downstream sequencing effort of the traditional dPCR application for identifying low fraction variants. In this study, identification of candidate HRM profiles indicating the presence of a low fraction somatic mutation reduced the sequencing effort by 90% compared to the traditional approach of re-sequencing all replicates.

Figure 3

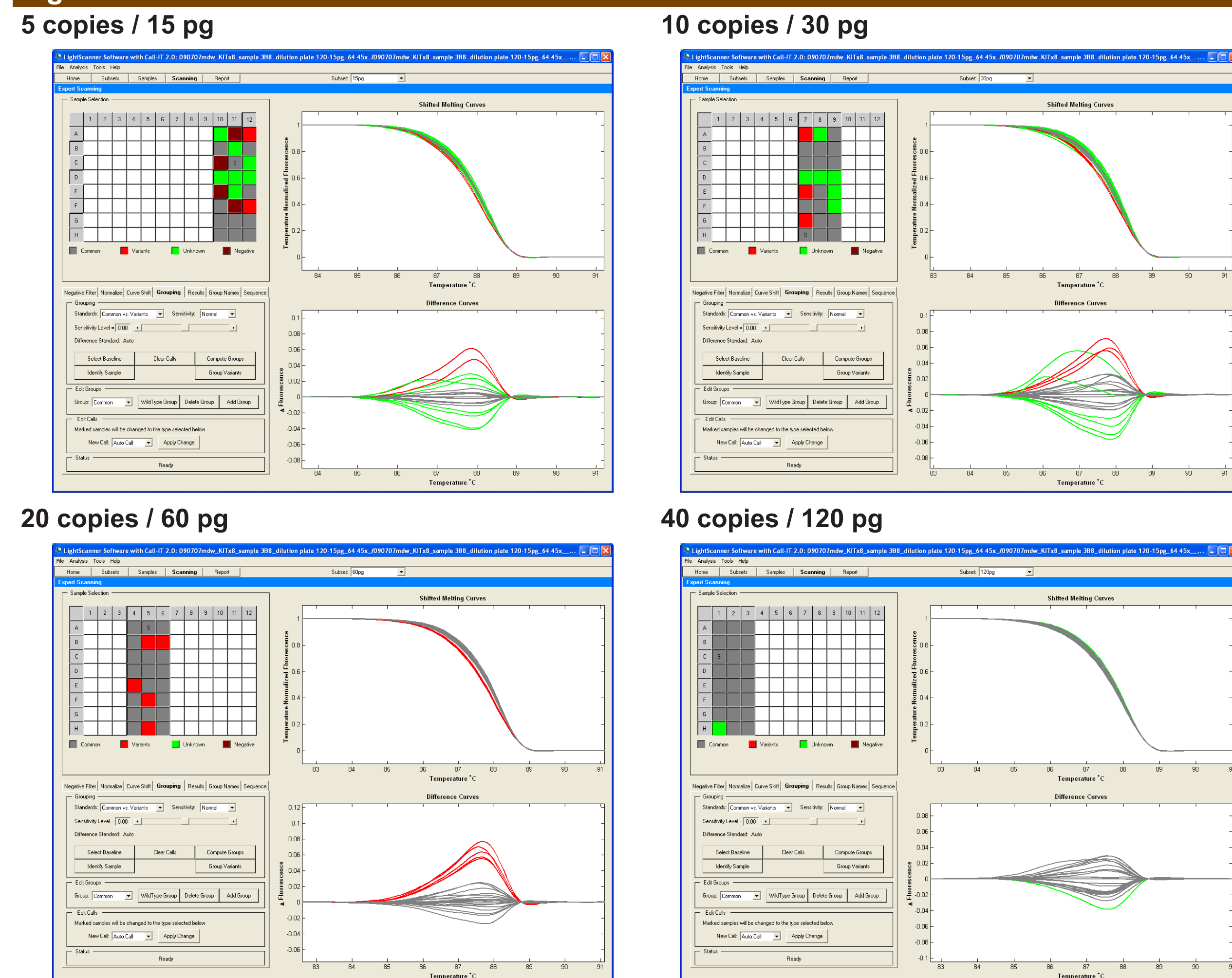


Figure 4

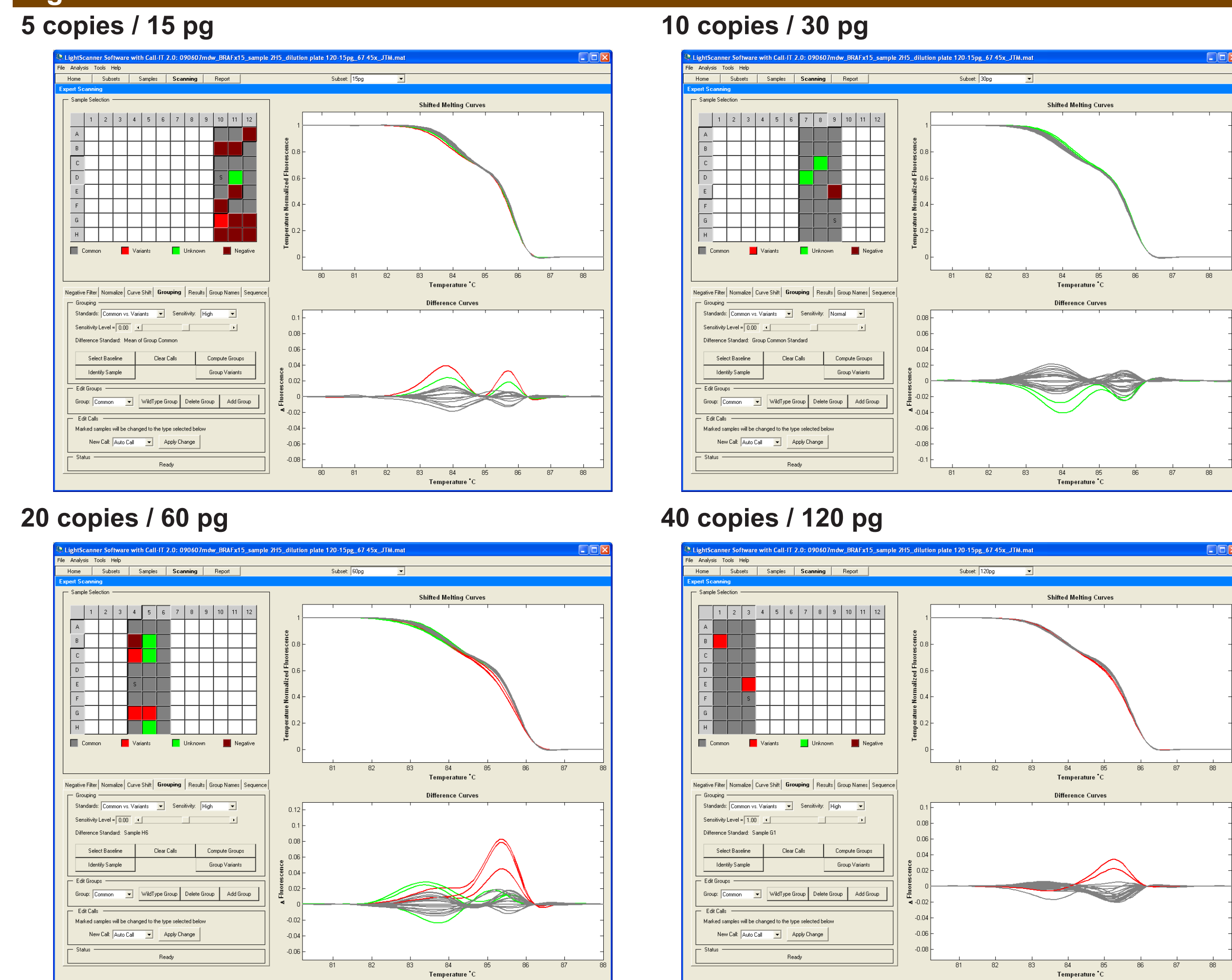


Figure 5

