

Methylated CpG Dinucleotides and Hi-Res Melting™

Cytosines found in CpG dinucleotides can be heterogeneously methylated in cells for a variety of reasons. Using Idaho Technology's (ITI) high-resolution melting (Hi-Res Melting™) process and the sophisticated analysis tools we offer, you can have a cost-efficient alternative to sequencing for validating and studying biologically relevant CpG islands.

In the process of methylation, cytosines are converted to uracil and base pair with adenosine during PCR. This results in a GC percentage difference in the amplified fragment. Keeping all else constant, melting temperature is determined by GC percentage; thus amplicon T_m is a function of the average percent methylation found initially in the tissue of interest. Calibration using our recently launched *High-Sensitivity Genotyping Master Mix* kit further improved the data. Figure 1 shows that T_m is a function of percent methylation across a 152 bp amplicon with five CpG sites. In addition, the extent of heterogeneity can be assessed by curve shape, with more homogeneously methylated samples displaying sharper peaks. This can also be seen in Figure 2. The most highly methylated samples (pink) correspond to 100% methylation across the CpG sites. The least methylated samples (light and dark blue) also present sharper curves and are the most homogeneously hypomethylated of the samples shown. Primer design for Hi-Res Melting methylation analysis is simple. In contrast to methylation-specific PCR, in methylation sensitive melting analysis, primers should not be designed over CpG sites. This will help fully amplify all samples present. The melting, not the primers, provides the specificity. Excellent results can be obtained with amplicons

of 40–200 bp, depending on the needs of the study and the number of CpG sites to be analyzed per assay.

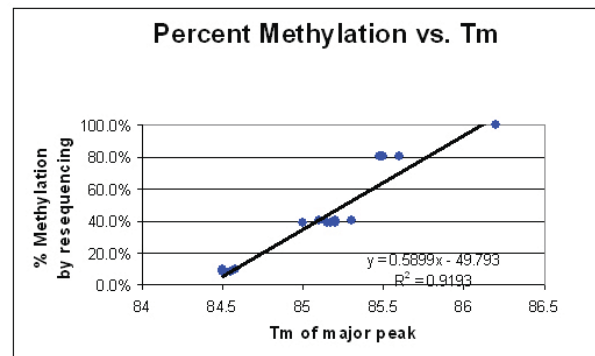


Figure 1. Amplicon T_m is a function of the average percent methylation across the tissues from which the DNA was extracted.

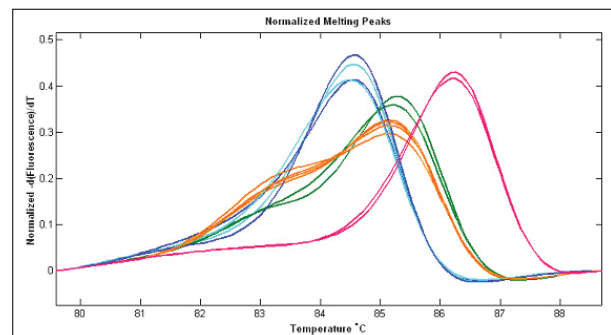


Figure 2. Hi-Res Melting derivative melting curves of several tumor samples representing hypo- and hypermethylated samples. Percentages across all five CpG sites are as follows: pink (100%), green (40%), orange (39%), dark blue (8%), and light blue (9.7%).

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Using our core Hi-Res Melting technology, you can achieve great results. Easily design your own high-resolution melting assay for methylation detection or use our assay design service.

For more information, please contact Cameron Gundry at cameron_gundry@idahotech.com or at (801) 736-6354 x. 444.

Using Digital PCR to Identify Low-fraction Mutations

Digital PCR (dPCR) was first described by Vogelstein and Kinzler (1999) as a means to identify mutations in a minor cell fraction. By diluting samples to a single copy of DNA, it was possible to transform PCR into a digital signal. When PCR from a single copy of template was successful, sequencing would display a somatic mutation as a rare homozygous change rather than a low amplitude peak potentially obscured by noise. By sequencing a sufficient number of replicates from a single sample, the minor cell fraction can be calculated.

We applied Hi-Res Melting using a modified approach to dPCR to: 1) identify low fraction somatic mutations indicated by an abnormal melt profile from the primary tumor sample and 2) Estimate the mutant allele fraction. 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. To apply Hi-Res Melting, a more robust amplification is desired across replicates to obtain reliable melt profile grouping, thus we chose a dilution of 5 copies. This dilution target is based on downstream sequencing being approximately 20% sensitive to detect low fraction variants. Hi-Res Melting has demonstrated sensitivity down to 2–5% mutant allele fraction; therefore, one copy of the mutant allele in five copies would be sufficient to observe a difference in the melting profile. Seventeen samples representing suspected low fraction somatic mutations (12) or known variants (5) were assayed in 14 cancer gene targets. For samples with known variants, low fraction mixtures were created containing 5%, 2.5%, and 1.25% of the minor allele. Samples were diluted to the target concentration and amplified in replicates of 24. Replicates that did not amplify robustly were excluded from the Hi-Res Melting analysis, while replicates that displayed a suspected variant profile were selected for sequencing confirmation.

Low fraction somatic mutations were verified by independent observations in 9 of 12 tumor samples, whereas all 5 samples with known variants were successfully identified down to the 1.25% dilution mixture. Figure 1 shows the results of a full 96-well plate of replicates from a single sample at a dilution target of 5 copies per reaction. Table 1 illustrates how the actual template concentration can be estimated by applying Poisson probabilities. The number of negative amplifications (14 out of 96) suggests the effective concentration of this dilution is actually ~2 copies. Four replicates (red profiles) were chosen for sequencing. The same mutation was identified in each independent PCR reaction. Further, sequencing indicated that wild type and mutant alleles were present at equal ratio's in these replicates, further validating the effective concentration estimate of 2 copies per reaction. Based on these results,

a mutant allele fraction can be calculated at 2.5% (4 mutant copies divided by 160 copies across the plate).

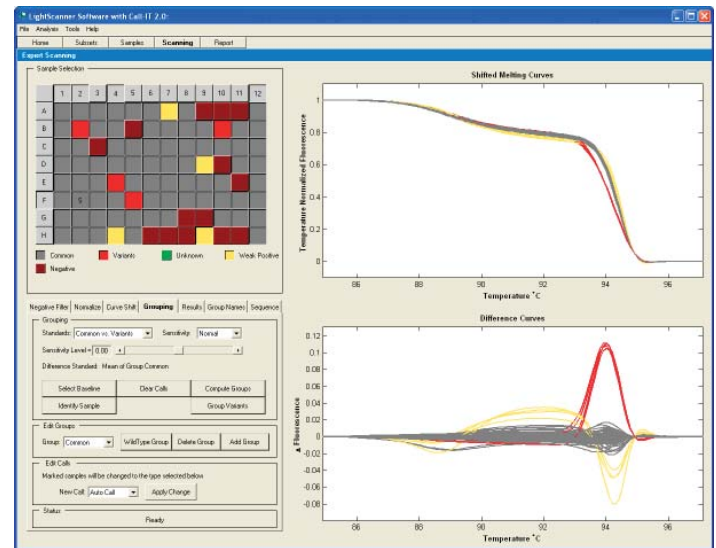


Figure 1. Single sample at a dilution target of 5 copies per reaction.

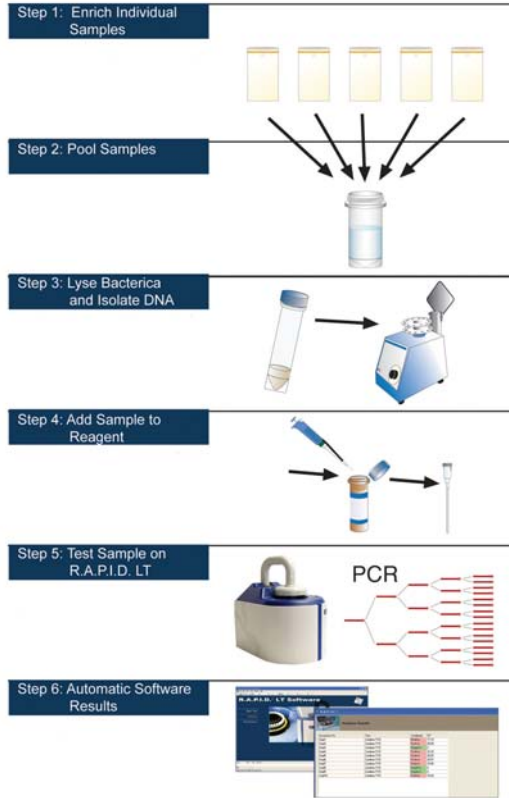
Table 1. Template concentration estimate.

# molecules per well	fraction of wells with 0 molecules	# of empty wells out of 96
0.25	77.9%	75
0.5	60.7%	58
0.75	47.2%	45
1	36.8%	35
1.5	22.3%	21
2	13.5%	13
2.5	8.2%	8
3	5.0%	5
3.5	3.0%	3
4	1.8%	2
4.5	1.1%	1
5	0.7%	1
6	0.2%	0
7	0.1%	0
8	0.0%	0
9	0.0%	0
10	0.0%	0

These results show that Hi-Res Melting using a modified dPCR approach can: 1) Identify low-fraction somatic mutations in a high background of normal alleles, 2) Reduce downstream sequencing of the traditional dPCR, and 3) Provide an estimate of the mutant allele fraction based on Poisson probabilities and total replicates. In this study, identifying aberrant melt profiles indicating the presence of a low fraction somatic mutation reduced the sequencing effort by >95% compared to sequencing all replicates. For more information about Hi-Res Melting and dPCR, please contact Jason McKinney at jasonm@idahotech.com or at (801) 736-6354 x. 411.

Pooling in Food Testing

Post-enrichment sample pooling is an excellent way to increase throughput and decrease costs. When most samples are negative, it is easy to quickly screen up to 5 samples in just one test. Samples are individually en-



riched then pooled into a composite sample. The composite sample is then tested as an individual sample. Using this method, negative samples are quickly screened. If the composite sample is positive, the individually enriched samples may be quickly tested to determine which of the samples is responsible

for the positive. The process takes less than 1 hour, even if there is a positive sample, thanks to the very short 35-minute PCR run. This method is routinely used with the MA-TRIX MicroScience Pathatrix® instrument.

In *Salmonella* experiments using Idaho Technology's R.A.P.I.D.® LT Food Security System, pooling samples yielded the same results as individual samples and demonstrated no false positives or false negatives. For food producers and manufacturers, this could mean big labor and cost savings as pooling takes less time to achieve a result than running several individual tests.

Testing Method	500 samples/day \$5/test	With a 10% positive rate
Individual Testing	\$2500.00	\$2500.00
Pooling	500.00	1750.00
Savings	\$2000.00	\$ 250.00

The R.A.P.I.D. LT Food Security System is approved by the AOAC for both single and pooled samples. For more information about the R.A.P.I.D. LT, pooling protocols, and assays, please contact Haleigh Millward at haleigh@idahotech.com or at 801-736-6354 x. 420.



Photo of the Quarter

Bryce Canyon
(Kathy Jedrzejczyk,
Research Associate)

Dates to Remember

August	
12-13	Force Health Protection (ARMY) Albuquerque, New Mexico http://chppm-www.apgea.army.mil/fhp/
September	
22-24	CBRNe World Conference and Exhibition Bucharest, Romania http://www.tcp-events.co.uk/cbrneworld/
October	
30-2 Nov	Association for Molecular Pathology Grapevine, Texas http://www.amp.org/2008/
November	
3-6	HazMat Explo Las Vegas, Nevada http://www.hazmatexplo.org
10-13	qPCR Symposium Millbrae, California http://www.qpcrsymposium.com/
12-14	American Society of Human Genetics Philadelphia, Pennsylvania http://www.ashg.org/2008meeting/exhibits/

Editor's Note: If you have comments or suggestions for articles, please e-mail the editor at loretta_organ@idahotech.com.

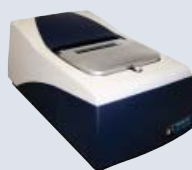
Department of State Note: The R.A.P.I.D. System and RAZOR Instrument are controlled for export under the International Traffic in Arms Regulations (ITAR), administered by the U.S. Department of State, Directorate of Defense Trade Controls (DDTC) and may not be exported or transferred to any foreign national without prior approval of the DDTC.

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R.A.P.I.D.® and RAZOR® Systems Training

ITI offers training courses for the R.A.P.I.D. and RAZOR systems. Training for two people is included with the purchase of the R.A.P.I.D. or RAZOR instruments, and more can attend for an additional cost. The training courses are three days for the R.A.P.I.D. and one day for the RAZOR. Courses focus on concepts of operation, sample preparation, reagent setup, and software. If you would like to attend or schedule a training course, please contact our training staff at 1-800-735-6544 x. 439.



FilmArray™ Corner

Each year 15–60 million people get influenza in the United States, and of those infected, 36,000 die. To properly treat influenza, an accurate diagnosis is needed to make critical and timely patient care decisions. Unfortunately, respiratory illnesses are difficult to diagnose because several pathogens cause very similar symptoms.

Recognizing the need to identify several pathogens from one sample, ITI has developed a highly multiplexed, real-time PCR system with integrated sample preparation called the FilmArray. Using a unique “lab-in-a-pouch” design, the current FilmArray system uses a single-sealed disposable pouch to automatically purify nucleic acids from a human specimen and then test that sample for over 20 different respiratory pathogens in less than 1 hour. This high level of multiplexing includes several common pathogens associated with similar clinical presentations eliminating the need for healthcare workers to order multiple tests. For more information about the FilmArray, please contact Wade Stevenson at wade_stevenson@idahotech.com or at (801) 736-6354 x. 463.

*The FilmArray is scheduled for clinical trials in November 2009.