Figure 1S. Derivative plot from the HRMA assay of the ROS1 gene promoter, resulting from the amplification of untreated (A) and BS-treated (B) DNA templates. The dark and red cursor (vertical line) in (B) indicate the $T_m$ value of melting peaks of standard fragment ($UMstd$) and clone 23sub.cl4 sample, respectively.
Figure 2S. Theoretical view of the resolution of homogeneous and/or heterogeneous methylated patterns as determined by HRMA of amplified PCR products from bisulfite-treated DNA template. In A are samples with homogeneous methylation that commonly display a single melting peak, with an increasing T$_m$ value with the increase of the number of methylated sites. Rarely, a homogeneous methylated amplicon having GC domain(s) might exhibit a complex curve shape with multiple melting domain. In B are represented samples with heterogeneous methylation that consist of a mixture of two or more epialleles with different methylation degree. Commonly, a mixture of unmethylated and fully methylated epialleles can be distinguished because the T$_m$ value of every allele is different, which unequivocally enables to identify the two melting peaks. When the sample is composed of a mixture of partial methylated epialleles, the melting profiles became very complex, depending on the number and proportion of the epialleles classes present. This last case becomes hardly distinguishable from the case of a complex melting curve in homogenous methylated samples.
Example of MethylExpress (or MSP-HTPrimer) software output. CpG site are bolded in red.

```
5'►AACAACAGGCTTGCCATGGAGTATAGGATTTTAT
ATTTGTGGGCCGCGGAAATTTTGAGAGGGCGAGGATTTTGGGAGGGAGG
GGTGCCGGGGAAGCCGAAATTTCTGCTAAGGAGATTGGAATTT
TGACGTGACGGTCCGAAAGCTTTTTGACCTTTGGGACCTTGATTT
GGTGGAGGAGCTTCTTTGGGTGATTTGGGCTGGTCTATGCΤΤTT
3'
```

Step 1. Manual identification of CpHpG and CpHpH sites (where H = A, C or T). In the cases where more than one cytosine occurs in a CpHpH site (underlined in black), only one should be randomly chosen (highlighted in blue).

```
5'►CAAAACAGGCTTGCCGATGGGAGTATAGGATTTTAT
ATTTGTGGGCCGCGGAAATTTTGAGAGGGCGAGGATTTTGGGAGGGAGG
GGTGCCGGGGAAGCCGAAATTTCTGCTAAGGAGATTGGAATTT
TGACGTGACGGTCCGAAAGCTTTTTGACCTTTGGGACCTTGATTT
GGTGGAGGAGCTTCTTTGGGTGATTTGGGCTGGTCTATGCΤΤTT
3'
```

Step 2. Finally, convert the remaining cytosine (C) in thymine (†), to obtain the fully methylated sequence.

```
5'►CAAAACAGGCTTGCTTTAGGGAGTATAGGATTTTAT
ATTTGTGGGCCGCGGAAATTTTGAGAGGGCGAGGATTTTGGGAGGGAGG
GGTGCCGGGGAAGCCGAAATTTCTGCTAAGGAGATTGGAATTT
TGACGTGACGGTCTCGAAAGCTTTTTGACCTTTGGGACCTTGATTT
GGTGGAGGAGCTTCTTTGGGTGATTTGGGCTGGTCTATGCΤΤTT
3'
```

**Figure 3S.** On the identified methylation sites (CpG) by Methyl Express tool the fine-tuned by hand steps are displayed from top to bottom.
Figure 4S. In A, the UT_DNA genomic sequence of the CMT3 amplicon is shown aligned with the unmethylated (0%) and the full methylated (100%) sequence after in silico bisulfite-treatment. Sequences of primers were excluded from the analysis. In panel B are shown the derivative melting plots predicted with uMELT software and MELTSIM algorithm after the adjustment of \( \sigma \) parameter highlighted in figure. In panel C is shown the CyMATE visualization of representative configurations used to build the calibration model for each class of epialleles. The classes of epialleles are indicated by the percentage of methylated Cytosine (\( mC\% \)) and methylated site number (\( #mC \)). The configurations in each class differed for the distribution of methylation at E (CpG) and/or L (CpHpG/CpHpH) sites. In panel D \( #mC \) values as function of the predicted mean T_{m-s} (±SD) of each epiallele class is shown.
**Figure 5S.** In A, the UT_DNA genomic sequence of the ROS1 amplicon (UT_DNA) is shown aligned with the unmethylated (0%) and the full-methylated (100%) sequence after *in silico* bisulfite-treatment. The primer sequences were excluded from the alignment. In panel B are shown the derivative melting plots predicted with the uMELT$^{\text{SM}}$ software and MELTSIM algorithm. In panel C is shown the CyMATE visualization of representative configurations used to build the calibration model for each class of epialleles. The classes of epialleles are indicated by the percentage of methylated Cytosine ($^{m}\text{C} \%$) and methylated site number ($^{\#}^{m}\text{C}$). The configurations in each class differed for the distribution of methylation at E (CpG) and/or L (CpHpG/CpHpH) sites. In panel D $^{\#}^{m}\text{C}$ values as function of the predicted mean $T_{\text{m-s}}$ (±SD) of each epiallele class is shown.
Figure 6S. The GC-rich domain is enclosed within the empty black box (A). The broadening of the derivative melting curve can be modulated though the value of σ parameter. Derivative plot of *in silico* predicted melting curve of *CMT3* amplicon for extreme configurations of the epiallele classes with 0, 5, 10 and 15 methylated cytosine, using default value (0.00021) of σ parameter (B), and σ value of 0.25 (C). The adjustment of σ parameters reduced the complexity of melting curve in the configurations 14E1L and 10E, as also experimental validated by the *in tube* HRMA of the respective synthetic amplicons (D).
Figure 7S. *In silico* $T_{m-s}$ values (black squares) and in tube $T_{m-o}$ values (red/green circles) for $^{#m}C$ as a function of $(T_m - UMstd T_m)$ for *ROS1*. The red line is the result of the linear fitting procedure of *in silico*-predicted data [$^{#m}C=a+b (T_m - UMstd T_m)$] (see text). Fitting parameters (for n=116): $a = -0.2\pm0.3$, $b = 5.65\pm0.17(°C)^{-1}$ with $R = 0.991$ with a corresponding p-value lower than 0.0001, which confirm the high significance of the hypothesized linear relationship.
**Figure 8S.** In silico $T_{m-s}$ values obtained for the DDM1 amplicon from melting curve profiles as generated by uMELT$^\text{SM}$ using the three selected thermodynamic sets (see text). The $T_{m-s}$ values are shown as function of $^mC$ number.
**Figure 9S.** MS-HRMAs tests for the *DDM1* gene promoter region quantification of epialleles proportions in the heterogeneously methylated clone sub18.c11. The melting peak with low $T_{m\text{-o}}$ value (78.61°C) was generated by the epialleles sub18.c11a (see *Figure 3*) with 2 methylated sites, whereas the melting curve with higher $T_{m\text{-o}}$ (81.04°C) derived from the epialleles sub18.c11b, with 11 methylated sites. The two alleles are present at an approximate proportion of 40 and 60% for allele $\alpha$ and $\beta$, respectively.
**Figure 10S.** In panel A are shown the derivative melting curves generated from the set of samples with different $^{3}H$C analysed for the $CMT3$ locus. The $T_{m-o}$ values correspond to the curve peaks for each $DDM1$ amplicons (coloured curves). Dark and pink curves for $UMstd$ and sub23.c14 sample, respectively. In panel B, the table shown the $Tm-o$ values detected for each sample and that generated from the comparison between numbers and percentages of the predicted $mC$. In the panel C is shown the position of methylated sites, as observed through the sequencing of amplicons using CyMATE representation.
Figure 11S. Derivative melting curves that each show the $T_{m-o}$ value corresponding to the curve peak for each ROS1 amplicon (A). In the table shown in panel B are reported the data generated from the comparison between numbers and percentages of the predicted $m^C$ and those observed in sequenced amplicons. In the panel C is showed an overview of the $m^C$ profiles as observed in the sequenced amplicons.
Figure 12S. The workflow of EpiHRMAssay. The sequential analysis steps are displayed from top to bottom. The box of the inputs has orange background, intermediate steps green, and the outputs have a grey background. *Download and preparation of the reference sequence and annotation from Peach Genome v1.0 browser step runs only once. **Bisulfite modification is only applicable for BSP, MSP, and COBRA methods. ***Use synthetized standard epialleles as double-stranded DNA fragments.