COLD-PCR HRM: A HIGHLY SENSITIVE DETECTION METHOD FOR IDH1 MUTATION

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Key Words:	IDH1 mutations, COLD PCR, High Resolution Melting, glioma, diagnostic marker

COLD PCR HRM: A HIGHLY SENSITIVE DETECTION METHOD FOR *IDH1* MUTATION

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Abbreviations:

AII: astrocytoma; COLD PCR: co-amplification al lower denaturation temperature PCR; HRM: high resolution melting; IDH1: isocitrate dehydrogenase 1; OAII: oligoastrocytoma; OII: oligodendroglioma.

Disclosures

The authors report no disclosures.

Competing interests

No potential conflicts of interest were disclosed.

Abstract

Isocitrate dehydrogenase 1 R132H (*IDH1*^{R132H}) mutation is a frequent alteration and a major prognostic marker in gliomas. However, direct sequencing of highly contaminated tumor samples may fail to detect this mutation. Our objective was to evaluated the sensitivity of a newly described amplification method, coamplification at lower temperature-PCR (COLD PCR), combined with high resolution melting (HRM) for the detection of *IDH1*^{R132H} mutation. To this end, we used serial dilutions of mutant DNA with wild type DNA. PCR-HRM assay detects *IDH1*^{R132H} mutation at an abundance of 25%, similar to the detection limit of direct Sanger sequencing. Introducing a run of COLD PCR allows the detection of 2% mutant DNA. Using two consecutive runs of COLD PCR, we detected 0.25% mutant DNA in a background of wild type DNA, that mimicks a tumor sample highly contaminated by normal DNA. We then analyzed ten biopsies of tumor edges, considered free of tumor cells by histological analysis, and showed that immunohistochemistry of *IDH1*^{R132H} was positive in 3 cases (30%), whereas double COLD PCR HRM was positive in the ten cases studied (100%). In summary, COLD PCR HRM analysis is 100-fold more sensitive than Sanger sequencing, rendering this rapid and powerful strategy particularly useful for samples highly contaminated with normal tissue.

Keywords: *IDH1*^{R132H} mutation, COLD PCR, High Resolution Melting, glioma

Introduction

In recent years, recurrent mutations of the gene encoding isocitrate dehydrogenase 1 (*IDH1*; MIM# 147700) have been described at a high frequency in gliomas [Balss, et al., 2008; Bleeker, et al., 2009; Hartmann, et al., 2009; Ichimura, et al., 2009; Parsons, et al., 2008; Sanson, et al., 2009; Yan, et al., 2009]. Mutations in the *IDH1* gene always affect the codon 132, which in more than 90% of cases result in a substitution of arginine by a histidine residue, $IDH1^{R132H}$ [Ichimura, et al., 2009; Sanson, et al., 2009; Yan, et al., 2009]. Since it is almost exclusively found in gliomas, *IDH1* mutation has a strong diagnostic value [Yan, et al., 2009]. *IDH1* mutation is also a powerful independent prognostic factor in gliomas [Dubbink, et al., 2009; Nobusawa, et al., 2009; Sanson, et al., 2009]: patients with an $IDH1^{R132H}$ mutated tumor having a longer survival when compared with those non mutated for the same grade. Given its diagnostic and prognostic potential, the determination of *IDH1* status in glioma samples is gaining increasing attention in clinical practice.

Conventional PCR followed by Sanger sequencing is currently the gold standard in identifying *IDH1* mutations in tumor DNA. Typically, clinical tumor samples are contaminated by normal tissue, thereby diluting the total amount of genetic that is present, and techniques are often required to identify low level alterations within an excess of wild-type DNA. This is particularly true for gliomas which are highly infiltrating tumors [Furnari, et al., 2007]. Conventional PCR enables mutation detection from a very small amount of sample but does not selectively amplify the mutant sequences. Therefore, unless the mutation exceeds a 20-25% abundance relative to wild-type alleles, conventional PCR followed by downstream methods such as Sanger sequencing will fail to detect mutations in clinical samples [Li, et al., 2009; Zuo, et al., 2009]. Enrichment methods are thus necessary to increase the mutant DNA /wild type DNA *ratio*. Tumor-cell enrichment methods, such as cell

sorting or microdissection, are expensive and time-consuming [Zuo, et al., 2009], and therefore inadequate for clinical routine use.

Recently, co-amplification at lower denaturation temperature PCR (COLD PCR) has been described as a powerful method to identify low-level mutations in the *TP53* gene [Li, et al., 2008; Milbury, et al., 2009]. This approach uses a critical temperature (Tc) during the PCR process in order to enrich mutations at any position of the amplified sequence. Indeed, a single nucleotide mismatch anywhere along a double-stranded DNA sequence generates a small change in the melting temperature for that sequence, with mutated sequences melting at a lower temperature than wild-type sequences [Li, et al., 2008]. During COLD PCR, the denaturation temperature is set to Tc, thereby mutation-containing sequences are preferentially denatured and available for primers binding and subsequent amplification. High Resolution DNA Melting (HRM) analysis is a relatively new and rapid method for detecting DNA sequence variants following an initial amplification [Erali, et al., 2008; Vossen, et al., 2009; Wittwer, 2009]. Gene scanning by HRM depends on the recognition of changes in the shape of the amplicon melting curve that result from heterozygous sequence alterations [Erali, et al., 2008]. At the end of PCR amplification, samples are heated to 95°C to ensure a complete denaturation. Next, the temperature is reduced to allow cross-hybridization between wild-type and mutant sequences, leading to the formation of mismatched heteroduplexes, which melt at a lower temperature than wild-type and mutant homoduplex [Vossen, et al., 2009]. In this study, we evaluated the sensitivity of different COLD PCR HRM assays for the detection of *IDH1*^{R132H} mutation in glioma samples.

Material and methods

DNA and tumors samples

Tumor samples were selected from the Pitié-Salpêtrière brain tumor database. Tumor DNA was extracted from frozen tissues using the QIAmp DNA according to the manufacturer instructions (Qiagen). Samples used in this work have previously been tested for the presence of *IDH1* mutation (reference sequence NM_005896.2) by direct sequencing, as already described [Parsons, et al., 2008; Yan, et al., 2009].

Determination of T_c

We applied the fast COLD PCR assays for detecting *IDH1* mutation based on the methodology described in the literature [Li, et al., 2008], with several modifications. We first determined the new denaturation temperature Tc for the reaction. We used the same primers as for conventional PCR assay, which produced an amplicon of 172 bp. To identify the optimal critical denaturation temperature Tc, a series of COLD PCR reactions at graded temperatures below Tm were performed. We then set the Tc at 81°C, the lowest temperature that reproducibly yielded a substantial PCR. In fast COLD PCR, the reaction protocol began with 20 cycles of conventional amplification for an initial build-up of all amplicons, followed by 30 COLD PCR cycles to selectively enrich for Tm-reduced mutant sequences (Figure 1). COLD PCR assays were performed on a LightCycler480 (Roche Diagnostics Corporation) to ensure a precise control of temperature during the experiments.

Comparison of sensitivities of the different assays to detect *IDH1* mutation

To determine if COLD PCR could enhance the sensitivity of *IDH1*^{R132H} mutation detection, we compared the sensitivity of three experimental protocols. Exon 4 of the *IDH1*

gene was first amplified either by conventional PCR or by fast COLD PCR. A second round of amplification was then performed either by PCR HRM, or by fast COLD PCR HRM (Figure 2).

To compare the sensitivity of the different assays, we performed a serial dilution study using a gDNA sample from a grade II oligoastrocytoma patient containing an *IDH1*^{R132H} mutation as the source of the mutant allele. This mutation-containing gDNA sample was serially diluted into wild-type gDNA (ie blood constitutive gDNA from the same patient) to the following percentages: 25%, 10%, 8%, 5%, 4%, 2%, 1%, 0.5%, 0.25%, 0.1%, and 0.05%. In addition, wild-type gDNAs (n=7) were included in each experiment. The same experiment was reproduced with several other mutated DNA samples: another CGT→CAT mutation (Arg132His), and also with other, less frequent mutations, such as CGT→CTT (Arg132Leu) and CGT→AGT (Arg132Ser).

First round of amplification

Each PCR amplification reaction contained 50ng gDNA. PCR cycling conditions for the first round consisted of an initial denaturation step at 94°C for 5 min; 40 cycles of 94°C for 30s; 60°C for 1 min; and 72°C for 1 min30; and final extension at 72°C for 7 min. The reactions were carried out using a Mastercycler (Eppendorf). Conventional PCR reactions contained final reagent concentrations as follows: 1x PCR Master Mix (Abgene), 0.25 µM forward and reverse primers (Invitrogen; Table I) and DNA template. COLD PCR cycling conditions on LightCycler480 (Roche Diagnostics Corporation) are summarized as follows: 96°C, 10 min; 20 cycles of 95°C, 15s; 60°C, 30s, then 30 cycles of 81°C, 15s; 60°C, 30s.

COLD PCR assays contained final reagent concentrations as follows: LightCycler480 HRM Master (Roche Diagnostics Corporation), 0.25 µM forward and reverse primers (Invitrogen), 3 mM $MgCl_2$ and DNA template.

Second round of amplification

PCR amplification was performed with the LightCycler480 (Roche Diagnostics Corporation). Each reaction contained diluted PCR amplicons (1/1000), 0.25 µM forward and reverse primers (Invitrogen, Table I), 3 mM $MgCl_2$ and LightCycler480 HRM Master (Roche Diagnostics Corporation). PCR HRM cycling conditions were as follows: 96°C, 10min; 40 cycles of 95°C, 30s; 60°C, 30s. COLD PCR HRM cycling conditions are summarized as follows: 96°C , 10min; 20 cycles of 95°C, 15s; 60°C, 30s, then 30 cycles of 81°C (Tc), 15s; 60°C, 30s. After amplification, a post amplification melting curve program was initiated by heating to 95°C for 1 min, cooling to 40°C for 1 min, and increasing the temperature to 95°C while continuously measuring fluorescence at 25 acquisitions per degree. Each PCR run contained a negative (no template) control and each amplification was duplicated.

High Resolution Melting (HRM) analysis and direct sequencing

At the end of the second round of amplification, fluorescent melting curves were analyzed usingLC480 Gene Scanning software V1.2.9 (Roche Diagnostics Corporation). All curves were analyzed following normalization, temperature shifting, automated grouping, and the inspection of difference plots. The grouping software uses a curve shape-matching algorithm in order to identify wild type from mutant samples. The 0.2 value was chosen for the grouping sensitivity in all experiments.

Products of each assay were then submitted to the sequencing reaction using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems) as previously described [Sanson, et al., 2009]. After a purification step using BigDye Xterminator Purification Kit (Applied Biosystems), both forward and reverse sequences were determined on an ABI prism 3730 DNA analyzer (Perkin Elmer).

Tumor sample selection and immunohistochemical detection of *IDH1*^{R132H}

Tumors were retrieved from the neuropathology department database according to the following criteria: histological diagnosis of WHO grade II gliomas; presence of *IDH1*^{R132H} mutation confirmed by Sanger sequencing; availability of frozen and paraffin embedded samples of tumor core and tumor edge, with edge biopsies considered as free of infiltrated tumor cells by standard HE staining.

Immunohistochemical staining for IDH1^{R132H} was performed on 4 µm paraffin sections of formalin-fixed tumor samples using mouse monoclonal anti-R132H-IDH1 antibody culture supernatant (a generous gift from Pr A. von Deimling), as previously published [Capper, et al., 2010; Capper, et al., 2009]. Local immunohistochemistry protocol was validated on gliomas samples which were previously analysed by Sanger sequencing for *IDH1*^{R132H} mutation (positive and negative controls). Labelling was defined as positive (at least one cluster of positive tumor cells) or negative (no positive tumor cells detected).

Results

Determination of the sensitivity with dilution studies

To identify the most sensitive method for detecting *IDH1* mutation, we evaluated two techniques after a first stage of conventional PCR: the PCR HRM assay and the COLD PCR HRM assay. In a third assay, we replaced the first conventional PCR by a run of COLD PCR (Figure 2). The sensitivity of the three assays was compared using a dilution series obtained by mixing DNA from heterozygous positive control carrying the *IDH1* R132H mutation with wild-type DNA from peripheral blood.

Conventional PCR HRM assay detects mutant DNA at a concentration of 25% in a background of wild-type DNA (Figure 3A). In contrast, the COLD PCR HRM assay detects *IDH1*^{R132H} mutation at a much lower concentration: mutant DNA diluted into wild-type DNA to a 2% abundance was still clearly differentiated from normal sequences (Figure 3B). Therefore, the COLD PCR HRM assay produced an approximately 10-fold improvement in the *IDH1* mutation detection, as compared to the conventional PCR HRM analysis. Replacing the first conventional PCR amplification by a run of COLD PCR further enhanced selective enrichment of the mutant DNA and improved the detection of *IDH1*^{R132H} mutation. In these conditions, we were able to detect mutant DNA at a concentration as low as 0.25% in mixture with wild-type DNA (Figure 3C). Thus, the double COLD PCR HRM assay for the detection of *IDH1*^{R132H} mutation was 100-fold more sensitive than the PCR HRM assay.

To both confirm the selective mutation enrichment and to exclude false positive results, amplicons produced at the end of the three assays were submitted to direct sequencing using the Sanger method. The sequencing chromatograms are presented in Figure 3D. In all three cases, direct sequencing confirmed the presence of the *IDH1*^{R132H} mutation. After

double COLD PCR HRM with 0.25% of mutant DNA/wild DNA, *IDH1*^{R132H} mutation was evident as clearly as for 25% with conventional PCR HRM and 2% with COLD PCR HRM.

The same results were obtained with another glioma with CGT→CAT mutation (Arg132His), and also with less frequent IDH1^{132} mutations CGT→CTT (Arg132Leu) and CGT→AGT (Arg132Ser) (supplementary figure).

Comparison of PCR HRM, COLD PCR HRM and double COLD PCR HRM and *IDH1*^{R132H} immunohistochemistry in biopsies of tumor edges.

To definitely validate the clinical interest of our technique, we selected 10 pairs of tumor core and tumor edge (one astrocytoma, 5 oligodendrogliomas, and 4 oligoastrocytomas), and performed *IDH1*^{R132H} mutation detection by both immunohistochemistry and molecular techniques (Table II). *IDH1*^{R132H} immunohistochemistry was positive in 3 cases (30%, 2 grade II oligodendrogliomas, and one grade II oligoastrocytoma) showing a cytoplasmic granular staining as previously described [Capper, et al., 2010]. Figure 4A presents the results of immunohistochemistry staining for patient 8 in the tumor core and in the biopsy edge (lower panel). PCR HRM was positive in 8 cases out of 10 (80.0%; Figure 4B upper panel). In contrast, both COLD PCR HRM (data not shown) and double COLD PCR HRM were positive in all cases (100%; Figure 4B, lower panel, and table II).

Discussion

Gliomas are characterized by a highly invasive phenotype, with tumor cells invading the brain at a distance far from the bulk of tumor [Furnari, et al., 2007]. Therefore biopsy samples are often contaminated by normal brain tissue, and mutated DNA is diluted in a the background of wild-type DNA from surrounding brain tissue, vasculature, and infiltrating lymphocytes [Dobrowolski, et al., 2009]. Because *IDH1*^{R132H} mutation is almost restricted to gliomas [Yan, et al., 2009], it has a major diagnostic potential. Therefore a fast and reliable scanning technique to detect this mutation in clinical samples with very few tumor cells (and appearing therefore normal at neuropathological analysis) is particularly important. In addition *IDH1*^{R132H} mutation is a major prognostic factor [Sanson, et al., 2009; van den Bent, et al., 2010]. Simple HRM has been very recently reported as a fast and sensitive strategy, detecting IDH1 and IDH2 mutations in a 90% normal DNA background [Horbinski, et al., 2010]. In this study, we used fast COLD PCR to selectively amplify *IDH1*^{R132H} mutated DNA and we showed that double COLD PCR HRM assay is a highly reliable method to detect *IDH1* mutation in samples with very few tumor cells (up to 0.25% mutated/non mutated DNA). In addition, this assay is fast (less than three hours) and therefore particularly suitable for routine diagnosis purposes in neuropathology.

Despite *IDH1*^{R132H} accounts for more than 90% of *IDH1* mutations reported in gliomas, other mutations have been described: CGT→AGT, CGT→CTT CGT→GGT, CGT→TGT [Balss, et al., 2008; Gravendeel, et al., 2010; Sanson, et al., 2009; Yan, et al., 2009]. Fortunately, all these mutations, except the CGT→GGT change, result in a lower Tm, rendering them theoretically detectable by our simple double COLD PCR assay. Indeed we were able to detect some of these less frequent mutations (supplementary figure). For the detection of CGT→GGT, a full COLD PCR assay –allowing the enrichment of all possible

mutations- could be performed, though the selective amplification *ratio* will probably be lower than in fast COLD PCR, because amplification, and thus enrichment in mutant sequences, begins earlier during cycling in fast COLD PCR than in full COLD PCR [Li, et al., 2008; Milbury, et al., 2009].

We showed that double COLD PCR HRM (followed by sequencing in case of aberrant HRM profiles) is a powerful method to evidence the presence of tumor cells with IDH1 mutation in apparently "blank" biopsies of grade II gliomas, and is much more sensitive than immunohistochemistry for *IDH1*^{R132H}. Such procedure may be particularly useful in lesions radiologically highly suggestive of glioma, whose biopsy appears non contributive because of the very low proportion of tumor cells. In such cases, finding an *IDH1* mutation will confirm the diagnosis of glioma, avoiding a further invasive procedure to establish diagnosis. Moreover, such approach could also be suitable for *IDH1*^{R132H} mutation detection in the cerebral spinal fluid thus confirming glioma diagnosis without the need for surgery.

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Figures legends

Figure 1: Principle of fast COLD PCR performed for the detection of *IDH1*^{R132H} mutation in glioma samples.

Figure 2: Assays performed to detect *IDH1*^{R132H} mutation in tumor samples.

Figure 3: Comparison of conventional PCR HRM (A), COLD HRM (B) and double COLD HRM (C) for the detection of *IDH1*^{R132H} mutation. Mutant DNA was serially diluted with wild type DNA and each mixture was submitted to the three assays. Sequencing chromatograms after conventional PCR HRM, COLD HRM and double COLD HRM (D).

Figure 4: Detection of *IDH1*^{R132H} mutation in grade II gliomas edges. A. *IDH1*^{R132H} immunohistochemistry in patient 8 tumor core (upper panel) and in tumor edge (lower panel); magnification x200. **B.** *IDH1*^{R132H} detection by PCR HRM (upper panel) and double COLD PCR HRM (lower panel)

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Abbreviations:

AII: astrocytoma; COLD PCR: co-amplification al lower denaturation temperature PCR; HRM: high resolution melting; IDH1: isocitrate dehydrogenase 1; OAII: oligoastrocytoma; OII: oligodendroglioma.

Disclosures

The authors report no disclosures.

Competing interests

No potential conflicts of interest were disclosed.

Abstract

Isocitrate dehydrogenase 1 R132H (*IDH1*^{R132H}) mutation is a frequent alteration and a major prognostic marker in gliomas. However, direct sequencing of highly contaminated tumor samples may fail to detect this mutation. Our objective was to evaluated the sensitivity of a newly described amplification method, coamplification at lower temperature-PCR (COLD PCR), combined with high resolution melting (HRM) for the detection of *IDH1*^{R132H} mutation. To this end, we used serial dilutions of mutant DNA with wild type DNA. PCR-HRM assay detects *IDH1*^{R132H} mutation at an abundance of 25%, similar to the detection limit of direct Sanger sequencing. Introducing a run of COLD PCR allows the detection of 2% mutant DNA. Using two consecutive runs of COLD PCR, we detected 0.25% mutant DNA in a background of wild type DNA, that mimicks a tumor sample highly contaminated by normal DNA. We then analyzed ten biopsies of tumor edges, considered free of tumor cells by histological analysis, and showed that immunohistochemistry of *IDH1*^{R132H} was positive in 3 cases (30%), whereas double COLD PCR HRM was positive in the ten cases studied (100%). In summary, COLD PCR HRM analysis is 100-fold more sensitive than Sanger sequencing, rendering this rapid and powerful strategy particularly useful for samples highly contaminated with normal tissue.

Keywords: *IDH1*^{R132H} mutation, COLD PCR, High Resolution Melting, glioma

Introduction

In recent years, recurrent mutations of the gene encoding isocitrate dehydrogenase 1 (*IDH1*; MIM# 147700) have been described at a high frequency in gliomas [Balss, et al., 2008; Bleeker, et al., 2009; Hartmann, et al., 2009; Ichimura, et al., 2009; Parsons, et al., 2008; Sanson, et al., 2009; Yan, et al., 2009]. Mutations in the *IDH1* gene always affect the codon 132, which in more than 90% of cases result in a substitution of arginine by a histidine residue, *IDH1*^{R132H} [Ichimura, et al., 2009; Sanson, et al., 2009; Yan, et al., 2009]. Since it is almost exclusively found in gliomas, *IDH1* mutation has a strong diagnostic value [Yan, et al., 2009]. *IDH1* mutation is also a powerful independent prognostic factor in gliomas [Dubbink, et al., 2009; Nobusawa, et al., 2009; Sanson, et al., 2009]: patients with an *IDH1*^{R132H} mutated tumor having a longer survival when compared with those non mutated for the same grade. Given its diagnostic and prognostic potential, the determination of *IDH1* status in glioma samples is gaining increasing attention in clinical practice.

Conventional PCR followed by Sanger sequencing is currently the gold standard in identifying *IDH1* mutations in tumor DNA. Typically, clinical tumor samples are contaminated by normal tissue, thereby diluting the total amount of genetic that is present, and techniques are often required to identify low level alterations within an excess of wild-type DNA. This is particularly true for gliomas which are highly infiltrating tumors [Furnari, et al., 2007]. Conventional PCR enables mutation detection from a very small amount of sample but does not selectively amplify the mutant sequences. Therefore, unless the mutation exceeds a 20-25% abundance relative to wild-type alleles, conventional PCR followed by downstream methods such as Sanger sequencing will fail to detect mutations in clinical samples [Li, et al., 2009; Zuo, et al., 2009]. Enrichment methods are thus necessary to increase the mutant DNA /wild type DNA *ratio*. Tumor-cell enrichment methods, such as cell

sorting or microdissection, are expensive and time-consuming [Zuo, et al., 2009], and therefore inadequate for clinical routine use.

Recently, co-amplification at lower denaturation temperature PCR (COLD PCR) has been described as a powerful method to identify low-level mutations in the *TP53* gene [Li, et al., 2008; Milbury, et al., 2009]. This approach uses a critical temperature (Tc) during the PCR process in order to enrich mutations at any position of the amplified sequence. Indeed, a single nucleotide mismatch anywhere along a double-stranded DNA sequence generates a small change in the melting temperature for that sequence, with mutated sequences melting at a lower temperature than wild-type sequences [Li, et al., 2008]. During COLD PCR, the denaturation temperature is set to Tc, thereby mutation-containing sequences are preferentially denatured and available for primers binding and subsequent amplification. High Resolution DNA Melting (HRM) analysis is a relatively new and rapid method for detecting DNA sequence variants following an initial amplification [Erali, et al., 2008; Vossen, et al., 2009; Wittwer, 2009]. Gene scanning by HRM depends on the recognition of changes in the shape of the amplicon melting curve that result from heterozygous sequence alterations [Erali, et al., 2008]. At the end of PCR amplification, samples are heated to 95°C to ensure a complete denaturation. Next, the temperature is reduced to allow cross-hybridization between wild-type and mutant sequences, leading to the formation of mismatched heteroduplexes, which melt at a lower temperature than wild-type and mutant homoduplex [Vossen, et al., 2009]. In this study, we evaluated the sensitivity of different COLD PCR HRM assays for the detection of *IDH1*^{R132H} mutation in glioma samples.

Material and methods

DNA and tumors samples

Tumor samples were selected from the Pitié-Salpêtrière brain tumor database. Tumor DNA was extracted from frozen tissues using the QIAmp DNA according to the manufacturer instructions (Qiagen). Samples used in this work have previously been tested for the presence of *IDH1* mutation (reference sequence NM_005896.2) by direct sequencing, as already described [Parsons, et al., 2008; Yan, et al., 2009].

Determination of T_c

We applied the fast COLD PCR assays for detecting *IDH1* mutation based on the methodology described in the literature [Li, et al., 2008], with several modifications. We first determined the new denaturation temperature Tc for the reaction. We used the same primers as for conventional PCR assay, which produced an amplicon of 172 bp. To identify the optimal critical denaturation temperature Tc, a series of COLD PCR reactions at graded temperatures below Tm were performed. We then set the Tc at 81°C, the lowest temperature that reproducibly yielded a substantial PCR. In fast COLD PCR, the reaction protocol began with 20 cycles of conventional amplification for an initial build-up of all amplicons, followed by 30 COLD PCR cycles to selectively enrich for Tm-reduced mutant sequences (Figure 1). COLD PCR assays were performed on a LightCycler480 (Roche Diagnostics Corporation) to ensure a precise control of temperature during the experiments.

Comparison of sensitivities of the different assays to detect *IDH1* mutation

To determine if COLD PCR could enhance the sensitivity of *IDH1*^{R132H} mutation detection, we compared the sensitivity of three experimental protocols. Exon 4 of the *IDH1*

gene was first amplified either by conventional PCR or by fast COLD PCR. A second round of amplification was then performed either by PCR HRM, or by fast COLD PCR HRM (Figure 2).

To compare the sensitivity of the different assays, we performed a serial dilution study using a gDNA sample from a grade II oligoastrocytoma patient containing an *IDH1*^{R132H} mutation as the source of the mutant allele. This mutation-containing gDNA sample was serially diluted into wild-type gDNA (ie blood constitutive gDNA from the same patient) to the following percentages: 25%, 10%, 8%, 5%, 4%, 2%, 1%, 0.5%, 0.25%, 0.1%, and 0.05%. In addition, wild-type gDNAs (n=7) were included in each experiment. The same experiment was reproduced with several other mutated DNA samples: another CGT→CAT mutation (Arg132His), and also with other, less frequent mutations, such as CGT→CTT (Arg132Leu) and CGT→AGT (Arg132Ser).

First round of amplification

Each PCR amplification reaction contained 50ng gDNA. PCR cycling conditions for the first round consisted of an initial denaturation step at 94°C for 5 min; 40 cycles of 94°C for 30s; 60°C for 1 min; and 72°C for 1 min30; and final extension at 72°C for 7 min. The reactions were carried out using a Mastercycler (Eppendorf). Conventional PCR reactions contained final reagent concentrations as follows: 1x PCR Master Mix (Abgene), 0.25 µM forward and reverse primers (Invitrogen; Table I) and DNA template. COLD PCR cycling conditions on LightCycler480 (Roche Diagnostics Corporation) are summarized as follows: 96°C, 10 min; 20 cycles of 95°C, 15s; 60°C, 30s, then 30 cycles of 81°C, 15s; 60°C, 30s.

COLD PCR assays contained final reagent concentrations as follows: LightCycler480 HRM Master (Roche Diagnostics Corporation), 0.25 µM forward and reverse primers (Invitrogen), 3 mM $MgCl_2$ and DNA template.

Second round of amplification

PCR amplification was performed with the LightCycler480 (Roche Diagnostics Corporation). Each reaction contained diluted PCR amplicons (1/1000), 0.25 µM forward and reverse primers (Invitrogen, Table I), 3 mM $MgCl_2$ and LightCycler480 HRM Master (Roche Diagnostics Corporation). PCR HRM cycling conditions were as follows: 96°C, 10min; 40 cycles of 95°C, 30s; 60°C, 30s. COLD PCR HRM cycling conditions are summarized as follows: 96°C , 10min; 20 cycles of 95°C, 15s; 60°C, 30s, then 30 cycles of 81°C (Tc), 15s; 60°C, 30s. After amplification, a post amplification melting curve program was initiated by heating to 95°C for 1 min, cooling to 40°C for 1 min, and increasing the temperature to 95°C while continuously measuring fluorescence at 25 acquisitions per degree. Each PCR run contained a negative (no template) control and each amplification was duplicated.

High Resolution Melting (HRM) analysis and direct sequencing

At the end of the second round of amplification, fluorescent melting curves were analyzed usingLC480 Gene Scanning software V1.2.9 (Roche Diagnostics Corporation). All curves were analyzed following normalization, temperature shifting, automated grouping, and the inspection of difference plots. The grouping software uses a curve shape-matching algorithm in order to identify wild type from mutant samples. The 0.2 value was chosen for the grouping sensitivity in all experiments.

Products of each assay were then submitted to the sequencing reaction using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems) as previously described [Sanson, et al., 2009]. After a purification step using BigDye Xterminator Purification Kit (Applied Biosystems), both forward and reverse sequences were determined on an ABI prism 3730 DNA analyzer (Perkin Elmer).

Tumor sample selection and immunohistochemical detection of *IDH1*^{R132H}

Tumors were retrieved from the neuropathology department database according to the following criteria: histological diagnosis of WHO grade II gliomas; presence of *IDH1*^{R132H} mutation confirmed by Sanger sequencing; availability of frozen and paraffin embedded samples of tumor core and tumor edge, with edge biopsies considered as free of infiltrated tumor cells by standard HE staining.

Immunohistochemical staining for IDH1^{R132H} was performed on 4 µm paraffin sections of formalin-fixed tumor samples using mouse monoclonal anti-R132H-IDH1 antibody culture supernatant (a generous gift from Pr A. von Deimling), as previously published [Capper, et al., 2010; Capper, et al., 2009]. Local immunohistochemistry protocol was validated on gliomas samples which were previously analysed by Sanger sequencing for *IDH1*^{R132H} mutation (positive and negative controls). Labelling was defined as positive (at least one cluster of positive tumor cells) or negative (no positive tumor cells detected).

Results

Determination of the sensitivity with dilution studies

To identify the most sensitive method for detecting *IDH1* mutation, we evaluated two techniques after a first stage of conventional PCR: the PCR HRM assay and the COLD PCR HRM assay. In a third assay, we replaced the first conventional PCR by a run of COLD PCR (**Figure 2**). The sensitivity of the three assays was compared using a dilution series obtained by mixing DNA from heterozygous positive control carrying the *IDH1* R132H mutation with wild-type DNA from peripheral blood.

Conventional PCR HRM assay detects mutant DNA at a concentration of 25% in a background of wild-type DNA (**Figure 3A**). In contrast, the COLD PCR HRM assay detects *IDH1*^{R132H} mutation at a much lower concentration: mutant DNA diluted into wild-type DNA to a 2% abundance was still clearly differentiated from normal sequences (**Figure 3B**). Therefore, the COLD PCR HRM assay produced an approximately 10-fold improvement in the *IDH1* mutation detection, as compared to the conventional PCR HRM analysis. Replacing the first conventional PCR amplification by a run of COLD PCR further enhanced selective enrichment of the mutant DNA and improved the detection of *IDH1*^{R132H} mutation. In these conditions, we were able to detect mutant DNA at a concentration as low as 0.25% in mixture with wild-type DNA (**Figure 3C**). Thus, the double COLD PCR HRM assay for the detection of *IDH1*^{R132H} mutation was 100-fold more sensitive than the PCR HRM assay.

To both confirm the selective mutation enrichment and to exclude false positive results, amplicons produced at the end of the three assays were submitted to direct sequencing using the Sanger method. The sequencing chromatograms are presented in Figure 3D. In all three cases, direct sequencing confirmed the presence of the *IDH1*^{R132H} mutation. After

double COLD PCR HRM with 0.25% of mutant DNA/wild DNA, *IDH1*^{R132H} mutation was evident as clearly as for 25% with conventional PCR HRM and 2% with COLD PCR HRM.

The same results were obtained with another glioma with **CGT→CAT mutation (Arg132His), and also with less frequent IDH1^{132} mutations CGT→CTT (Arg132Leu) and CGT→AGT (Arg132Ser) (supplementary figure).**

Comparison of PCR HRM, COLD PCR HRM and double COLD PCR HRM and *IDH1*^{R132H} immunohistochemistry in biopsies of tumor edges

To definitely validate the clinical interest of our technique, we selected 10 pairs of tumor core and tumor edge (one astrocytoma, 5 oligodendrogliomas, and 4 oligoastrocytomas), and performed *IDH1*^{R132H} mutation detection by both immunohistochemistry and molecular techniques (**Table II**). *IDH1*^{R132H} immunohistochemistry was positive in 3 cases (30%, 2 grade II oligodendrogliomas, and one grade II oligoastrocytoma) showing a cytoplasmic granular staining as previously described [Capper, et al., 2010]. **Figure 4A** presents the results of immunohistochemistry staining for patient 8 in the tumor core and in the biopsy edge (lower panel). PCR HRM was positive in 8 cases out of 10 (80.0%; **Figure 4B** upper panel). In contrast, both COLD PCR HRM (data not shown) and double COLD PCR HRM were positive in all cases (100%; **Figure 4B**, lower panel, and **table II**).

Discussion

Gliomas are characterized by a highly invasive phenotype, with tumor cells invading the brain at a distance far from the bulk of tumor [Furnari, et al., 2007]. Therefore biopsy samples are often contaminated by normal brain tissue, and mutated DNA is diluted in a the background of wild-type DNA from surrounding brain tissue, vasculature, and infiltrating lymphocytes [Dobrowolski, et al., 2009]. Because *IDH1*^{R132H} mutation is almost restricted to gliomas [Yan, et al., 2009], it has a major diagnostic potential. Therefore a fast and reliable scanning technique to detect this mutation in clinical samples with very few tumor cells (and appearing therefore normal at neuropathological analysis) is particularly important. In addition *IDH1*^{R132H} mutation is a major prognostic factor [Sanson, et al., 2009; van den Bent, et al., 2010]. Simple HRM has been very recently reported as a fast and sensitive strategy, detecting IDH1 and IDH2 mutations in a 90% normal DNA background [Horbinski, et al., 2010]. In this study, we used fast COLD PCR to selectively amplify *IDH1*^{R132H} mutated DNA and we showed that double COLD PCR HRM assay is a highly reliable method to detect *IDH1* mutation in samples with very few tumor cells (up to 0.25% mutated/non mutated DNA). In addition, this assay is fast (less than three hours) and therefore particularly suitable for routine diagnosis purposes in neuropathology.

Despite *IDH1*^{R132H} accounts for more than 90% of *IDH1* mutations reported in gliomas, other mutations have been described: CGT→AGT, CGT→CTT CGT→GGT, CGT→TGT [Balss, et al., 2008; Gravendeel, et al., 2010; Sanson, et al., 2009; Yan, et al., 2009]. Fortunately, all these mutations, except the CGT→GGT change, result in a lower Tm, rendering them theoretically detectable by our simple double COLD PCR assay. Indeed we were able to detect some of these less frequent mutations (supplementary figure). For the detection of CGT→GGT, a full COLD PCR assay –allowing the enrichment of all possible

mutations- could be performed, though the selective amplification *ratio* will probably be lower than in fast COLD PCR, because amplification, and thus enrichment in mutant sequences, begins earlier during cycling in fast COLD PCR than in full COLD PCR [Li, et al., 2008; Milbury, et al., 2009].

We showed that double COLD PCR HRM (followed by sequencing in case of aberrant HRM profiles) is a powerful method to evidence the presence of tumor cells with IDH1 mutation in apparently "blank" biopsies of grade II gliomas, and is much more sensitive than immunohistochemistry for *IDH1*^{R132H}. Such procedure may be particularly useful in lesions radiologically highly suggestive of glioma, whose biopsy appears non contributive because of the very low proportion of tumor cells. In such cases, finding an *IDH1* mutation will confirm the diagnosis of glioma, avoiding a further invasive procedure to establish diagnosis. Moreover, such approach could also be suitable for *IDH1*^{R132H} mutation detection in the cerebral spinal fluid thus confirming glioma diagnosis without the need for surgery.

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Figures legends

Figure 1: Principle of fast COLD PCR performed for the detection of *IDH1*^{R132H} mutation in glioma samples.

Figure 2: Assays performed to detect *IDH1*^{R132H} mutation in tumor samples.

Figure 3: Comparison of conventional PCR HRM (A), COLD HRM (B) and double COLD HRM (C) for the detection of *IDH1*^{R132H} mutation. Mutant DNA was serially diluted with wild type DNA and each mixture was submitted to the three assays. Sequencing chromatograms after conventional PCR HRM, COLD HRM and double COLD HRM (D).

Figure 4: Detection of *IDH1*^{R132H} mutation in grade II gliomas edges. A. *IDH1*^{R132H} immunohistochemistry in patient 8 tumor core (upper panel) and in tumor edge (lower panel); magnification x200. **B.** *IDH1*^{R132H} detection by PCR HRM (upper panel) and double COLD PCR HRM (lower panel)

Table I: Primers used for first and second rounds of amplification

	Primer		**Amplicon length**
First amplification	Forward	CGGTCTTCAGAGAAGCCATT	172 bp
	Reverse	CACATACAAGTTGGAAATTTCTGG	
Second amplification	Forward	CGGTCTTCAGAGAAGCCATT	129 bp
	Reverse	GCAAAATCACATTATTGCCAAC	

Table II: Comparison of IDH1^{R132} immunohistochemistry with PCR HRM and double COLD PCR HRM for the detection of *IDH1*^{R132H} mutation in tumor edges. For all techniques, results are expressed as positive (+) or negative (-). COLD PCR HRM (data not shown) gave the same results as double COLD PCR HRM.

Patients	Histology	IDH1^{R132} immunohistochemistry		PCR HRM		Double COLD PCR HRM	
		Tumor core	Tumor edge	Tumor core	Tumor edge	Tumor core	Tumor edge
1	OAII	+	-	+	+	+	+
2	OII	+	-	+	-	+	+
3	AII	+	-	+	+	+	+
4	OAII	+	-	+	+	+	+
5	OAII	+	+	+	+	+	+
6	OII	+	-	+	+	+	+
7	OII	+	+	+	+	+	+
8	OII	+	+	+	+	+	+
9	OAII	+	-	+	+	+	+
10	OII	+	-	+	-	+	+

AII: grade II oligoastrocytoma; OAII: oligoastrocytoma; OII: oligodendroglioma.

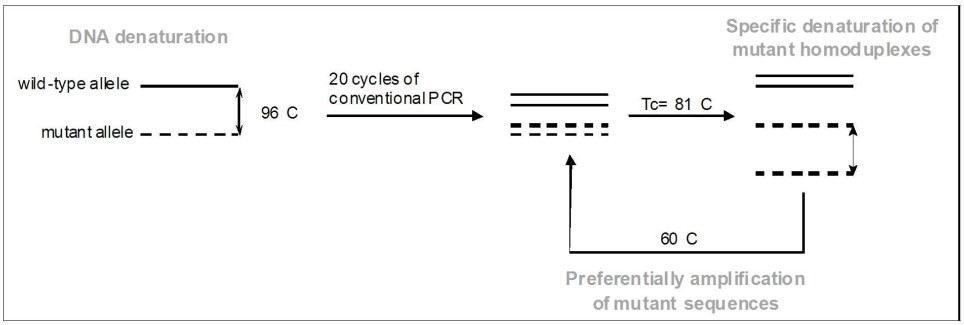

Figure 1
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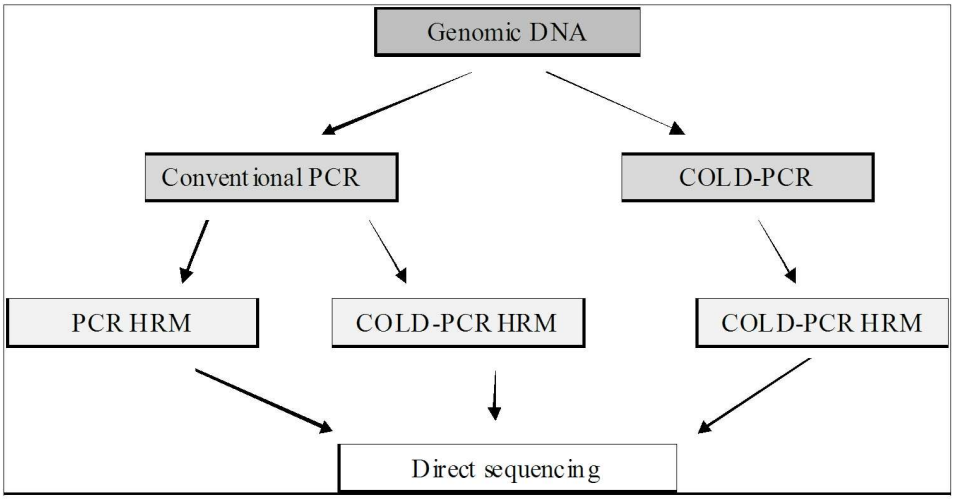

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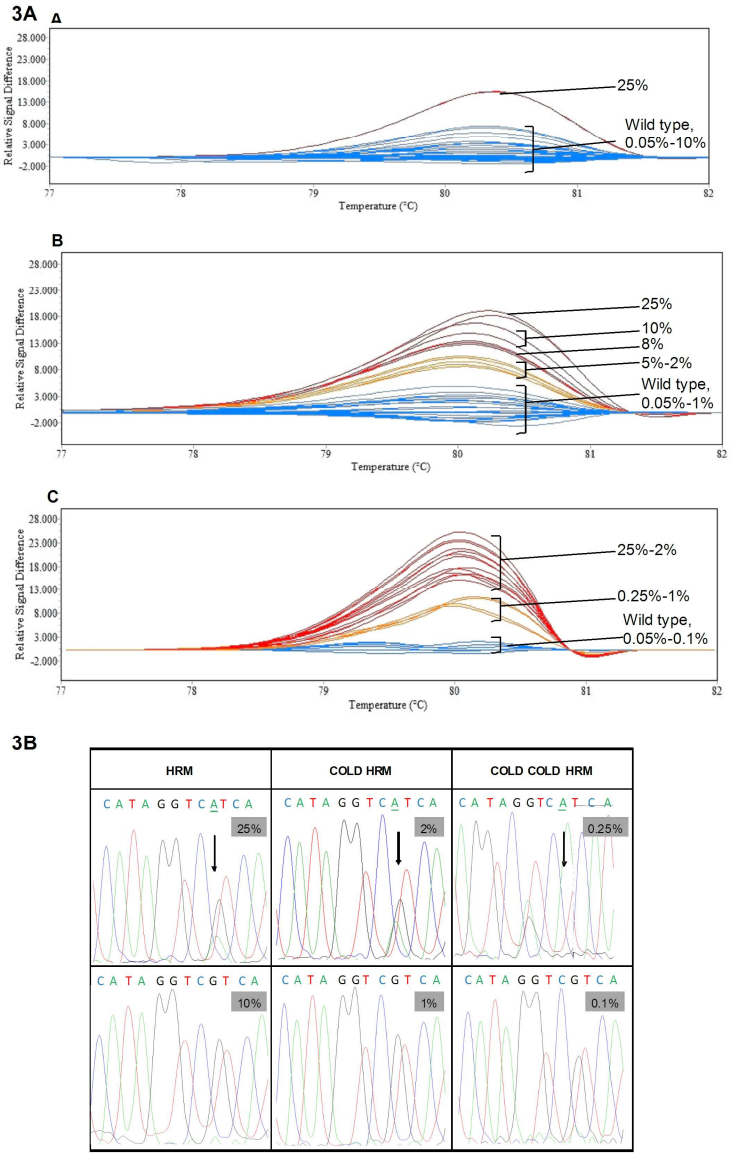

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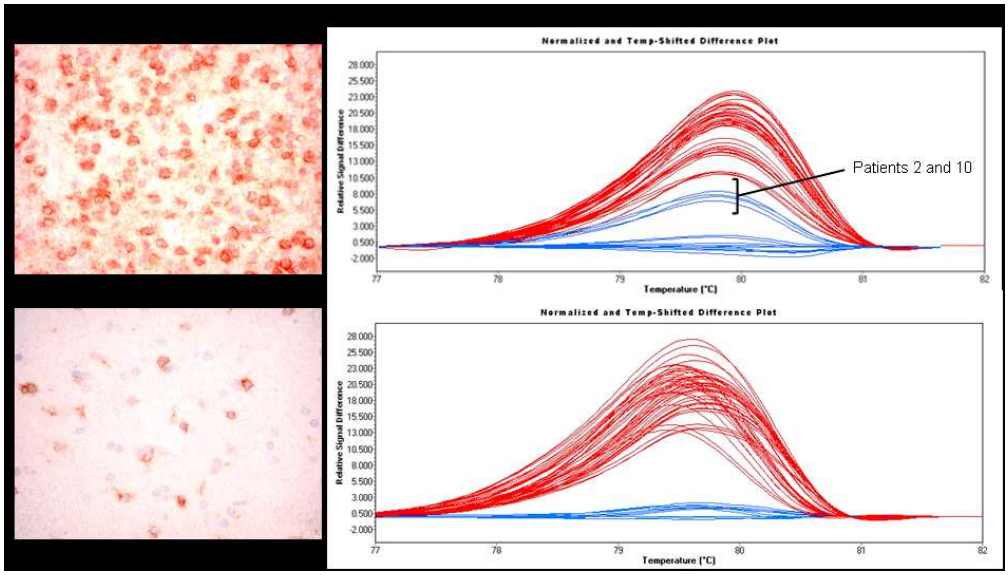

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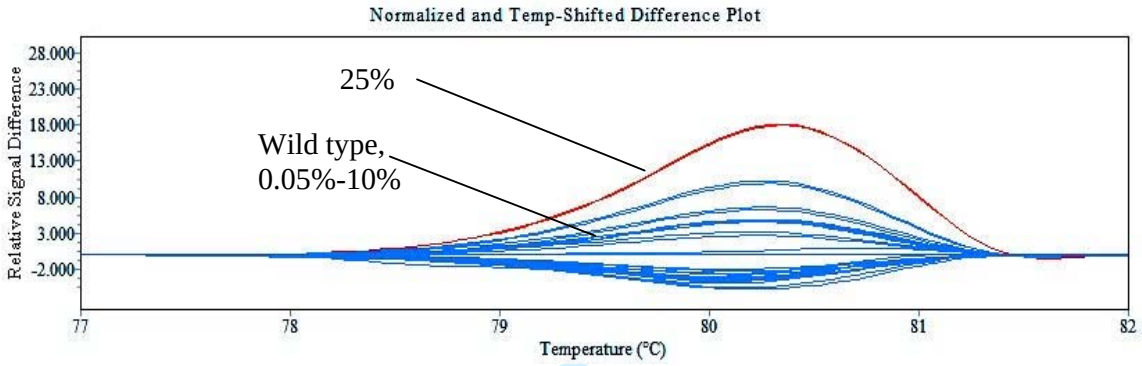
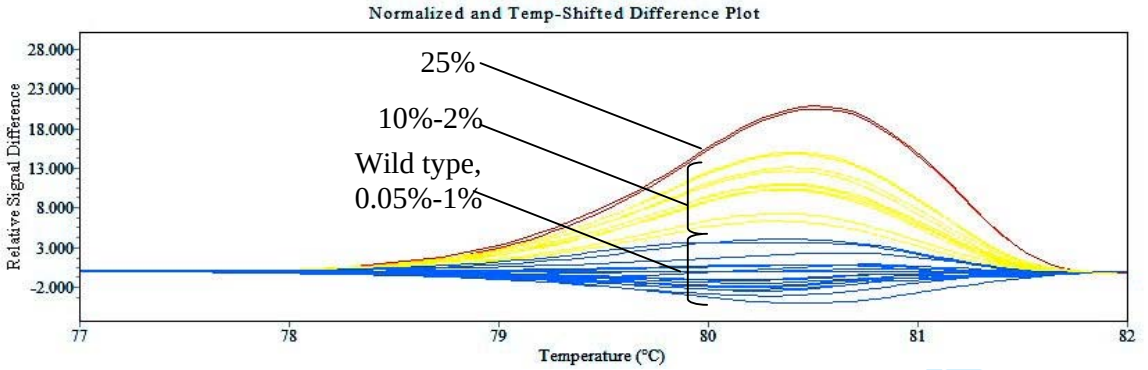
Supplementary figure showing conventional PCR HRM (top), COLD HRM (middle) and double COLD HRM (bottom) for several different mutations of IDH1 codon 132.

MUTATION CGT → CTT

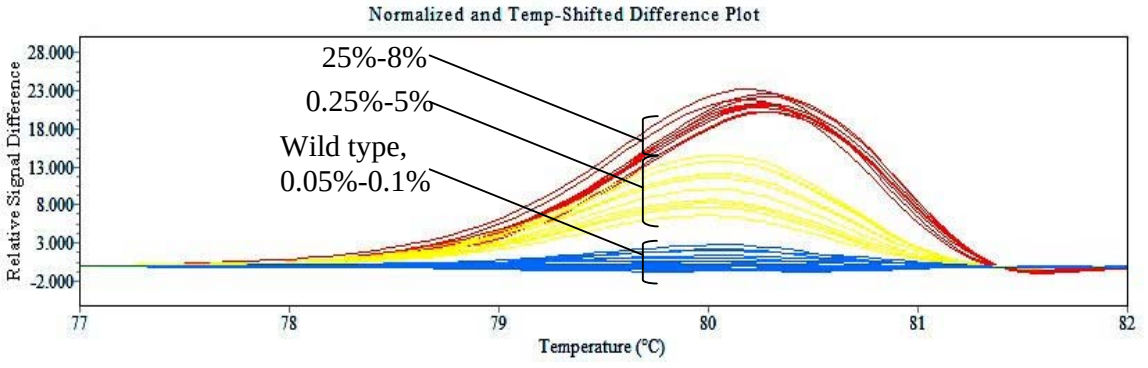
PCR HRM



COLD HRM

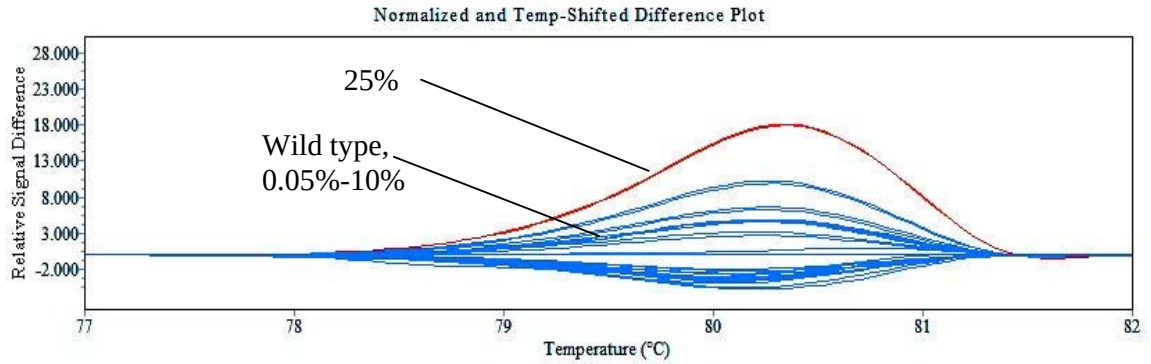


Double COLD HRM

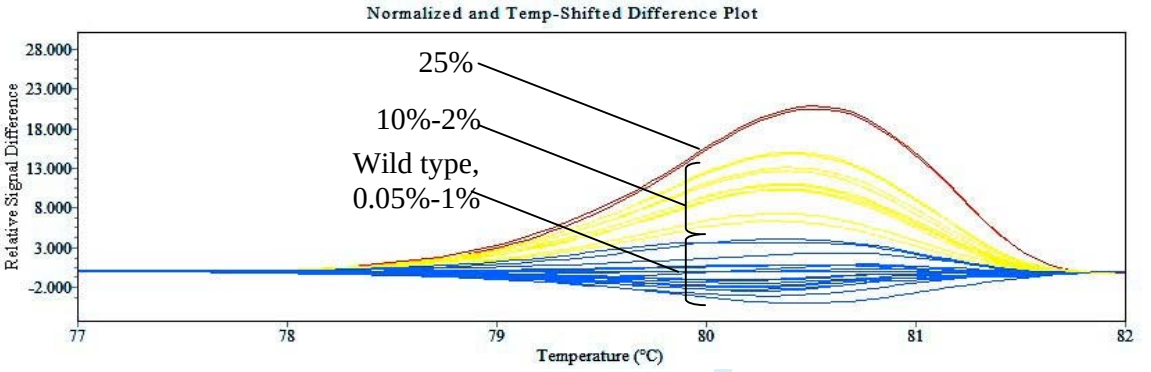


MUTATION CGT → CTT

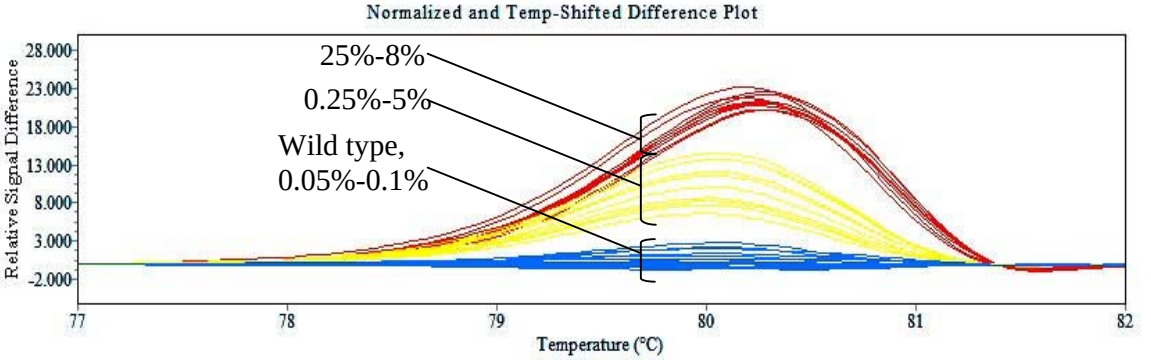
PCR HRM



COLD HRM

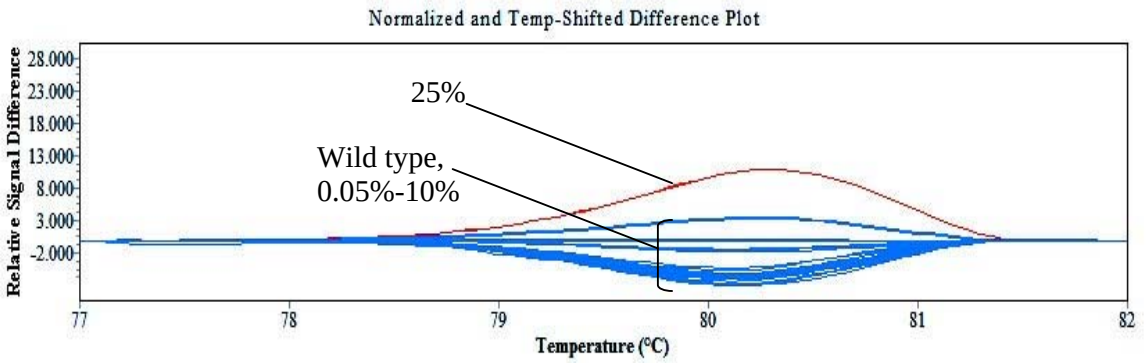


Double COLD HRM

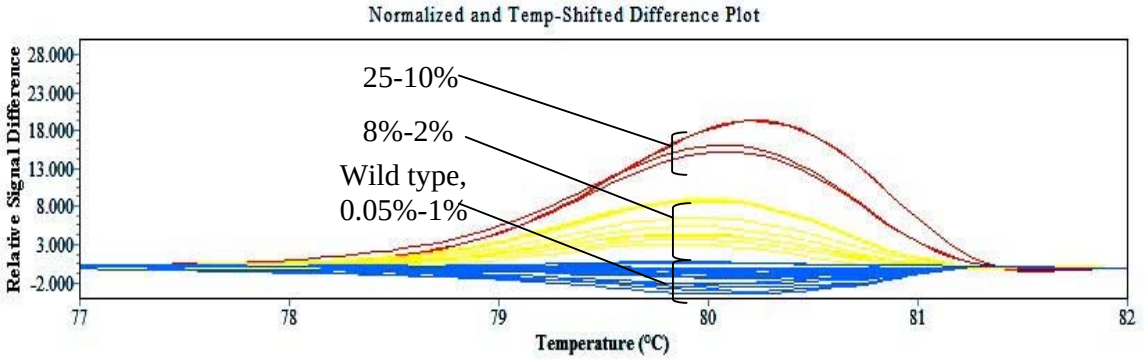


MUTATION CGT → AGT

PCR HRM



COLD HRM



Double COLD HRM