Single-tube reaction using peptide nucleic acid as both PCR clamp and sensor probe for the detection of rare mutations

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The detection of rare mutant DNA from a background of wild-type alleles usually requires laborious manipulations, such as restriction enzyme digestion and gel electrophoresis. Here, we describe a protocol for homogeneous detection of rare mutant DNA in a single tube. The protocol uses a peptide nucleic acid (PNA) as both PCR clamp and sensor probe. The PNA probe binds tightly to perfectly matched wild-type DNA template but not to mismatched mutant DNA sequences, which specifically inhibits the PCR amplification of wild-type alleles without interfering with the amplification of mutant DNA. A fluorescein tag (which undergoes fluorescence resonance energy transfer with the adjacent fluorophore of an anchor probe when both are annealed to the template DNA) also allows the PNA probe to generate unambiguous melting curves to detect mutant DNA during real-time fluorescent monitoring. The whole assay takes about only 1 h. This protocol has been used for detecting mutant K-*ras* DNA and could be applied to the detection of other rare mutant DNAs.

INTRODUCTION

Detection of rare mutant DNA from a background of wild-type alleles is important but sometimes difficult in the laboratory. Such rare mutations are commonly found in a cancer-related gene, a mitochondrial gene at a low heteroplasmic frequency or a gene from a small subpopulation of bacteria or viruses, for example. They may only consist of a single change in the DNA sequence (e.g., a point mutation) and usually exist in very low abundance in the samples compared with the wild type. Therefore, assays to detect such mutant DNA must be very specific and sensitive. In addition, if mutations are identified as useful markers, a simple and rapid method for their detection is needed to facilitate the screening of a large number of samples. Conventional methods to enrich the mutant signal include allele-specific amplification[1], sequence-specific ligation[2] and restriction enzyme digestion of wild type DNA[3–5]. These methods usually require subsequent detection procedures, such as gel electrophoresis[6–8], hybridization[9], mass spectrometry[10,11] or denaturing high-performance liquid chromatography[12], which can be laborious and increase the risk of contamination.

Recently, we have developed a method to detect rare mutant K-*ras* DNA in a single closed tube[13], which can also be applied to the detection of other rare mutant DNAs in clinical samples. The method uses a PNA as both PCR clamp and fluorescent sensor probe. This allows PNA-mediated PCR clamping to be undertaken, followed by fluorescent melting curve analysis, which enables the selective PCR amplification of mutant templates and their detection in a single tube without further laborious procedures (see **Fig. 1** for a schematic diagram).

Fluorescent probes and melting curve analysis

The use of fluorescent probes and melting curve analysis for genotyping was first introduced by Lay *et al.*[14] and was soon modified to include a pair of hybridization probes[15]. The probes, namely a sensor and an anchor, are two oligonucleotides labeled with different fluorophores between which fluorescence resonance energy transfer (FRET) can occur (see **Fig. 1b**): when these two fluorophores are close to each other, one of them (the donor) absorbs excitation light and transfers the energy to the other fluorophore (the acceptor), which in turn emits a specific wavelength of light. This acceptor emission reveals the status of probe binding because FRET occurs only when these two probes anneal to adjacent sites of a complementary DNA strand. Conventionally, a longer probe with a higher melting temperature (T_m) serves as an anchor because it remains annealed to the complementary DNA at temperatures that cause the dissociation of a shorter probe with a lower T_m. Monitoring the acceptor emission generated by FRET along with temperature change produces a melting curve that displays the interaction between probes and the complementary DNA (see **Fig. 1c**). Because the shorter probe dissociates from the complementary DNA first, resulting in a drop in the intensity of the acceptor emission, the melting curve profile actually reveals the behavior of the shorter probe. When the shorter probe is positioned over the variable region, it serves as a sensor because any change in DNA sequence in this region results in a shift in its T_m, altering the melting curve profile. Today, a combination of real-time PCR and hybridization probes has become a powerful tool for the detection of single-nucleotide polymorphisms causing inherited diseases[16–18].

Genotyping single-nucleotide polymorphisms responsible for inherited diseases using hybridization probes is easy, as these kinds of sequence variations can comprise up to 50% (if the mutation is heterozygous) or even 100% (in the case of a homozygous mutation) of the total alleles present. However, applying hybridization probes to the detection of somatic mutations in clinical samples (the most frequently observed of which occur in cancer cells, such as the K-*ras* or *p53* mutations) can be difficult, because the wild-type alleles from normal cells usually

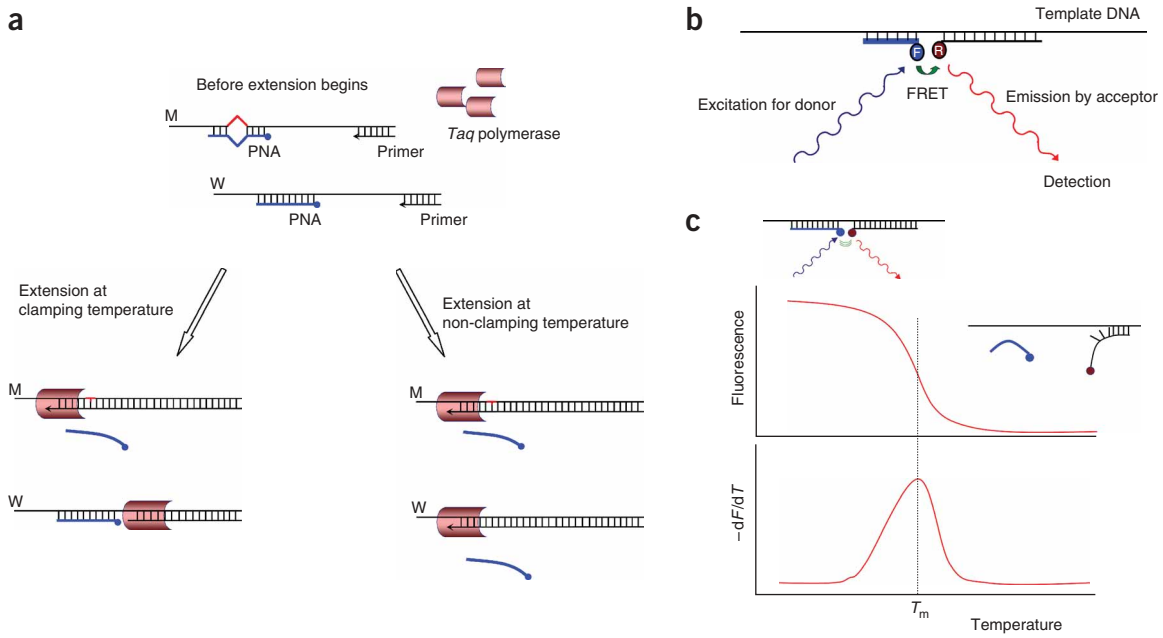

Figure 1 | Schematic diagram of the role of the PNA probe. This diagram reveals the role of the PNA probe in (**a**) PCR clamping to allow selective amplification of mutant alleles, (**b**) the generation of FRET signal in combination with an anchor probe and (**c**) detection of mutations by fluorescent melting curve analysis. In **a**, PNA probes (shown as thick blue lines) bind to the wild-type template (labeled with a W) perfectly but bind to the mutant template (labeled with an M) with a mismatch at the mutation site (shown in red on the mutant template). Under non-clamping PCR conditions (see the right-hand side of the diagram), *Taq* polymerase easily repels PNA from both templates. Therefore, both wild-type and mutant alleles are amplified. Under clamping PCR conditions, however (see the left-hand side of the diagram), the PNA can be repelled from the mutant template but remains tightly bound to the wild-type template, blocking its extension by *Taq* polymerase. This is known as PNA-mediated PCR clamping and ensures selective PCR amplification of mutant template to enrich for rare mutant alleles. For simplification, the anchor probe and the other template strand that are also present within the reaction tube are not shown in this diagram. The PNA also acts as one of a pair of fluorescent hybridization probes (known as sensor and anchor probes) to allow the detection of mutations by fluorescent melting curve analysis. In **b**, a FRET signal is generated when both the PNA sensor probe (shown as a thick blue line) and the oligonucleotide anchor probe (shown as a thick black line) anneal to the template DNA, bringing their fluorophores into close proximity. When excitation light (represented by a blue arrow) is applied, the donor fluorophore (in this case, fluorescein, labeled with an F) absorbs the light and transfers the energy to the acceptor fluorophore (in this case, Red640, labeled with an R) by FRET, which in turn generates acceptor emission (represented by a red arrow). In **c**, monitoring the acceptor emission along with temperature change generates a melting curve; at lower temperature, both the sensor and the anchor probes anneal to the template and FRET occurs, which generates intense acceptor emission. As the temperature increases, first the sensor probe then the anchor probe dissociate from the template, which abolishes FRET and results in a drop in the intensity of acceptor emission (see top graph). The temperature at which the PNA sensor probe dissociates from the template, abolishing FRET signal, is known as the T_m of the PNA/template complex and is represented as a peak on a graph plotting the negative derivative of fluorescence over temperature ($-dF/dT$) against the temperature change (see lower graph). This T_m is different for wild-type/PNA complexes and mutant/PNA complexes, which allows mutant sequences to be distinguished from wild-type alleles during melting curve analysis.

account for the majority of DNA and only relatively small amounts of mutant alleles exist. The wild-type template can exhaust essential reagents during PCR, and its product will mask the mutant signal during melting curve analysis. Conventional methods to enrich the mutant PCR products, such as restriction enzyme digestion or sequence-specific primer extension, are not compatible with hybridization probes as they need a PCR primer that extends to the variable region of the template, which will compete with a probe that binds to the same region.

PNA-mediated PCR clamping

PNA is a synthetic DNA analog in which the normal phosphodiester backbone is replaced with an *N*-(2-aminoethyl)glycine chain. Its nucleobases complement DNA or RNA in the normal A-T and G-C geometry[19–21]. With the artificial backbone, PNA is resistant to nuclease activities. The PNA–DNA duplex is more stable than the DNA–DNA duplex. However, a single mismatch in the PNA–DNA duplex causes a drop in T_m much greater than that in the DNA–DNA duplex[19]. Therefore, within an appropriate temperature range, PNA can specifically bind to wild type template and inhibit primer annealing or chain elongation of perfectly matched template, without interfering with the amplification of mismatched template, which is known as "PNA-mediated PCR clamping" (see **Fig. 1a**)[22]. PNA-mediated PCR clamping has been widely used for enrichment of rare mutant alleles, including K-*ras* mutations[23] and mutations in mitochondrial DNA[24], the *uidA* gene of *Escherichia coli* O157:H7 strain[25] and the DNA polymerase gene of hepatitis B virus[26,27]. However, electrophoretic analysis was still required in these assays.

The addition of a PNA clamp into a PCR that contains a pair of hybridization probes[28–30] or hydrolysis probes (also known as TaqMan probes)[31] allows homogeneous detection of rare mutant DNA in a closed tube. However, in those designs, the added PNA competes with the sensor probe for DNA binding. The sensor probe, therefore, should be mutation specific, that is, it complements one of the mutant alleles instead of the wild-type allele. Some researchers introduced a DNA analog, the locked nucleic acid (LNA), as the mutation-specific probe for better competition

with PNA clamp (LNA probes have a higher affinity and better discrimination between wild-type and mutant templates than DNA probes)[31]. The mutation-specific probe allows the mutation type to be determined, as it correlates to the specific probe sequence. However, the disadvantage is that if more than one type of mutation occurs in the target region, several probes would need to be synthesized and tested for their efficiency and compatibility when combined in the same reaction.

We have now introduced a novel design that uses PNA as the sensor probe. There are several advantages of this design: first, as the sensor probe is complementary to the wild-type sequence, only one probe is sufficient to distinguish nearly all mutation types within the probe-binding region from the wild type by their T_m shift. Second, the T_m of PNA with both wild-type and mutant sequences can be initially determined by melting curve analysis following PCR amplification to provide useful information for the optimization of thermal conditions for PCR clamping. Third, a PNA probe shows a greater T_m difference between perfectly matched templates and mismatched templates than a DNA probe, which facilitates the identification of mutant melting peaks.

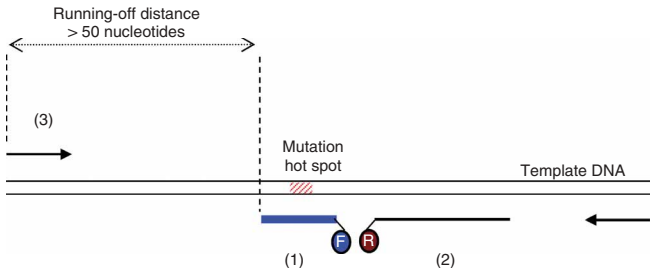

Figure 2 | Probe and primer design. The figure shows (i) a PNA sensor probe (represented by a thick blue line), labeled at the 5′ end with fluorescein (represented by an F), spanning the region of interest (mutation hot spot, represented by a checked box), (ii) an oligonucleotide anchor probe (represented by a thin black line), labeled with a fluorophore at its 3′ end, for example, LC-Red640 (represented by an R) annealing within 2–3 bp of the PNA probe and (iii) oligonucleotide PCR primers (represented by arrows). The primer annealing to the opposite strand as the PNA probe should anneal at least 50 nucleotides away from the PNA-binding site to give an adequate running-off distance for *Taq* DNA polymerase (see the Experimental Design section of INTRODUCTION for further explanation).

Possible applications and limitations

This method is very useful to detect rare alleles at "hotspots" of sequence variation. Most previously reported assays that used PNA for mutant enrichment could be modified using our design to reduce the effort of subsequent analysis, including, for example, the detection of cancer-related mutations (e.g., mutations in the *ras* oncogene or *p53* tumor suppressor gene[11,23]), mitochondrial DNA mutations during aging[24], mutations related to drug resistance in a subpopulation of cancer (e.g., the mutation in the epidermal growth factor receptor gene[31]) and mutations related to virulence or drug resistance in a bacterial or viral subpopulation[25–27]. This method may also have the potential to detect epigenetic changes such as methylation and can be applied to DNA obtained from blood samples (whole blood, serum or plasma), other body fluids, tissue biopsies, paraffin-embedded samples or cultured cells.

However, only mutations in the region covered by the PNA probe can be detected. Therefore, the protocol is not suitable for genes without known mutation hotspots and cannot be used to screen for unknown mutations. In addition, the protocol only differentiates mutants from the wild type. To identify the individual mutation types, subsequent DNA purification and sequence analysis are required. Moreover, detecting mutations that affect more than 2 bp may be difficult, as the melting peak may shift to a temperature too low to be observed, although the enrichment of mutant alleles still occurs.

In addition to these limitations, all PCR-based methods (including this protocol) for mutant enrichment face a common problem: *Taq* DNA polymerase is error prone when incorporating nucleotides. Its error rate is estimated at around or higher than 1×10^{-5} per nucleotide per amplification cycle[32], which means it can be expected to generate one mutation in 10,000 amplifications of a 10-bp target region. When using a small amount of DNA as template or when mutant template DNA represents less than 0.1% of total alleles present, PCR error may, therefore, lead to a false-positive result. For these kinds of samples, PCR should be performed in duplicate and the products should be sequenced to check if the mutation type is as expected. Theoretically, using a thermal stable polymerase with proofreading activity (e.g., *Pfu* polymerase) may generate fewer mutations during PCR and therefore increase the assay sensitivity. Unfortunately, *Pfu* polymerase has poor PCR efficiency in our reaction buffer: the reaction conditions for these polymerases may require further optimization.

Experimental design

Probes and primers. The success of this protocol greatly relies on good design of probes and primers. The key features of the design are depicted in **Figure 2**: the PNA sensor probe (complementary to

TABLE 1 | An example design of primers and probes for detection of K-*ras* mutations at codons 12 and 13 (ref. 13).

Description	Sequence (5′ → 3′ for oligonucleotide or N-terminus → C-terminus for PNA)	Length (%GC)	Estimated T_m[a]
PNA sensor probe[b]	(Fluorescein)-O-CCTACGCCACCAGCTCC	17-mer (70%)	79 °C at 1 μM[c]; 68 °C at 1 nM[c]
Oligonucleotide anchor probe[b]	GTCCACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACTCT-(Red 640)	44-mer (41%)	73.6 °C
Oligonucleotide primer (opposite)[d]	ATTAACCTTATGTGTGACAT[e]	20-mer (30%)	54.2 °C
Oligonucleotide primer	CAAGATTTACCTCTATTGTT[e]	20-mer (30%)	52.4 °C

[a]The T_m for PNA was estimated on the website: http://www.appliedbiosystems.com/support/pnadesigner.cfm; those for oligonucleotides were calculated using the software PerlPrimer (see Experimental Design). [b]The two probes separate from each other by two nucleotides when annealing to the complementary strand. [c]The actual T_m is 69 °C in the buffer of this protocol. [d]The oligonucleotide primer that anneals to the opposite strand from the PNA probe (labeled "opposite") anneals 97 nucleotides away from the PNA-binding site. [e]This primer pair generates an amplicon of 191 bp.

TABLE 2 | Program setting of LightCycler.

Segment	Target temperature (°C)	Incubation time (s)[c]	Transition rate (°C s^{-1})[d]	Acquisition mode[f]	Description
Program: preincubation (one cycle; analysis mode: none)					This program is for denaturation of template DNA and activation of hotstart *Taq*
1	94	120	20	None	
Program: thermal cycling (50 cycles; analysis mode: quantification)					This program is for PCR amplification of template
1	94	0	20	None	1. For denaturation
2	70	5	20	None	2. For PNA binding
3	54[a]	5	20	Single	3. For annealing and generation of amplification curve
4	72[b]	7–15	2	None	4. For extension
Program: melting curve analysis (one cycle; analysis mode: melting curves)					This program is for generation of melting curves
1	95	2	20	None	1. For denaturation
2	45[e]	0	0.7	Step	2. For generation of melting curves

[a]The annealing temperature (52–60 °C) should be tested for each PCR primer. [b]Set the extension temperature at 72 °C for non-clamping PCR and at a temperature 5–10 °C lower than the wild-type T_m for clamping PCR. [c]An incubation time of 7–15 s is required for extension of a 200-bp amplicon. For the other segments of the thermal cycling program, a much shorter incubation time (0–5 s) is sufficient, as the reactions usually complete during temperature transition. [d]The transition rate for denaturation must be set at 20 °C s^{-1} (as discussed in Experimental design) but it is not critical for other segments. [e]The melting curve is generated by a downward temperature change to 45 °C, as this temperature allows complete annealing of the sensor probe. [f]The LightCycler provides several acquisition modes for fluorescence signals. In the thermal cycling program, the mode "Single" is used during the annealing segment to measure once per cycle the fluorescent signal generated by FRET. In the melting curve analysis program, the mode "Step" (means stepwise) is used to measure the fluorescent signal after each temperature transition (e.g., each 0.7 °C temperature shift).

the wild-type sequence) should be 15–18 bp in length, labeled with fluorescein at its N-terminus via an O-linker and cover the variable region of a target gene. This PNA probe should have a T_m around 70 °C (this is concentration dependant, which can be estimated on the website http://www.appliedbiosystems.com/support/pnadesigner.cfm); the oligonucleotide anchor probe should be 40–45 bp in length, anneal to the same template strand 2–3 nucleotides away from the PNA probe and have a fluorophore label (which will undergo FRET with the fluorescein of the PNA probe) at its 3′ end, for example, LC-Red640. It is crucial that the fluorophore label is not placed next to a guanine, as the base may quench the fluorescence. The anchor probe must have a T_m at least 5 °C higher than that of the PNA probe (introduction of LNAs or minor groove binder into this oligonucleotide can increase its T_m (refs. 31,33,34)); a pair of oligonucleotide PCR primers (20–25 bp in length with T_m around 52–60 °C) should be designed to amplify a 140–200 bp fragment of DNA containing the mutation hotspot of interest (PCR primers can be designed through the assistance of computer software, e.g., PerlPrimer, which can be downloaded on the website http://perlprimer.sourceforge.net/). The primer that anneals to the opposite strand from the PNA probe should be positioned at least 50 nucleotides away from the PNA-binding site. The position of this "opposite primer" determines the running-off distance of *Taq* polymerase (i.e., the distance for polymerase to proceed from PNA-binding site to the end of the amplicon). The longer the distance, the more efficient the clamping of wild-type amplification (see below for further discussion). However, to increase PCR efficiency, the whole amplicon should not be larger than 200 bp. For assays using DNA purified from plasma or paraffin-embedded samples, the amplicon size should be further reduced, as such DNA is often fragmented. An example primer and probe set is detailed in **Table 1**.

PCR conditions. In addition to the design of probes and primers, several important parameters must be considered during

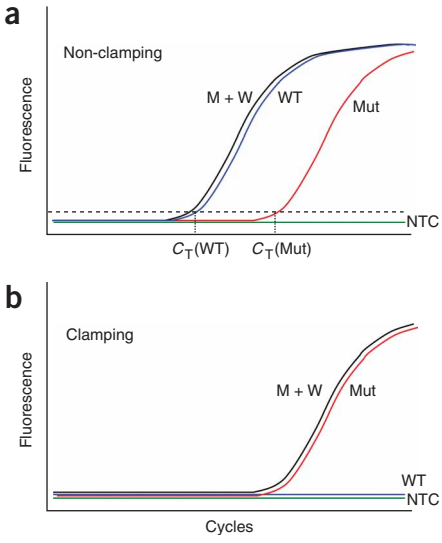

Figure 3 | Amplification curves of control PCR reactions under (**a**) non-clamping or (**b**) clamping conditions. The control PCR reactions use four different templates: distilled water (NTC; shown as green lines), 300 copies of a mutant allele (Mut; shown as red lines), 30,000 copies of the wild-type allele (WT; shown as solid black lines) and a mixture of mutant (300 copies) and wild-type (30,000 copies) alleles (M+W; shown as blue lines). The fractional cycle number at which the fluorescence passes the fixed threshold (shown as a dashed black line) is designated as C_T. Under non-clamping PCR conditions (**a**), reactions with templates WT and M+W should generate the same amplification curves, as they have nearly the same amount of amplifiable alleles (differing by only 1%). The reaction with template Mut will generate a curve in parallel but with a threshold cycle (C_T) 6–7 cycles higher (because $\log_2 100=6.7$; here 100 represents that the amount of template WT is 100 fold of template Mut). Under clamping PCR conditions (**b**), as the wild-type amplification is inhibited, reactions with template Mut and M+W should both generate the same amplification curves, whereas the amplification of template WT should be the same as an NTC.

PCR. First, the extension temperature should be 5–10 °C lower than the T_m of the PNA/perfectly matched template duplex (known as the wild-type T_m) but not lower than the T_m of the PNA/mismatched template duplex (known as the mutant T_m). This ensures that PNA remains bound to wild type templates during PCR extension steps but dissociates or is easily repelled by *Taq* polymerase from mutant templates, which completely inhibits chain elongation from the wild-type template but allows the elongation of mutant templates (see **Fig. 1a**). Second, the temperature ramp from the extension step to the next denaturing step during PCR should be as steep as possible to avoid the wild-type template from escaping PNA clamping during the temperature increase: when the temperature reaches the wild-type T_m, PNA dissociates from the wild-type template and polymerization can occur until the polymerase also dissociates at an even higher temperature. During this narrow "window of chance," if polymerase dissociates before running off the wild-type amplicon, the extension step generates only a truncated product, which cannot serve as a template in the next cycle of amplification, as this shorter product lacks the binding site of the reverse primer. In this case, there is no "chain reaction," but only linear amplification occurs on the template strand without PNA binding, which could not generate detectable signals. In contrast, if polymerase runs off the wild-type amplicon, clamping fails. Increasing the temperature ramp and the running-off distance ensures successful clamping of wild-type amplification. Among the commercial real-time PCR machines, the air-heated thermal cyclers with capillary tubes, such as Roche's LightCycler systems, have the steepest temperature ramp. Their transition rate of temperature can reach 20 °C s^{-1} and are chosen for this experiment.

Measurement of fluorescent signal. During PCR amplification, the fluorescent signal generated by FRET (see **Fig. 1b**) is measured once per cycle (see **Table 2**) and increases along with accumulation of PCR products, as the PCR products provide complementary DNA strand for annealing of both probes. These data, therefore, generate an amplification curve (on the graph "Fluorescence vs Cycles"; see **Figs. 3** and **4**), which can be used for measurement of PCR efficiency and for quantification of templates. During the melting curve analysis that follows PCR amplification of template, the fluorescent signal is measured after each temperature transition (e.g., each 0.7 °C temperature shift in this protocol; see **Table 2**) to generate a melting curve (on the graph "Fluorescence vs Temperature"; see **Fig. 1c**). The LightCycler converts the melting curve into another format, the curve on the graph "–dF/dT versus temperature" (where dF/dT means derivative of fluorescence over temperature; see **Figs. 1c** and **5**), in which the inflection point of the reduction in fluorescence that occurs in the melting curve at the probe T_m is displayed as a peak.

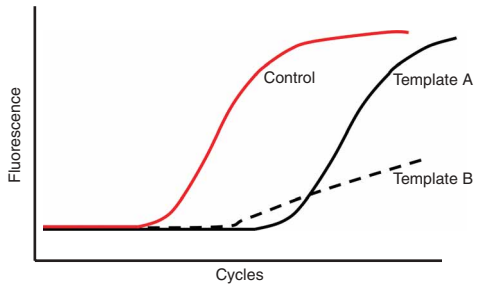

Figure 4 | Determination of PCR efficiency as well as template quality through the amplification curves: the quality of template A is good, as its amplification curve (represented by the solid black line) is similar to that of the control template (represented by the red line). The steep amplification curves suggest that both reactions are efficient. In contrast, the reaction with template B (represented by the dashed black line) is inefficient, probably because the template contains impurities.

Template DNA. Good-quality DNA is required for this assay. DNA must be free of contaminants that may interfere with PCR reactions, such as hemoglobin, polysaccharides, alcohol, humic acid or high salt concentrations. DNA with high molecular weight is not necessary, as the amplicon size is shorter than 200 bp. Several methods can be used to prepare good-quality DNA. The silica membrane-based spin columns provided by Qiagen are convenient tools for DNA purification, for example, QIAamp DNA Blood Kits for blood samples or DNeasy Tissue Kits for animal tissues. Qiagen also provides guidelines for purifying DNA from different sources of samples (http://www1.qiagen.com/Products/GenomicDnaStabilizationPurification/), including those hard to handle, such as plasma and paraffin-embedded tissues. The quality of DNA can be measured by a spectrophotometer: the absorbance ratio at 260 nm/280 nm should be around 1.8. However, for clinical samples, it is sometimes difficult to obtain sufficient DNA for spectrophotometry, in which case quality can be determined through the analysis of amplification curves: under non-clamping PCR, an efficient reaction with high quality template will generate a steep amplification curve in parallel with a curve generated from control DNA (see DISCUSSION) of known high quality. Inefficient PCR will, however, produce a curve with a much shallower gradient (see **Fig. 4**). If reaction conditions are the same as those of control reactions, inefficient PCR is usually due to low quality of sample DNA.

This assay can detect mutant DNA from at least a 100-fold excess of wild-type alleles (and perhaps even from a 1,000–10,000-fold excess of wild type template, in our experience). Less than ten copies of mutant template have also been successfully amplified and have generated detectable signals in both the amplification curve and the melting curve. The detection of a mutation in sample DNA is determined by two important parameters that should be determined for each sample source: the quantity of template DNA used in PCR and the percentage of mutant present in the total alleles. For example, if 10 ng of human genomic DNA (equivalent to 3,000 copies of the haploid genome) is used as the template, the mutation of a single-copy gene with abundance greater than 1/300 is detectable. Increasing the template to 100 ng means that mutants with abundance greater than 1/3,000 can be identified. However, for certain clinical samples, for example, plasma DNA, using more than 100 ng of DNA as template for a screening test is not practicable (as the concentration of plasma DNA in most cancer patients ranges from 10 to 100 ng ml^{-1}; ref. 35). On the other hand, using too much DNA (e.g., more than 1 μg) as template may result in a lower PCR efficiency, and reaction volumes are limited by the capacity of the capillary tubes. It is therefore difficult to specify the amount of template DNA to include, but we usually include the total DNA extracted from 0.1 to 0.2 ml plasma as template, split into two duplicate reactions.

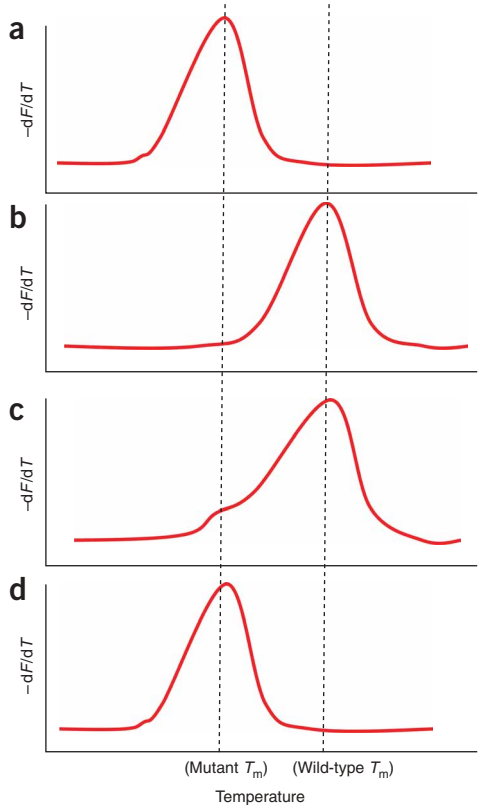

Figure 5 | Typical melting curves: (**a**) a mutant peak; (**b**) a wild-type peak; (**c**) the melting peak generated by a non-clamping PCR using a mixture of abundant wild-type DNA and rare mutant DNA as template; and (**d**) the melting peak generated by a clamping PCR using a mixture of abundant wild-type DNA and rare mutant DNA as template. The vertical axis is the negative derivative of fluorescence over temperature ($-dF/dT$).

Control experiments. Template DNA for control experiments can be purified from cell lines or microbial strains with known sequence in the target gene, through which high-quality and unlimited template can be easily obtained. However, it is sometimes difficult to find a cell line or microbial strain that harbors the sequence variation of interest. In this case, the mutant template can be generated by site-directed mutagenesis on a wild-type template that has been cloned into a suitable plasmid (e.g., using the Quick-Change II Site-Directed Mutagenesis Kits provided by Stratagene). Sequences of template DNAs should be checked before being used for control experiments. Usually, we use a group of control reactions to optimize the conditions during the initial assay setup and to check the quality of the assay during sample screening. These control templates are (i) distilled water (no template control (NTC)), (ii) 300 copies of the mutant allele to be detected (Mut), (iii) 30,000 copies of the wild-type allele (WT) and (iv) a mixture of mutant (300 copies) and wild-type (30,000 copies) alleles (M+W). If the target is a single-copy gene in human cells, the amount of 300 copies is equivalent to 1 ng of genomic DNA. Examples of amplification and melting curves from such control reactions can be seen in **Figures 3** and **5**. A flow diagram to illustrate the stages of assay design and sample analysis is shown in **Figure 6**.

MATERIALS

REAGENTS

- PNA sensor probe with fluorescein label at the N-terminus (Panagene)
- Oligonucleotide anchor probe with fluorescent dye LC-Red640 at the 3′ end (TIB Molbiol)
- Oligonucleotide PCR primers (MWG Biotech or Purigo Biotech)
- Native *Taq* DNA polymerase supplied with *Taq* antibody for hot start, for example, Platinum *Taq* (Invitrogen, cat. no. 10966-030) or Titanium *Taq* (BD Bioscience, cat. no. S1792) ▲ **CRITICAL** Modified hot start *Taq* such as Roche's FastStart *Taq* or ABI's AmpliTaq Gold is not compatible with the buffer system of this protocol.
- dNTPs (New England Biolabs, cat. no. NO446S)
- Tris(hydroxymethyl)-aminomethane (Merck, cat. no. 1.08382)
- $MgCl_2$ (Sigma, cat. no. M8266)
- Bovine serum albumin (BSA; Calbiochem, cat. no. 126609)
- DNA preparation kit: DNeasy Tissue kit (Qiagen, cat. no. 69504) ▲ **CRITICAL** This kit can be used to purify DNA from cell lines. For preparation of DNA from other types of samples, other suitable kits can be used. Other purification methods that generate high-quality DNA are also acceptable.
- DNA template

EQUIPMENT

- LightCycler real-time PCR system version 1.0 or 2.0 (Roche)
- Carousel centrifuge (Roche, cat. no. 2189682)
- Capillary tube (Roche, cat. no. 11909339001)
- Amber polypropylene tubes (Labcon, cat. no. 3016-871-000)

REAGENT SETUP

PCR buffer (10×) 500 mM Tris-HCl (pH 8.5), 30 mM $MgCl_2$, 5 mg ml^{-1} BSA, 2 mM each dNTP. Aliquot and store at −20 °C. It is stable for at least 6 months.

Probe dilution buffer 1 mM Tris (pH 8.5) containing 50 μg ml^{-1} BSA. Sterilize it with a 0.2-μm syringe filter and store at −20 °C. It is stable for at least 1 year.

PCR primers Dissolve primers in sterile water to 100 μM as a stock. Dilute the stock solution with sterile water to 10 μM as a working solution. Both the stock and the working solution are stored at −20 °C. The stock solution is stable for 1 year.

PNA probe Dissolve PNA probe in sterile water to a concentration of 40 μM, warm the solution for 5–10 min at 50 °C, aliquot into amber polypropylene tubes and store at −80 °C. The stock solution is stable for at least 6 months. Dilute the solution to 10 μM with sterile water before use.

▲ **CRITICAL** PNA aggregates easily during storage. If so, warm it at 50 °C for 5 min before dilution. Also, avoid glass tube for storage because PNA has high affinity to glass surface.

Anchor probe Resuspend anchor probe in probe dilution buffer to a concentration of 20 μM, aliquot into amber tubes and store at –80 °C. It is stable for at least 3 months. ▲ **CRITICAL** The probe is not stable when stored at 4 °C or diluted in water.

PROCEDURE

Determine the mutant and wild-type T_m under non-clamping PCR conditions

1| Turn on the Lightcycler and the computer. Set up the programs according to **Table 2** (using non-clamping extension temperature).

2| In a clean reservoir, assemble the PCR master mix according to the following table.

Reagents	Amount per reaction	Final amount or concentration in PCR
PCR buffer (10×)	2 μl	1×
Forward primer (10 μM)	1 μl	0.5 μM
Reverse primer (10 μM)	1 μl	0.5 μM
PNA probe (10 μM)	0.5 μl	0.25 μM
Anchor probe (20 μM)	0.2 μl	0.2 μM
Platinum *Taq* (5 U μl^{-1})	0.1 μl	0.5 U
dH_2O	10.2 μl	–
Total	15 μl	

3| Pipette 15 μl of the above mix into four capillary tubes.

4| Pipette 5 μl of template into each tube (for this control experiment, use H_2O, wild-type DNA, mutant DNA and wild-type plus mutant DNA as templates; see Control Experiments in Experimental Design for details).

5| Cap the capillary tubes and place them into the carousel.

6| Centrifuge the samples using the default program for the carousel centrifuge specified in the EQUIPMENT section (3000 r.p.m., 15 s).

7| Place the carousel into the LightCycler.

8| Enter the information of samples (name, type and concentration, etc.) in Edit Samples and run the programs.

9| After PCR is completed, analyze the amplification curves and melting curves using the correct display mode, for example, F2/F1 for LC-Red640 on the LightCycler version 1.0.

10| Identify the wild-type and mutant T_m on the melting curve graph "Fluorescence – d(*F2/F1*)/d*T* versus Temperature" (see **Fig. 5a,b**).

11| (Optional) Check the PCR products by gel electrophoresis and direct sequencing to make sure the amplification is efficient and the products are correct.
▲ CRITICAL STEP To recover the PCR product from a capillary tube, remove the cap, invert it into a 1.5-ml Eppendorf tube and centrifuge briefly at 100–200 r.p.m. on a desktop centrifuge.

Optimize the conditions for clamping PCR

12| Use the four control templates as described in Step 4 and perform Steps 1–9 but set the extension temperature (the target temperature of segment 4 of the "thermal cycle" program) at 5 °C lower than the wild-type T_m and vary the concentration of PNA probe at 0.05, 0.1, 0.25, 0.5 and 1 μM.
▲ CRITICAL STEP Set the highest transition rate at segment 1 of "thermal cycle" program for optimal clamping efficiency.

13| Perform Step 12 but test the extension temperature at 7, 9 or 11 °C lower than the wild-type T_m.

14| Determine the C_T on the amplification curve and measure the relative peak area of wild-type and mutant on the melting curve. Choose a combination of PNA concentration and extension temperature that has little influence on the mutant C_T but efficiently enriches mutant peaks and suppresses the wild-type peaks on the melting curve graphs (see **Fig. 5d**).

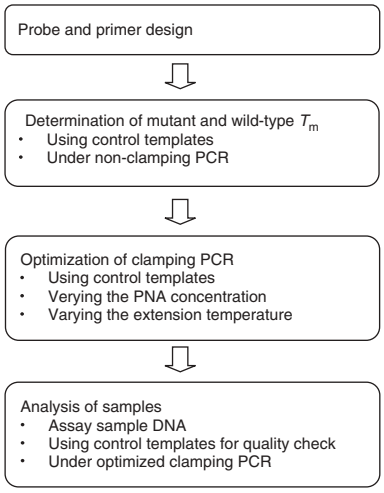

Figure 6 | Flowchart detailing the stages involved in the optimization and use of this single-tube assay for rare mutation detection using a PNA probe. Mutant and wild-type T_m refer to the melting temperature (T_m) of the PNA/mismatched template duplex and the PNA/perfectly matched template duplex, respectively. Non-clamping PCR conditions use an extension temperature higher than both the mutant and wild-type T_m to allow amplification of both wild type and mutant alleles. Clamping PCR conditions, however, use an extension temperature lower than the wild-type T_m but higher than the mutant T_m, so that the PNA probe remains bound to wild-type templates only, preventing their amplification and allowing selective amplification of mutant alleles.

Assay samples under optimized clamping PCR conditions

15| Use sample DNA as template and perform Steps 1–9 but set the extension temperature and use the PNA probe concentration determined to be optimal in Step 14. Reactions carried out in duplicate are helpful to rule out a false–positive result (see TROUBLESHOOTING).

▲ CRITICAL STEP To monitor the assay quality, also include the four control templates detailed in Step 4 (see Control Experiments in Experimental Design).

16| Identify the samples containing a mutant allele by the melting curves that have a known mutant T_m.

● TIMING

Steps 1–7 for program setup and reagent assembly, 20 min

Steps 8–10 for PCR and melting curve analysis, 45 min

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Problem	Possible reason	Solution
All the reactions reveal only background noise	The wrong display mode was chosen	Select a correct mode, for example, F2/F1 for LC-Red640 on the LightCycler V1.0
	Inefficient PCR reaction	Check the PCR efficiency by gel electrophoresis for PCR products. Inefficient PCR may be due to a bad reagent or a machine failure. Replace the reagent or fix the machine. Also, avoid PCR primers that generate primer dimers or nonspecific products
	Inefficient fluorescent probes	Check the efficacy of the probes by adding them into an existing PCR product and running a melting curve analysis. If the probes are ineffective, replace or redesign them and be sure to carefully handle and store the probes correctly
Wild-type amplification cannot be clamped	Not enough PNA	Try a higher PNA concentration. A titration experiment may be helpful. Sometimes, the lower PNA concentration is because of its precipitation. If so, warm an aliquot of PNA stock at 50 °C for 5 min and make new working solution
	Extension temperature too high	Try to set a lower extension temperature
	The opposite PCR primer not far enough away from PNA	Redesign an opposite primer at a longer distance from the PNA binding-site
	The temperature ramp becomes low	Check the setup and actual transition rate at segment 1 of the "thermal cycle program" (see **Table 2**). It should be 20 °C s^{-1}
Wild-type control or samples have weak mutant signals; the peak height is usually lower than the most diluted mutant control	Probably a polymerization error	This happens in around 1/6 of control reactions, perhaps because polymerization error occurs on the template strand without PNA binding or when the polymerase encounters an obstacle, that is, the PNA. These PCR products usually have unexpected mutation type and its signal is weak. However, it can lead to confusion. You may set a suitable threshold of peak height to differentiate this background signal from the real mutant signal. The threshold can be chosen by a statistical analysis of these background signals in repeated control reactions. Repeat the test in duplicate and consider it a false-positive result if the mutant signal or mutation type is not reproducible
Wild-type control and no-template control have strong mutant signals	Contamination with mutant alleles	Use clean equipment to prepare DNA samples and comply with good PCR manipulation procedures

ANTICIPATED RESULTS

Typical melting curves generated from mutant and wild-type PCR products should look like **Figure 5a** and **b**, respectively. The melting peak of the mutant will occur at the mutant T_m and that of the wild type is at the wild-type T_m. When using a template mixture containing abundant wild-type and rare mutant DNA (greater than 100:1), non-clamping PCR only generates a major peak of wild-type (see **Fig. 5c**). Only when PCR clamping is successfully conducted, can the mutant allele be fully amplified and the mutant melting peak be seen (see **Fig. 5d**). In our experience, this assay can detect as few as three copies of mutant K-*ras* DNA in a background of 10,000-fold wild type alleles[13].

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