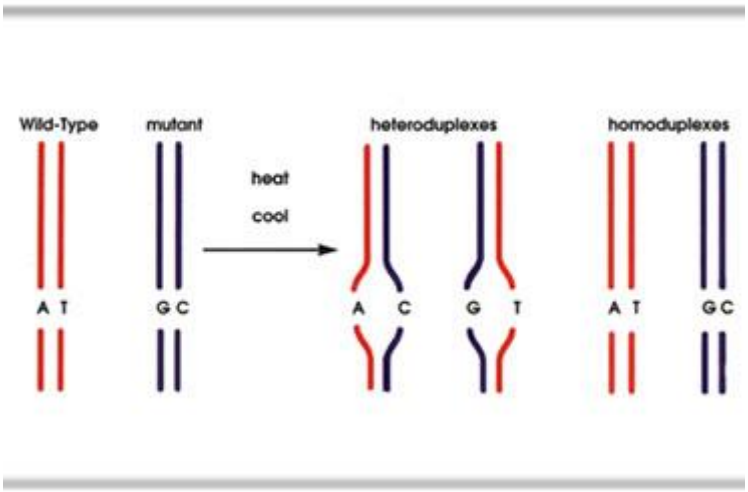
Detection of TP53 mutations

by Denaturing High Performance Liquid Chromatography (DHPLC)

(IARC protocol)

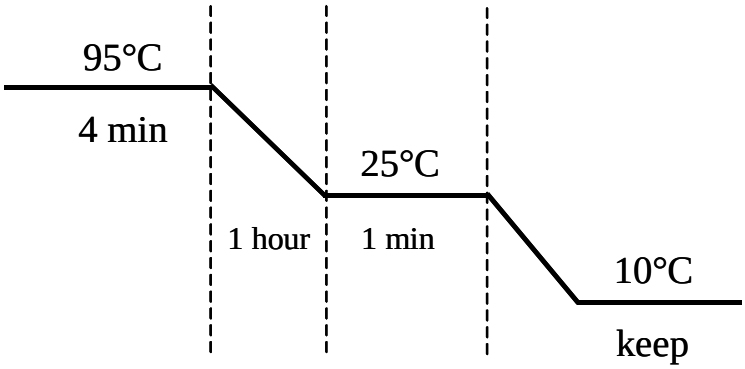
Principle

DHPLC analysis is based on heteroduplexes (HD) formation between a wild-type and a mutant sequence through hybridization after heating and cooling of PCR products.



Heteroduplex (HD) formation

PCR products (see PCR conditions in tables below) are heated at 95°C for 4min and then cooled at room temperature for one hour.



DHPLC analysis

5 to 10µl of the PCR products are then injected into a preheated reverse-phase column (DNASep Column, Transgenomic) equilibrated by an ion pairing agent TEAA 0.1M (Triethylammonium acetate). DNA is removed from the column at a constant flow rate of 0.9ml/min by a linear acetonitrile gradient, achieved by mixing a buffer A (TEAA 0.1M) with a buffer B (TEAA 0.1M and acetonitrile 25%) with 2% per minute gradient increase.

The temperature for optimum separation of heteroduplex from homoduplex was calculated by Transgenomic software so that 75% of the length of PCR product remain in double stranded form. For each screening temperature, a positive control, containing a specific mutation, is injected. These controls consist either in DNA isolated from cell lines that contain a mutation diluted with equal quantity of wild-type DNA (to enable heteroduplex formation, because only mutant sequences are present in the cell lines used here), or in DNA isolated from tumor samples (which contain mutant sequences but also wild-type sequences coming from contaminating non-tumor cells).

The eluted DNA is detected at 260nm. When heteroduplex are detected, sequencing is performed on an independent PCR product (see PCR sequencing conditions below).

Conditions for the analysis of exons 4-9 of human TP53 are summarized in the following tables:

			Exon 4		
PCR conditions	Primers (5'3')	Forward (*326*)	TgAggACCTggTCCTCTgAC		
		Reverse (*327*)	AgAggAATCCCAAAgTTCCA		
	Fragment length		413 bp		
	Mix		Taq Platinum*, 1.5mM $MgCl_2$, 0.2mM each dNTP, 1uM each primer		
	PCR program *(see below)*		Program 1 + HD		
DHPLC conditions	DHPLC temperature		62°C	65°C	68°C
	Time shift (min)		0.5	3	6
	Acetonitril gradient (% B)		57	52	46
	Controls used at IARC	Cell lines	*Tumor DNA*	*Tumor DNA*	Raji
		Mutation	*Polymorphism at codon 36: CC**G**-->CC**A***	*Mutation at codon 91: TG**G**-->TG**A***	Polymorphism at codon 72: C**G**C-->C**C**C

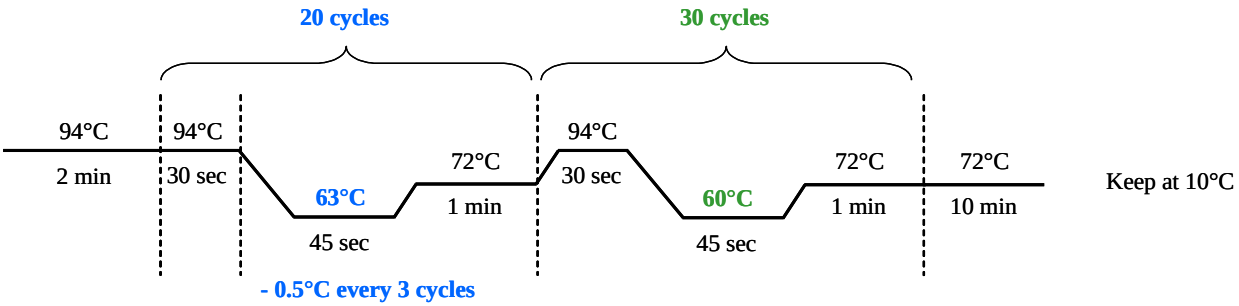
			Exon 5-6		
PCR conditions	Primers (5'3')	Forward (*236*)	TgTTCACTTgTgCCCTgACT		
		Reverse (*240*)	TTAACCCCTCCTCCCAgAgA		
	Fragment length		467 bp		
	Mix		Taq Platinum*, 1.5mM $MgCl_2$, 0.2mM each dNTP, 0.4uM each primer		
	PCR program *(see below)*		Program 1 + HD		
DHPLC conditions	DHPLC temperature		62°C	66°C	68°C
	Time shift (min)		0.5	3.5	4.5
	Acetonitril gradient (% B)		58	52	50
	Controls used at IARC	Cell lines	T47D	*Tumor DNA*	Hs578T
		Mutation	Codon 194: **C**TT-->**T**TT	*Codon 144 : **C**AG-->**T**AG*	Codon 157: **G**TC-->**T**TC

			Exon 7
PCR conditions	Primers (5'3')	Forward (*333*)	CTTgCCACAggTCTCCCCAA
		Reverse (*313*)	AggggTCAgCggCAAgCAgA
	Fragment length		237 bp
	Mix		HotStar Taq*, 1.5mM $MgCl_2$ 0.2mM each dNTP, 0.4uM each primer
	PCR program *(see below)*		Program 2 + HD
DHPLC conditions	DHPLC temperature		64°C
	Time shift (min)		0
	Acetonitril gradient (% B)		53
	Controls used at IARC	Cell lines	TE-11
		Mutation	Codon 237: AT**G**-->AT**T**

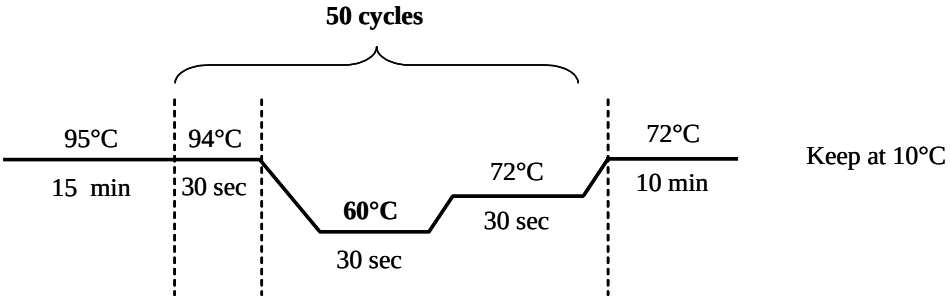
			Exon 8-9		
PCR conditions	Primers (5'3')	Forward (*314*)	TTgggAgTAgATggAgCCT		
		Reverse (*315*)	AgTgTTAgACTggAAACTTT		
	Fragment length		445 bp		
	Mix		Taq Platinum*, 2mM $MgCl_2$, 0.2mM each dNTP, 0.4uM each primer		
	PCR program *(see below)*		Program 1 + HD		
DHPLC conditions	DHPLC temperature		60°C	62°C	65°C
	Time shift (min)		1	2	4.5
	Acetonitril gradient (% B)		56	54	49
	Controls used at IARC	Cell lines	TE-1	TE-1	MDA-MB-231
		Mutation	Codon 272: **G**TG→**A**TG	Codon 272: **G**TG→**A**TG	Codon 280: A**G**A→A**A**A

* 0.8U/20ul mix

PCR program 1:



PCR program 2:



TP53 sequencing

Sequencing is performed by IARC common sequencing service. PCR products are analyzed by a 16-capillary automated sequencer (ABI PRISM® 3100 Genetic Analyzer, Applied Biosystems), based on the Sanger method (see principle at: http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Obenrader/sanger_method_page.htm)

Purification of PCR products

Prior sequence analysis, 5 µl of PCR products are purified with the enzyme ExoSap-IT (USB) for15min at 37°C and 15min at 80°C.

You may also use:

- columns (i.e. QIAquick PCR Purification kit, QIAGEN)
- plates (i.e. NucleoFast 96 PCR kit, Clontech)

Sequencing reaction

Sequencing reaction is done with BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the following protocol:

Mix:

- 7 µl of purified PCR product
- 1.25µl Buffer
- 0.5µl primer 10µM*
- 1.5µl Big Dye

Program:

96°C	10 sec	30 cycles
50°C	5 sec	
60°C	4 min	

* Same primers as the ones used for PCR amplification reactions (note that R primer for exon 11 does not work well for sequencing).

Purification of sequencing reaction

Before analysis, purification of the sequencing reaction products is done by the Sequencing Service with 96-well Multiscreen filtration plates (G50-Pharmacia-Millipore).

Sequencing analysis

PCR products are analyzed by a 16-capillary automated sequencer (ABI PRISM® 3100 Genetic Analyzer, Applied Biosystems), based on the Sanger method (see principle at: http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Obenrader/sanger_method_page.htm)

Result analysis and interpretation

Chromatograms are analyzed semi-automatically by visual inspection of sequences imported in a sequence analysis software using the reference sequence, NC_000017.9 (hg18 built), from Genbank (http://www-p53.iarc.fr/TP53sequence_NC_000017-9.html).

Variations are analyzed with the IARC TP53 database (http://www-p53.iarc.fr), to check whether the variation is a known polymorphism or a mutation, and get frequency and functional data for each specific variation.