Article title: Verifying Primers For Point Mutation Using Primers To Amplify DNA Region Containing Mutation Site

Authors: Halak Shukla[1]

Affiliations: Student, Government Biotech College, INDIA.[1]

Orcid ids: 0000-0002-6041-3793[1]

Contact e-mail: shuklask1942@gmail.com

License information: This work has been published open access under Creative Commons Attribution License <http://creativecommons.org/licenses/by/4.0/>, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. Conditions, terms of use and publishing policy can be found at <https://www.scienceopen.com/>.

Preprint statement: This article is a preprint and has not been peer-reviewed, under consideration and submitted to ScienceOpen Preprints for open peer review.

Funder: N/A

DOI: 10.14293/S2199-1006.1.SOR-.PPBAGT6.v1

Preprint first posted online: 28 June 2021

Keywords: PCR, BRAF, BRAF V600E, Point mutation , Trick method, Primers, Easy tools, Save costs , Dry lab, Amplify DNA sequence

USING PRIMERS TO AMPLIFY DNA REGION CONTAINING MUTATION SITE.

[Ms. Halak Shukla.](#)

[Student,](#)

[Government Biotech College,](#)

[Ahmedabad- Gujarat.](#)

[INDIA.](#)

[Mobile : +91 7698616354](#)

[E-mail: halakshukla0101@gmail.com](#)

Date: 27th July, 2021.

Method- Trick article for verifying primers for point mutation.

ABSTRACT:

Analysing DNA sequences for determining mutation point in PCR needs paid, Hi-tech software tools but we can confidently order the right piece of primer by this simple and easy method before we do PCR in lab. For instance, we here take BRAF V600E mutation. This method is to verify that the primers you've got or designed would successfully amplify the same region of DNA you want and not somewhere else.

INTRODUCTION:

By analysing the commercial Taqman single nucleotide polymorphism assay, assessing whether a mutation is there or not in the DNA sequence. For this, an alternative would be to simply amplify the region of DNA that contains the mutation site and perform the method of direct sequencing upon that DNA sequence.

For each mutation to be tested, specific sequences are located for primers that allow amplification of a certain target sequence also termed “segment”, while the primers for the first PCR harbour is a desired tested location [1]. For example, the amplicon size is substantially 200- 400 bp long, melting temperature (T_m) of primers is 63-65° C and primer length is 18- 26 bp. The mechanism of mutation is that the negative charge of the acidic glutamic acid residue causes it to be phosphomimetic [2]. The BRAF V 600E mutation includes melanoma[3], hairy cell leukemia[4], papillary thyroid carcinoma[5], colorectal cancer[6], non-small-cell lung cancer[7], Langerhans cell histiocytosis[8], Erdheim-Chester disease[9] and ameloblastoma[10].

PROCEDURAL STEPS :

For this method, I have taken a already designed forward and reverse primer from a patented paper [11] where they construct DNA for sequencing, which is an example here for specific primers to the BRAF mutation at amino acid position V 600. The valine (V) will be substituted by glutamic acid (E). So, I'll use that as an example for explaining my procedural steps. We'll see how the DNA sequence codes so primers amplify the right bits of DNA and don't bind somewhere other than that we want.

Dividing the method in two segments, first for identification of PCR product sequence and second to verify that DNA sequence present includes codon 600.

Starting method by listing the primers, I've taken :

Forward primer : cttcataatgcttgctctgatagg

Reverse primer : gcatctcagggccaaaaatt

SEGMENT 1 :- IDENTIFICATION OF PCR PRODUCT SEQUENCE

Part 1 : Listing.

- Google NCBI website (<https://www.ncbi.nlm.nih.gov>). [12]
- On the web page we can see “ popular resources” on the right hand side.

- There's "BLAST" listed in it, click.
- On BLAST, we scroll down to "Nucleotide BLAST", click.
- There, in "Enter Query Sequence" we type our primer sequence. We will first take the forward primer, copy it and paste there.
- Without changing any default settings, Scroll down and click on "BLAST".
- It will now take a minute to search all known nucleotide sequences.
- The result of BLAST for forward primer is shown to "descriptions" see top of some human links. (Refer Image :1).
- Taking another tab we can follow the same steps mentioned above by taking the reverse primer sequence.

select all 100 sequences selected

GenBank Graphics Distance tree of results [New MSA Viewer](#)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Homo sapiens B-Raf proto-oncogen...	Homo sa...	48.1	48.1	100%	0.005	100.00%	212438	NG_007873.3
Homo sapiens BRAF protein (BRAF)...	Homo sa...	48.1	48.1	100%	0.005	100.00%	529	HM459603.1
Homo sapiens v-raf murine sarcoma...	Homo sa...	48.1	48.1	100%	0.005	100.00%	194260	EU600171.1
Pan troglodytes BAC clone CH251-6...	Pan trogl...	48.1	48.1	100%	0.005	100.00%	192362	AC187613.3
Homo sapiens PAC clone RP4-726N...	Homo sa...	48.1	48.1	100%	0.005	100.00%	127447	AC006344.2
Homo sapiens serine/threonine-prot...	Homo sa...	44.1	44.1	91%	0.072	100.00%	227	KF481581.1
Haemonchus contortus strain NZ_H...	Haemon...	40.1	72.4	83%	1.1	100.00%	77295023	CP035801.1
Haemonchus contortus JSE/inbred I...	Haemon...	40.1	40.1	83%	1.1	100.00%	47382676	LS997563.1
PREDICTED: Corvus cornix cornix un...	Corvus c...	40.1	40.1	83%	1.1	100.00%	3071	XR_005602230.1
Acomys russatus genome assembly...	Acomys r...	40.1	40.1	83%	1.1	100.00%	47487087	LR877227.1
PREDICTED: Corvus moneduloides s...	Corvus ...	38.2	38.2	79%	4.5	100.00%	4647	XM_032116930.1
PREDICTED: Yersinia enterocolitica ATCC 4804...	Yersinia...	38.2	38.2	79%	4.5	100.00%	5708	Y14_031804001

Feedback

IMAGE 1 :- LISTING OF THE DESCRIPTION FOR HUMAN BRAF PROTEIN.

Both forward and reverse primer will bind somewhere near Each Other a few hundred base pair apart because that's where the primers are and they should bind to some piece of DNA. Once we know where the forward primer binds and where the reverse primer bind then that allows us to calculate how much DNA there is in between those two primers and it will also give us the total size of our PCR product.

Part 2 : Compare & select.

- We will select those links which have mentioned “human” in common name tag list and “100%” in query cover.
- Comparing this link of description on both the forward and reverse primer resultant tab, select the sequence that are available to both tabs and shows BRAF
- Follow it’s “accession”, click on it.
Accession are just large genomic fragment of chromosome which happens to be where the BRAF gene is.
- We will get a screen showing its details. Scrolling down we find long sequence, our job is to look at where within the primer is in that sequence. Click on FASTA given below NCBI reference sequence ID. FASTA will remove the gaps and numbers. (Refer Image:2)
- Which find in page or Control + F we will find the forward primer, type the first half of the forward primer sequence and check for the rest.

10:05

VoLTE LTE1

< cttcat aatgcttgct ctgatagg

X

1/1

COVID-19 Information[Public health information \(CDC\)](#) | [Research information \(NIH\)](#)[SARS-CoV-2 data \(NCBI\)](#) | [Prevention and treatment information \(HHS\)](#) | [Español](#)

GenBank

Send to:

Homo sapiens BRAF protein (BRAF) gene, exon 15 and partial cds

GenBank: HM459603.1

[FASTA](#) [Graphics](#)

Go to:

LOCUS HM459603 529 bp DNA linear PRI 25-JUL-2016

DEFINITION Homo sapiens BRAF protein (BRAF) gene, exon 15 and partial cds.

ACCESSION HM459603

VERSION HM459603.1

KEYWORDS

SOURCE Homo sapiens (human)

ORGANISM [Homo sapiens](#)

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 529)

AUTHORS Herrmann,I., Baeuerle,P.A., Friedrich,M., Murr,A., Filusch,S., Ruttinger,D., Majdoub,M.W., Sharma,S., Kufer,P., Raum,T. and Munz,M.

TITLE Highly efficient elimination of colorectal tumor-initiating cells by an EpCAM/CD3-bispecific antibody engaging human T cells

JOURNAL PLoS ONE 5 (10), E13474 (2010)

PUBMED [20976159](#)

REMARK Publication Status: Online-Only

REFERENCE 2 (bases 1 to 529)

AUTHORS Herrmann,I. and Munz,M.

TITLE Direct Submission

JOURNAL Submitted (31-MAY-2010) EpCAM Research, Micromet, Staffelsestr. 2, Munich, Bavaria 81477, Munich

FEATURES Location/Qualifiers

source 1..529
 /organism="Homo sapiens"
 /mol_type="genomic DNA"
 /db_xref="taxon:9606"
 /cell_line="HT-29"

gene <1..>529
 /gene="BRAF"

mRNA <210..>328
 /gene="BRAF"
 /product="BRAF protein"

CDS <210..>328
 /gene="BRAF"
 /codon_start=3
 /product="BRAF protein"
 /protein_id="ADQ00186.1"
 /translation="IFLHEDLTVKIGDFGLATVKSRSWSGSHQFEQLSGSILWM"

exon 210..328
 /gene="BRAF"
 /number=15

ORIGIN

```

1 aatcttaaaa gcaggttata taggctaaat agaactaatc attgttttag acatacttat
61 tgactctaag aggaaagatg aagtactatg ttttaaagaa tattatatta cagaattata
121 gaaattagat ctcttaccta aactcttcat aatgcttgct ctgataggaa aatgagatct
181 actgttttcc ttacttactt acactcaga tatatttctt catgaagacc tcacagtaaa
241 aataggtgat ttggtctag ctacagtga atctcgatgg agtgggtccc atcagtttga
301 acagttgtct ggatccattt tgggatggt aagaattgag gctatttttc cactgattaa
361 attttggcc ctgagatgct gctgagttac tagaaagtc tgaagggtct caactatagt
421 attttcatag ttccagatgt tcacaaaaat cagtgttctt atttttatg taaatagatt
481 tttaacttt ttctttacc cttaaaacga atattttgaa accagtttc
//

```

IMAGE 2:- MARKING OF FORWARD PRIMER IN *Homo sapiens* BRAF PROTEIN GENE.

- Select 5 lines up and 5 lines down from the sequence found. Copy them and paste in Notepad or MS Word. Highlight this forward primer sequence with yellow.
- To locate the reverse primer, we need to decode it. As, reverse primer is in reverse antisense.
So, we will type it "backwards" and "antisense". For example: ggccattaagc when decoded goes gcttaatggcc. (Refer Image- 3)

GenBank

Send to:

Change region s

Homo sapiens BRAF protein (BRAF) gene, exon 15 and partial cds

GenBank: HM459603.1

[FASTA](#) [Graphics](#)

Go to:

LOCUS HM459603 529 bp DNA linear PRI 25-JUL-2016
DEFINITION Homo sapiens BRAF protein (BRAF) gene, exon 15 and partial cds.
ACCESSION HM459603
VERSION HM459603.1
KEYWORDS

SOURCE Homo sapiens (human)

ORGANISM [Homo sapiens](#)

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 529)

AUTHORS Herrmann,I, Baeuerle,P.A., Friedrich,M., Murr,A., Filusch,S.,
Ruttinger,D., Majdoub,M.W., Sharma,S., Kufer,P., Raum,T. and
Munz,M.

TITLE Highly efficient elimination of colorectal tumor-initiating cells
by an EpCAM/CD3-bispecific antibody engaging human T cells

JOURNAL PLoS ONE 5 (10), E13474 (2010)

PUBMED [20976159](#)

REMARK Publication Status: Online-Only

REFERENCE 2 (bases 1 to 529)

AUTHORS Herrmann,I. and Munz,M.

TITLE Direct Submission

JOURNAL Submitted (31-MAY-2010) EpCAM Research, Micromet, Staffelseestr. 2,
Munich, Bavaria 81477, Munich

FEATURES Location/Qualifiers

source 1..529

/organism="Homo sapiens"

/mol_type="genomic DNA"

/db_xref="taxon:9606"

/cell_line="HT-29"

gene <1..>529

/gene="BRAF"

mRNA <210..>328

/gene="BRAF"

/product="BRAF protein"

CDS <210..>328

/gene="BRAF"

/codon_start=3

/product="BRAF protein"

/protein_id="ADQ00186.1"

/translation="IFLHEDLTVKIGDFGLATVKSRSWSGSHQFEQLSGSILWM"

exon 210..328

/gene="BRAF"

ORIGIN

Copy Select all Share Find

```
181 actgttttcc ttacttact acacctcaga tatatttct catgaagacc tcacagtaaa
241 aatagggtgat ttggctctag ctacagttaa atctcgatgg agtgggtccc atcagtttga
301 acagtgtgct ggtatcattt tgtggatggg aagaattgag gctatttttc cactgattaa
361 atttttgccc ctgagatgct gctgagttac tagaaagtc tgaaggtct cagtttct
421 attttcatag ttcccgatga gaaaaat cagtgttctt atttttatg taattttt
481 ttttaacttt tttcttaac cagtttga atattttgaa accagtttc
```

//

Customize view

Analyze this seq

Run BLAST

Pick Primers

Highlight Sequenc

Find in this Sequen

Articles about th

Cardiofac
synk [Zhong Nan D

Novel and Potent :
against Melanom

Activating mutatic
hypothalamo-pitui

Reference seque

RefSeq alternative
See 14 reference r
variants for the BF

More about the E

This gene encodes
to the RAF family
protein kinases. TI
in regulating the M
Also Known As: B-
N...

Homologs of the

The BRAF gene is
chimpanzee, Rhes
mouse, rat, chicke
mosquito, C.elega

Related informat

Protein

PubMed

Taxonomy

Full text in PMC

Gene

PubMed (Weighte

LinkOut to exterr

IMAGE 3:- MARKING OF REVERSE PRIMER IN *Homo sapiens* BRAF PROTEIN GENE .

- By find in page or Ctrl+ F, the decoded reverse primer sequence is found. Copy 5 lines up and down, paste in previous notepad or MS word. Highlight with red.

- Select the distance from forward to reverse primer and cut the rest out.
(Refer Image: 4)

No title

```
cttcataatgcttgctctgataggaaaatgagatctactgttttct
ttacttactacctcagatatatttcttcatgaagacctcacagt
aaaaataggtgatgttgggtctagctacagtgaatctcgatgga
gtgggtcccatcagtttgaacagttgtctggatccattttgtgga
tggtagaattgaggctattttccactgattaaattttggccct
gagatgc
```

IMAGE 4 :- HIGHLIGHTING NEEDED REGION IN NOTEPAD.

Hence, from the selected region of Forward primer to that of the region of reverse primer is the number of base pair in the PCR product we want to use. Checking word count of the selected region will tell us how long our PCR product is. Now, in this DNA sequence, it might be exons or introns, to know this here's SEGMENT 2.

SEGMENT 2 : TO VERIFY THAT DNA SEQUENCE PRESENT INCLUDES CODON 600.

Part 1: Gene Search.

- Go to NCBI website.
- By clicking pull down list of all databases, select "Gene".
- Type "BRAF" and click search.

- Search result shows us various options. The one we need is of *homo sapiens*. Click on that link.
 - BRAF *homo sapiens* description page will show up and scrolling down, select “Go to : reference sequence details”.
- Now, we have got link which shows NM number list. NM number are reference sequence for individual genes especially if there are splice variants.
- The general rule with reference sequences is a NM number with lowest number tends to be originally identified and the higher number tend to be more recently identified so we will go with the lowest one, it should be the lowest isoform.
 - We will click on the lowest isoform associated with NM number. Copy that NM number. (Refer Image: 5)

20:03

13. [NM_004333.6](#) → [NP_004324.2](#) serine/threonine-protein kinase B-raf isoform 1

[See identical proteins and their annotated locations for NP_004324.2](#)

Status: REVIEWED

Source sequence(s)	AC006006 , AC006344 , AW971583 , BC101757 , DB454480 , M95712
Consensus CDS	CCDS5863.1
UniProtKB/Swiss-Prot	P15056
Related	ENSP00000493543.1 , ENST00000646891.1

Conserved Domains (3) [summary](#)

cd01816	Raf_RBD; Ubiquitin domain of Raf serine/threonine kinases
Location:156 → 227	
cd14062	STKc_Raf; Catalytic domain of the Serine/Threonine Kinases, Raf (Rapidly Accelerated Fibrosarcoma) kinases
Location:463 → 715	
pfam00130	C1_1; Phorbol esters/diacylglycerol binding domain (C1 domain)
Location:235 → 280	

IMAGE 5:- NM NUMBER WITH LOWEST ISOFORM.

Part 2: Find

- Search for “ORF Finder” [\[13\]](#) or we can also use “EMBOSS SIXPACK” [\[14\]](#). ORF finder is basically a open reading frame finder website.
- We will copy paste our NM number and without changing any default settings, click on “Submit”

OR

We can copy paste the sequence by clicking on the NM number and run it in fasta format. Copy the sequence and paste it here in the ORF finder “enter query sequence” region.

- It will take a minute here, what it is doing is to scan that DNA sequence and look the possible open reading frame, which are basically regions of DNA which convert to copy mrna and translate them to longest runs of amino acids.
- Sequence page will appear. Here, in the right side we see column if “length (nt/aa)”, we will select the longest one as we need BRAF gene of at least 600 amino acids. Here, it’s the amino acid 600 that is changed there’s oncogenic mutation to BRAF gene.
- We will now click on “Display ORF as” and select “CDS translation”. It puts what amino acid codes for particular DNA.

The screenshot shows the NCBI ORF Finder tool interface. The left pane displays a DNA sequence with positions 1711, 1756, 1801, 1846, and 1891. The right pane lists Open Reading Frames (ORFs) with their start and end positions and lengths. ORF9 is highlighted at the bottom left.

ORF	Start	End	Length (nt/aa)
ORF65	-	-	-
ORF18	+	+	+
ORF58	-	-	-
ORF29	+	+	+
ORF45	-	-	-
ORF48	-	-	-
ORF32	-	-	-
ORF62	-	-	-
ORF22	+	+	+

IMAGE 6:- POINTING THE CODON 600

- Now, our needed codon is 600. So, approximately 1800 Nucleotide base pair. ($600 \times 3 = 1800$).
Scrolling down, we find around 1800.
- If for example there’s lysine at 1801, we will see the previous line. Here, it codes for GTG which stands for valine. (Refer image: 6)

This effectively tells us that DNA sequence of the code that copies from the M RNA which is translated gives us the amino acid around 600 is valine.

DURATION:

This method will take about 10-15 minutes maximum.

RESULT :

A quick check for the sequence. Copy few half of sequenced lines and by opening our previous notepad or Ms word, control+ f and paste we will find that it perfectly matches this sequence. We now know that it primers will amplify our region of BRAF gene and within that there is DNA that codes for codon 600 in our PCR product. So, if we amplify this DNA from Melanoma samples, we could then sequence this DNA and have our mutation site. Those 3 nucleotides are changed from valine to a glutamic acid in v600e mutation.

WEBSITES :

1. NCBI website : (<https://www.ncbi.nlm.nih.gov/>)
2. ORF FINDER website : (<https://www.ncbi.nlm.nih.gov/orffinder/>)
3. EMBOSS SIXPACK website :
(https://www.ebi.ac.uk/Tools/st/emboss_sixpack/)

CONCLUSION NOTE:

A reference paper I found (<https://pubmed.ncbi.nlm.nih.gov/17298986/>)^[15] is looking at thyrocarcinoma which has this same mutation within it. Here, we can see the GTG wild type when mutates, instead of "t", it's "a" on one of the alleles. Thus, this mutation of GTG to GAG (GTG codes for valine and "GAG" codes for glutamic acid) makes absolutely sure that we are dealing with right DNA.

By that, we can confidently order primers what we want to amplify for. We will not need any specialist DNA analysis software or tools, we can do simply like this and access everything easily, perfectly good for point mutation.

I hope this method for verifying the primers for point mutation will save costs before ordering PCR product assay and help to detect and amplify DNA region with mutation site.

RREFERENCES WITH LINKS:

1. Roi Feingersch. "DNA construct for sequencing and method for preparing the same". (2018) (<https://patents.google.com/patent/US20200080143A1>)
2. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, et al. (June 2002). "[Mutations of the BRAF gene in human cancer](#)" (PDF). *Nature*. **417** (6892): 949–54. doi:10.1038/nature00766. PMID 12068308.
3. Maldonado JL, Fridlyand J, Patel H, Jain AN, Busam K, Kageshita T, et al. (December 2003). "[Determinants of BRAF mutations in primary melanomas](#)". *Journal of the National Cancer Institute*. **95** (24): 1878–90. doi:10.1093/jnci/dig123. PMID 14679157.

4. Tiacci E, Trifonov V, Schiavoni G, Holmes A, Kern W, Martelli MP, et al. (June 2011). "[BRAF mutations in hairy-cell leukemia](#)". *The New England Journal of Medicine*. **364** (24): 2305–15. doi:[10.1056/NEJMoa1014209](#). PMC [3689585](#). PMID [21663470](#).
5. Puxeddu E, Moretti S, Elisei R, Romei C, Pascucci R, Martinelli M, et al. (May 2004). "[BRAF\(V599E\) mutation is the leading genetic event in adult sporadic papillary thyroid carcinomas](#)". *The Journal of Clinical Endocrinology and Metabolism*. **89** (5): 2414–20. doi:[10.1210/jc.2003-031425](#). PMID [15126572](#).
6. Li WQ, Kawakami K, Ruzsiewicz A, Bennett G, Moore J, Iacopetta B (January 2006). "[BRAF mutations are associated with distinctive clinical, pathological and molecular features of colorectal cancer independently of microsatellite instability status](#)". *Molecular Cancer*. **5**: 2. doi:[10.1186/1476-4598-5-2](#). PMC [1360090](#). PMID [16403224](#).
7. Sánchez-Torres JM, Viteri S, Molina MA, Rosell R (June 2013). "[BRAF mutant non-small cell lung cancer and treatment with BRAF inhibitors](#)". *Translational Lung Cancer Research*. **2** (3): 244–50. doi:[10.3978/j.issn.2218-6751.2013.04.01](#). PMC [4367599](#). PMID [25806238](#).
8. Badalian-Very G, Vergilio JA, Degar BA, Rodriguez-Galindo C, Rollins BJ (January 2012). "[Recent advances in the understanding of Langerhans cell histiocytosis](#)". *British Journal of Haematology*. **156**(2): 163–72. doi:[10.1111/j.1365-2141.2011.08915.x](#). PMID [22017623](#).
9. Haroche J, Cohen-Aubart F, Emile JF, Arnaud L, Maksud P, Charlotte F, Cluzel P, Drier A, Hervier B, Benameur N, Besnard S, Donadieu J, Amoura Z (Feb 2013). "[Dramatic efficacy of vemurafenib in both multisystemic and refractory Erdheim-Chester disease and Langerhans cell histiocytosis harboring the BRAF V600E mutation](#)". *Blood*. **121** (9): 1495–500. doi:[10.1182/blood-2012-07-446286](#). PMID [23258922](#).
10. Kurppa KJ, Catón J, Morgan PR, Ristimäki A, Ruhin B, Kellokoski J, Elenius K, Heikinheimo K (April 2014). "[High frequency of BRAF V600E mutations in ameloblastoma](#)". *The Journal of Pathology*. **232** (5): 492–8. doi:[10.1002/path.4317](#). PMC [4255689](#). PMID [24374844](#).
11. Sequencing primer pair for qualitatively detecting human BRAF V600E gene mutation and kit thereof (2012) (<https://patents.google.com/patent/CN102925555B>)
12. NCBI website (<https://www.ncbi.nlm.nih.gov/>)
13. ORF FINDER website (<https://www.ncbi.nlm.nih.gov/orffinder/>)
14. EMBOSS SIXPACK website (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/)
15. Leslie R Rowe *et al.* *Journal of Clinical Pathology* (November, 2007) " Detection of BRAF V600E activating mutation in papillary thyroid carcinoma using PCR with allele-specific fluorescent probe melting curve analysis. (<https://pubmed.ncbi.nlm.nih.gov/17298986/>)