LightMix® Kit NRAS 12-13, 59-61, 117, 146 and KRAS 117, 146

Cat.-No. 40-0654-16

2015-04: NRAS 12/13 improved. N-Uracil-DNA Glycosylase concentration changed.

Kit with reagents for the detection of *NRAS* and *KRAS* mutations in genomic FFPE DNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 / 480 II / cobas z 480 (UDF channel) Instruments.

Lyophilized mix of primers, probes and competitor for 96 reactions (6 vials each 16 rxns 20 µl each)
Store protected from light at room temperature (18-25°C), do NOT freeze!

1. Introduction

RAS mutations are commonly found in cancer tissue, causing a permanent activation of the signal transduction pathway downstream of the EGF receptor (EGFR), thus positive signaling for cell growth. Most frequent are KRAS mutations in codons 12-13 in the second (first transcribed) exon. Up to 45% of all colon cancer (CRC) patients exhibit KRAS 12-13 mutations, while another 10-15% may have mutations in KRAS exons 3 and 4 or in the respective regions of the homologous NRAS gene.

This kit allows to detect somatic mutations in and near NRAS codons 12-13, 59-61, 117 and 146 as well as KRAS codons 117 and 146 in a nucleic acid extract from FFPE material.

This LightMix® Kit is tested with the Roche Diagnostics 'LightCycler® FastStart DNA Master HybProbe' ready–to-use reaction mix in the LightCycler® 1.x / 2.0 / 480 / cobas z 480 Instruments.

2. Description

Six different sized PCR fragments from NRAS exons 2, 3, 4 and KRAS exon 4 are generated with specific primers. The PCR amplification of the respective wild type sequence is reduced using a Locked-Nucleic Acids (LNA) based competitor. The binding of the contained hybridization probes to the PCR products is monitored in a melting curve analysis, resulting in a melting peak in case of the presence of a mutation while a wild type will show a baseline curve. We recommend to treat the sample DNA with UNG to destroy cytosine oxidation products in order to avoid to detect artifacts.

Any mutations are identified by a melting peak while wild type samples will generate a baseline curve. This product is intended to be used for samples already tested for absence of KRAS exon 2 and 3 mutations and thus been confirmed to contain amplificable DNA. Samples showing a baseline curve for all six reactions are wild type for the analyzed mutations; however, samples may fail to amplify due to low amount of sample, inhibition, or other cause. This kit does not include an amplification control.

3. Contents

1 Vial with **orange** cap with 16 mutation-search-reagent (MSR) reactions for NRAS 12/13 mutations
1 Vial with **blue** cap with 16 MSR reactions NRAS 59-61
1 Vial with **red** cap with 16 MSR reactions NRAS 117
1 Vial with **purple** cap with 16 MSR reactions NRAS 146
1 Vial with **yellow** cap with 16 MSR reactions KRAS 117
1 Vial with **green** cap with 16 MSR reactions KRAS 146

1 Vial with **colorless** cap with 32 reactions Positive Control containing 1,000 copies mutation / rxn

Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at room temperature (18-25°C). See expiry date on t he product label.

4. Additional Reagents and Items Required

	Roche Diagnostics
LightCycler® FastStart DNA Master HybProbe (96 reactions)	Cat.-No. 03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 96, white (plate based Instrument)	Cat.-No. 04 729 692 001
Cobas® DNA Sample Preparation Kit	Cat.-No. 05 985 536 190
LightCycler® Uracil-DNA Glycosylase (UNG)	Cat.-No. 03 539 806 001

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5. Product Characteristics

Run time: PCR results are obtained within 110 min with LC 2.0 and 100 min with 480 systems.
Sensitivity: This kit detects 300 or less copies mutation in 100 ng (33,000 copies) total DNA (1%)

Productivity	Runs	Controls	Samples / Run	Reactions / Run	Instruments
14 samples	**1**	2 +	14	96	plate based instrument
12 samples	**2**	2 +	6	48	plate based instrument
9 samples	**3**	2 +	3	30	Any instrument
8 samples	**4**	2 +	2	24	Any instrument
5 samples	**5**	2 +	1	18	Any instrument

Table 1

Protein Variant - HGVS Mutation Code		Codon(s)	Tm values :	*in-vitro*	cobas z 480
NRAS Codons 12-13		GGT GGT	**baseline**	(59.4)	
p.G12S	c.34G>A	AGT GGT		not tested	
p.G12R	c.34G>C	CGT GGT		not tested	
p.G12C	c.34G>T	TGT GGT		not tested	
p.G12N	c.34_35GG>AA	AAT GGT		not tested	
p.G12P	c.34_35GG>CC	CCT GGT		not tested	
p.G12Y	c.34_35GG>TA	TAT GGT		not tested	
p.G12D	c.35G>A	GAT GGT		not tested	**58,6**
p.G12A	c.35G>C	GCT GGT	**Pos. Control**	not tested	**57.9**
p.G12V	c.35G>T	GTT GGT		not tested	**63,5**
p.G12G	c.36T>C	GGC GGT	wild type !	not tested	
p.G13G	c.39T>C	GGT GGC	wild type !	not tested	
p.G13S	c.37G>A	GGT AGT		not tested	
p.G13R	c.37G>C	GGT CGT		not tested	
p.G13C	c.37G>T	GGT TGT		not tested	
p.G13N	c.37_38GG>AA	GGT AAT		not tested	
p.G13Y	c.37_38GG>TA	GGT TAT		not tested	
p.G13D	c.38G>A	GGT GAT		not tested	
p.G13A	c.38G>C	GGT GCT		not tested	
p.G13V	c.38G>T	GGT GTT		not tested	
p.G13V	c.38_39GT>TC	GGT GTC		not tested	
NRAS Codons 59/60/61		GCT GGA CAA	**baseline**	(56.9)	
p.A59T	c.175G>A	ACT GGA CAA		not tested	
p.A59D	c.176C>A	GAT GGA CAA		not tested	
p.G60R	c.178G>C	GCT CGA CAA		not tested	
p.G60E	c.179G>A	GCT GAA CAA		not tested	
p.Q61K	c.181C>A	GCT GGA AAA		not tested	**57.2**
p.Q61E	c.181C>G	GCT GGA GAA		not tested	
p.Q61R	c.181_182CA>AG	GCT GGA AGA		not tested	
p.Q61L	c.181_182CA>TT	GCT GGA TTA		not tested	
p.Q61K	c.181_183CAA>AAG	GCT GGA AAG		not tested	
p.Q61P	c.182A>C	GCT GGA CCA		not tested	
p.Q61R	c.182A>G	GCT GGA CGA	**Pos. Control**	not tested	**61.6**
p.Q61L	c.182A>T	GCT GGA CTA		not tested	**56.2**
p.Q61R	c.182_183AA>GG	GCT GGA CGG		not tested	
p.Q61L	c.182_183AA>TG	GCT GGA CTG		not tested	
p.Q61H	c.183A>C	GCT GGA CAC		not tested	
p.Q61Q	c.183A>G	GCT GGA CAG	wild type !	not tested	
p.Q61H	c.183A>T	GCT GGA CAT		not tested	
NRAS Codon 117		AAG	**baseline**	(48.3)	
p.K117A	c.349_350AA>GC	GCG	**Pos. Control**	57.3	
NRAS Codon 146		GCC	**baseline**	(63.3)	
p.A146T	c.436G>A	ACC	**Pos. Control**	56.6	**56.1**
KRAS Codon 117		AAA	**baseline**	(48.4)	
p.K117E	c.349A>G	GAA		44.3	
p.K117N	c.351A>C	AAC		50.5	
p.K117N	c.351A>T	AAT	**Pos. Control**	55.9	**55.9**
KRAS Codon 146		GCA	**baseline**	(59.3)	
p.A146T	c.436G>A	ACA	**Pos. Control**	61.8	**61.4**
p.A146P	c.436G>C	CCA		56.2	
p.A146G	c.437C>G	GGA		55.2	
p.A146V	c.437C>T	GTA		54.0	
p.A146A	c.438A>G	GCG	wild type !	**55.6**	

Table 2. NRAS / KRAS mutations from the COSMIC database with Tm values in ℃ observed using synthetic targets (LC 2.0 system). Tm values observed with clinical samples will be reported where available (cobas z 480).

6. Experimental Protocol

Sample material: Use aqueous nucleic acid preparations for FFPE tissue (e.g. cobas® DNA Sample Preparation Kit, Roche Diagnostics. Use 20 - 100 ng (4 ng - 20 ng/µl) total DNA per reaction.
Note: The amount of DNA may be estimated by amplification of the globin gene (Kit 40-0085) since the spectrophotometric measurement does not always reflect the true amount of amplicable nucleic acids.

Negative control: Always run at least one no-template control (NTC) - replace the template DNA with water.

Positive control: Run a positive control - replace the template DNA with the provided control DNA.

6.1 Preparation of MSR reagents (16 reactions):

One reagent vial with a **colored** cap contains all primers and probes to run 16 reactions.
Add **34 µl** (**35 µl** permitted) water to each reagent vial, mix the solution (vortex) and spin down.
Dissolved reagents are stable for 21 days when stored protected from light and refrigerated (4°C).

►**Use 2 µl** reagent per reaction

6.2. Preparation of the Positive Control DNA

Add 160 µl PCR-grade water to the vial with a **colorless** cap containing the positive control.
Mix the target DNA (vortex) and spin down. The solution allows to run up to 32 control reactions.

►**Use 5 µl** control per reaction

Dissolved controls can be stored for 30 days at 4°C . Long time storage -20°C allowed. Avoid repeated f reezing thawing cycles. Discard control reagent not used. Note : Opening these vials may cause contamination of the work-space (aerosol).

6.3. Preparation of the Reaction Mix

In a cooled reaction tube, prepare the reaction mix by multiplying each volume for a single reaction by the number of reactions to be cycled, plus one additional reaction.

6.3.1 LightCycler® 1.x / 2.0 : MSR Single Reaction (six distinct reactions) - Roche FastStart		
1 rxn	**Component**	**16 rxns**
6,15 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart Master kit)	98.4 µl
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart Master kit)	25.6 µl
2.0 µl	**MSR reagent** - (mutation specific reagents containing primers and probes)	32.0 µl
2.0 µl	Roche FastStart Master (red cap, for preparation see Roche manual)	32.0 µl
0.25 µl	N-Uracil-DNA Glycosylase 0,5U/rxn (UNG)	4.0 µl
12.0 µl	Final volume of reaction mix	**192 µl**

Table 3

Mix gently, spin down and **transfer 12 µl** of the reaction mix per capillary. Add **5 µl** sample / control for a final volume of **17 µl**. Close the capillaries, centrifuge, and insert into the instrument. **Start run**

6.3.2 480 instruments : MSR Single Reaction (six distinct reactions) - Roche FastStart		
1 rxn	**Component**	**16 rxns**
9,15 µl	water, PCR-grade (colorless cap, provided with the Roche FastStart Master kit)	146.4 µl
1.6 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart Master kit)	25.6 µl
2.0 µl	**MSR reagent** - (mutation specific reagents containing primers and probes)	32.0 µl
2.0 µl	Roche FastStart Master (red cap, for preparation see Roche manual)	32.0 µl
0.25 µl	N-Uracil-DNA Glycosylase 0,5U/rxn (UNG)	4.0 µl
15.0 µl	Final volume of reaction mix	**240 µl**

Table 4

Mix gently, spin down and **transfer 15 µl** of the reaction mix per well. **Add 5 µl** of sample / control for a final volume of 20 µl. Seal plate with foil and centrifuge. Insert plate into the instrument. **Start run**

7. LightCycler® 1.x / 2.0 Instruments

Start programming before preparing the solutions. See the Instrument operator's manual for details.

7.1. Programming

The protocol consists of five program steps

1: UNG: Uracil-DNA Glycosylase treatment for removal of oxidize cytosine
2: Denaturation: Sample denaturation and enzyme activation
3: Amplification: Mutation-enrichment PCR-amplification of the target DNA
4: Melting: Melting curve analysis for identification product derived from the target DNA
5: Cooling: Cooling the instrument

For LightCycler® 1.2 and 1.5 instruments use software 4.10 or later.

Program Step:	UNG	Denat.	Mutation-Enrichment PCR Amplification						Melting				Cooling
Parameter													
Analysis Mode	None	None	Quantification mode						Melting Curves mode				None
Cycles	1	1	60						1				1
Target [℃]	40	95	95	95	58	58	72	72	95	58	40	85	40
Hold [hh:mm:ss]	00:10:00	00:10:00	00:00:10	00:00:30	00:00:05	00:00:05	00:00:05	00:00:10	00:00:20	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [℃/s]	20	20	20	20	20	20	20	20	20	20	20	0.2	20
Sec Target [℃]				81			58						
Step Size [℃]				0.7			0.7						
Step Delay (cyles)				1			1						
Acquisition Mode	None	None	None	None	Single	None	None	None	None	None	None	Cont.	None

Table 5

7.2 Data Analysis

Perform data analysis, as described in the instrument manual. View data in channel 640 "Tm Calling".

(1) The provided Positive Control must yield a peak for each of the six mutation-search reactions.
(2) The Negative Control (NTC) must show no signal for all six mutation-search reactions
(3) Analyze all samples and the Positive Control for each single mutation reaction.
Check for melting peaks for the clinical samples and compare with the peak for the Positive Control.
Tm values differ according to the respective mutation - Tm can not be used for mutation identification.

7.3. Interpretation of Data

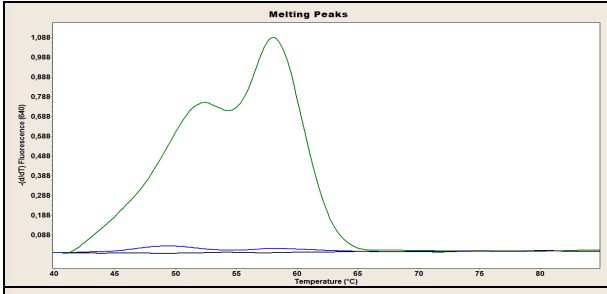
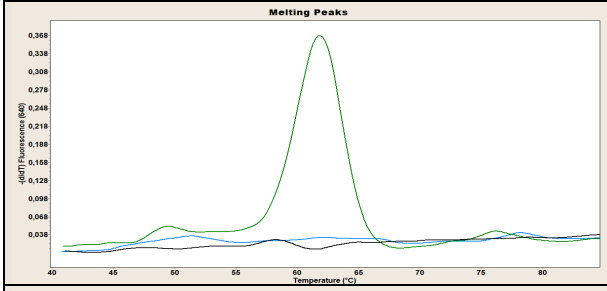
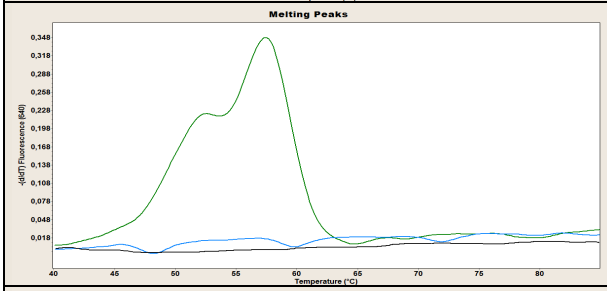
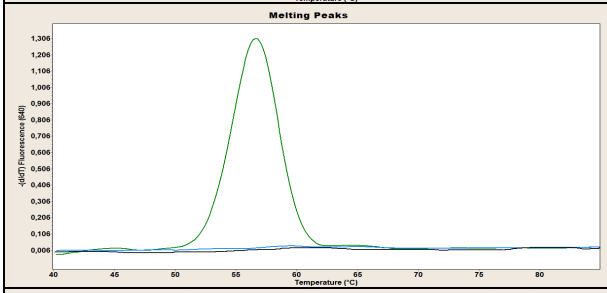
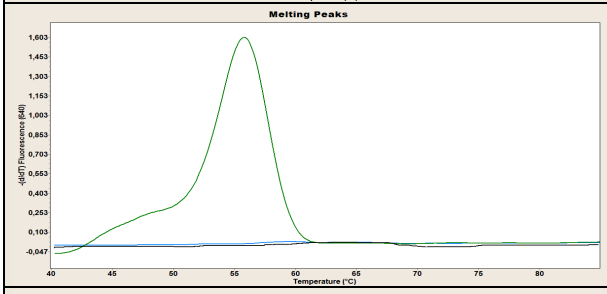
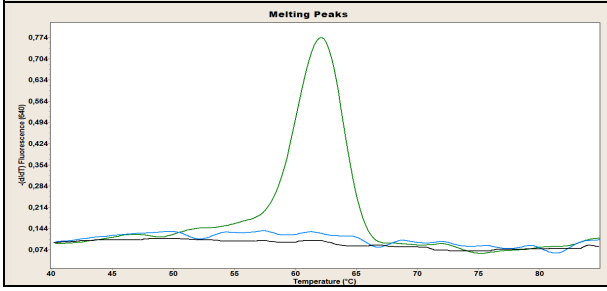
NRAS				KRAS		Result
12-13	59-61	117	146	117	146	
no peak	no peak	no peak	no peak	no peak	no peak	wild type
peak						mutation
	peak					mutation
		peak				mutation
			peak			mutation
				peak		mutation
					peak	mutation
58.1℃	61.8℃	57.3℃	56.6℃	56.2℃	62.0℃	Pos.Control

Table 6

Note: Compare the peak size with those of the positive controls. Analysis of only wild type samples will change automatic scaling of the melting curve window and baseline signals might look like a peak. Peaks with a size down to 50% of the controls are probably a mutation while peaks 20% and less are more likely wild type. The reagent detects also genomic mutations not causing an amino acid change. The sensitivity depends on the respective mutation and total amount of DNA and can be better than 1%. Low percentage mutations are not visible in Sanger sequencing and might be not detectable.
High amounts of DNA containing a single mutation might cause more than one peak; the presence of two or more significant peaks in one reaction is no evidence for the presence of several mutations.
This kit is not yet clinically validated. Verify identified mutations with a second method or submit extracted DNA samples to TIB Molbiol for verification. Contact us at: service@tib-molbiol.de

See section 10 for sample data and interpretation.

7.4. Sample Data – Typical Results obtained with the Positive Control

	**NRAS exon 2: Codons 12-13** **Green** : Variant c.35G>C Tm = 58.1°C **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) LightCycler® 2.0 pos control signal range **0.5 to 0.8**
	**NRAS exon 3: Codons 59-61** **Green** : Variant c.182A>G Tm = 61.8°C **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) LightCycler® 2.0 pos control signal range **0.2 to 0.4**
	**NRAS exon 4: Codon 117** **Green** : Variant c.349A>G Tm = 57.3°C **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) LightCycler® 2.0 pos control signal range **0.2 to 0.3**
	**NRAS exon 4: Codon 146** **Green** : Variant c.436G>A Tm = 56.6°C **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) LightCycler® 2.0 pos control signal range **0.8 to 1.1**
	**KRAS exon 4: Codon 117** **Green** : Variant c.351A>T Tm = 56.2°C **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) LightCycler® 2.0 pos control signal range **1.0 to 1.2**
	**KRAS exon 4: Codon 146** **Green** : Variant c.436G>A Tm = 62.0°C **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) LightCycler® 2.0 pos control signal range **0.5 to 0.7**

8. LightCycler® 480 II Instrument / cobas z 480 Analyzer

Start programming before preparing the solutions. See the Instrument operator's manual for details.

8.1. Programming

The protocol consists of four program steps:

- 1: Denaturation: Sample denaturation and enzyme activation
- 2: Amplification: Mutation-enrichment PCR-amplification of the target DNA
- 3: Melting: Melting curve analysis for identification product derived from the target DNA
- 4: Cooling: Cooling the instrument

Detection Format: 'Multi Color HybProbe'

LightCycler® 480 Instrument: 483-510 and 483-640
LightCycler® 480 II Instrument: 465-510 and 498-640
cobas z 480 Analyzer: 465-510 and 498-645

Program Step:	UNG	Denat.	2 : Mutation-Enrichment PCR Amplification				3 : Melting				Cooling
Parameter											
Analysis Mode	*None*	*None*	*Quantification mode*				*Melting Curves mode*				*None*
Cycles	1	1	60				1				1
Target [℃]	40	95	95	58	58	72	95	58	40	85	40
Hold [hh:mm:ss]	00:10:00	00:10:00	00:00:05	00:00:05	00:00:10	00:00:15	00:00:20	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [℃/s]	4.4	4.4	4.4	2.2	4.4	4.4	4.4	2.2	1.5	0.14	1.5
Sec Target [℃]											
Step Size [℃]											
Step Delay (cyles)											
Acquisition Mode	None	None	None	Single	None	None	None	None	None	Cont.	None
Acqu. per ℃										2	

Table 7

Note: In case that only one channel (640) is recorded change acquisition to 4 pictures / second to keep the slope of the melting curve in the same range.

8.2 Data Analysis

Perform data analysis, as described in the instrument manual. View data in channel 640 "Tm Calling".

(1) The provided Positive Control must yield a peak for each of the six mutation-search reactions.
(2) The Negative Control (NTC) must show no signal for all six mutation-search reactions
(3) Analyze all samples and the Positive Control for each single mutation reaction.
Check for melting peaks for the clinical samples and compare with the peak for the Positive Control.
Tm values differ according to the respective mutation - Tm can not be used for mutation identification.

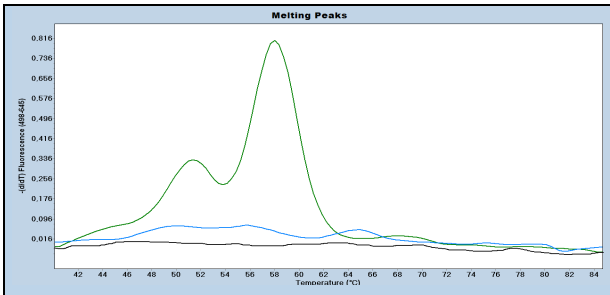
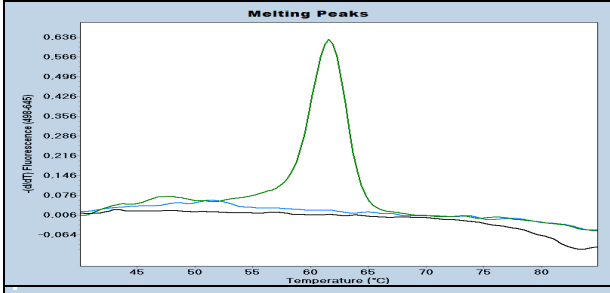
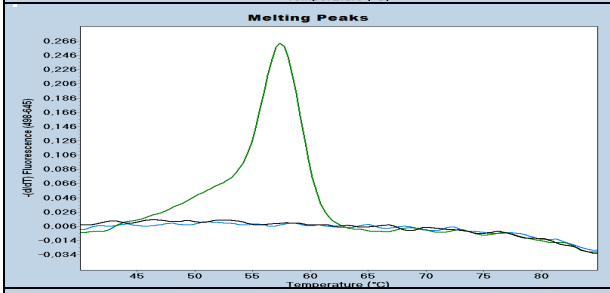
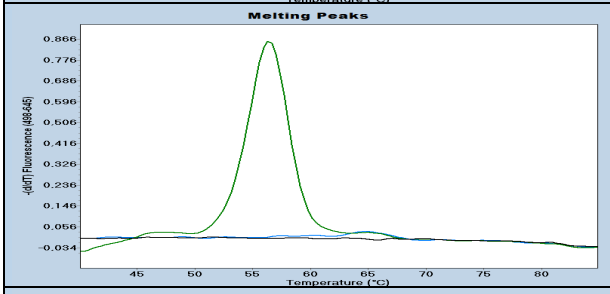
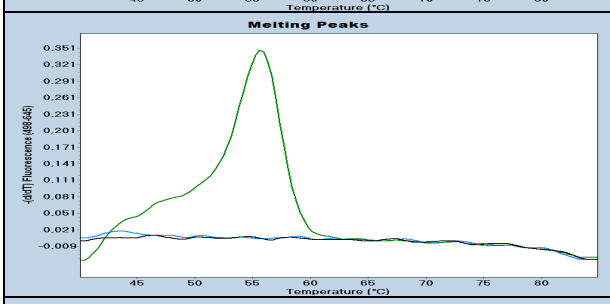
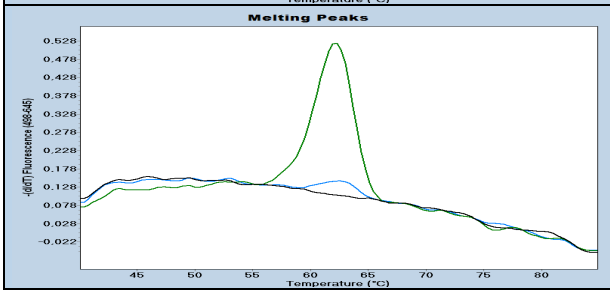
8.3. Sample Data – Typical Results

NRAS				KRAS		Result
12-13	59-61	117	146	117	146	
no peak	no peak	no peak	no peak	no peak	no peak	wild type
peak						mutation
	peak					mutation
		peak				mutation
			peak			mutation
				peak		mutation
					peak	mutation
57.9℃	61.6℃	57.4℃	56.1℃	55.9℃	62.0℃	Pos.Control

Table 8

See note in section 7.3
See section 10 for sample data and interpretation.

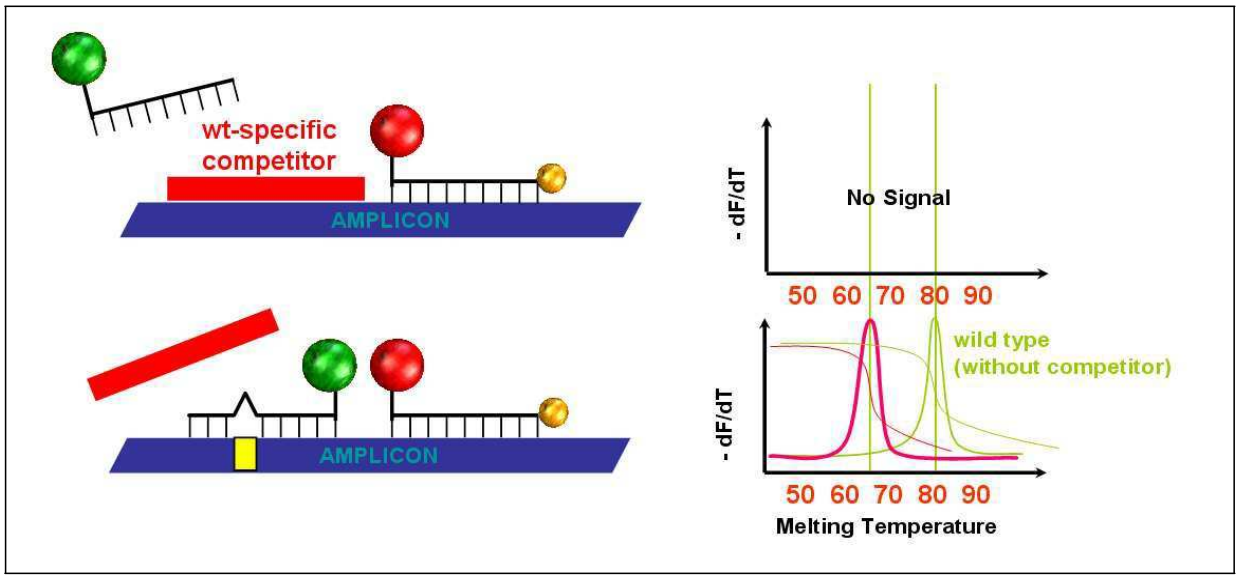
8.4. Sample Data – Typical Results obtained with the Positive Control

	**NRAS exon 2: Codons 12-13** **Green** : Variant c.35G>C Tm = 57.9℃ **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) cobas z 480 pos control signal range **0.6 to 0.9** LC480 II instruments report 25% higher signals
	**NRAS exon 3: Codons 59-61** **Green** : Variant c.182A>G Tm = 61.6℃ **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) cobas z 480 pos control signal range **0.4 to 0.6** LC480 II instruments report 25% higher signals
	**NRAS exon 4: Codon 117** **Green** : Variant c.349A>G Tm = 57.4℃ **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) cobas z 480 pos control signal range **0.2 to 0.3** LC480 II instruments report 25% higher signals
	**NRAS exon 4: Codon 146** **Green** : c.436G>A Tm = 56.1℃ **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) cobas z 480 pos control signal range **0.6 to 0.8** LC480 II instruments report 25% higher signals
	**KRAS exon 4: Codon 117** **Green** : Variant c.351A>T Tm = 55.9℃ **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) cobas z 480 pos control signal range **0.2 to 0.3** LC480 II instruments report 25% higher signals
	**KRAS exon 4: Codon 146** **Green** : Variant c.436G>A Tm = 62.0℃ **Light blue** : Wild type (not visible in MSR reaction) **Black** : No-Template-Control (NTC) cobas z 480 pos control signal range **0.4 to 0.6** LC480 II instruments report 25% higher signals

9. The Clamped Probe Assay - Test Principle and Interferences

This kit is based on a PCR amplification of the gene fragment containing the mutation and subsequent melting curve analysis with hybridization probes, reporting the presence of sequence variations in the probe binding region.

In order to reduce the amplification from the wild type target the reaction mix contains a wild type specific competitor oligonucleotide as published for conventional PCR first by Thiede et al., 1996. After the amplification reaction sensor and an anchor probe are hybridized to the mutation-enriched amplification product. The fluorescence decrease is measured during heating of the reaction mixture, reporting the dissociation of the sensor probe. Wild type target will generate a baseline curve while mutated samples will yield a melting peak. The observed melting temperature Tm is dependent on the number of mismatches between probe and target thus reporting different Tm values for different mutations. Since the Tm is also dependent on the amount of target DNA the Tm values may not be used for the identification of the respective mutation.



Clamped-Probe-Assay: Mit Locked-Nucleic-Acid (LNA) auf der Spurensuche. Landt, O. Biospektrum, 2005

The sensitivity of this test depends on the amount of input target DNA and on the respective mutation.

The values in this table have been determined for BRAF mutations using the same methodology, thus giving an estimate for the expected sensitivities in relation to the amount of target DNA used.

Table 9

DNA Input Amount	Minimum Fraction of Mutation Detected [Percent]					
ng / PCR	3.0	1.5	1.2	0.9	0.6	0.5
175						X
150					X	
100				X		
50			X			
20		X				
10	X					

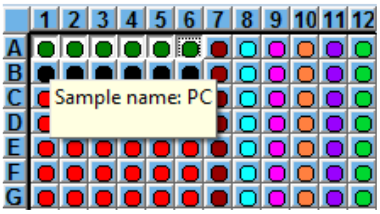
This type of assay can detect traces of sequence variations and is therefore extremly sensitive to (lab) contaminations as well as to bad quality of DNA. In particular prolonged formalin treatment may oxidize Cytosine bases generating Uracil which is homologue to Thymidin and will be detected like a mutation. UNG treatment of the FFPE extracted DNA will destroy Uracil and improve the results. However, also fragmented DNA may interfere with the reaction and cause an increased background or low temperature and low intensity peaks.

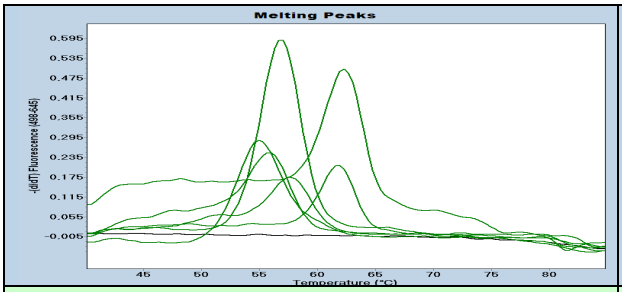
Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping
Thiede, C., Bayerdörffer, E., Blasczyk, R., Wittig, B. and Neubauer, A., NAR (1996) 24, 983-984

Detection of Point Mutations in Kirsten ras 2 Gene Using Locked Nucleic Acids Clamped PCR Beranek, Bures, Sacha, Sakra, Rajman, Jandik, Rudolf, Landt Chem. Listy 101, 738−741 (2007)

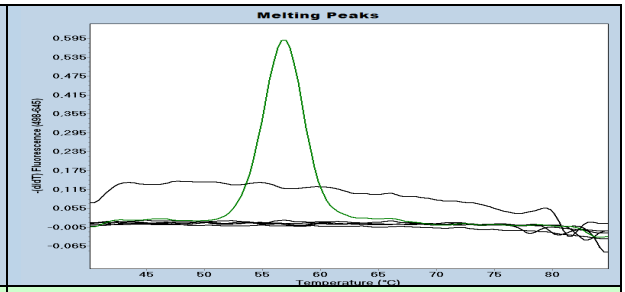
9. Sample Analysis Procedure cobas z 480 Instrument (compare section 8.2)

(1) The Positive Control must yield a peak for each of the six mutation-search reactions (**left**)
(2) The Negative Control (NTC) must show no signal for all mutation-search reactions (**right**)



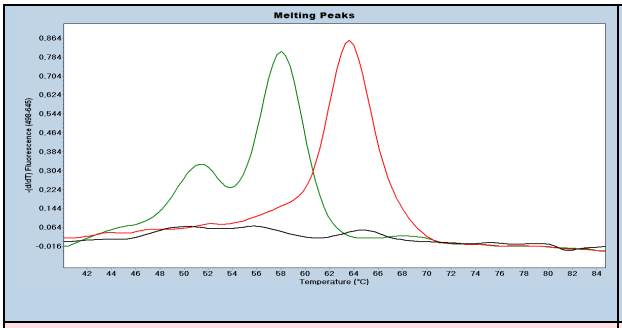


(1) Positive controls > Passed

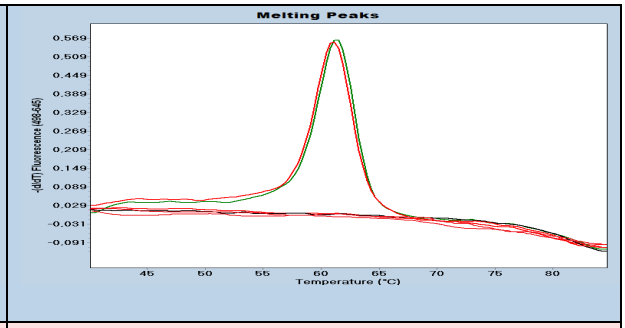


(2) NTC + one PC : Baseline > Passed

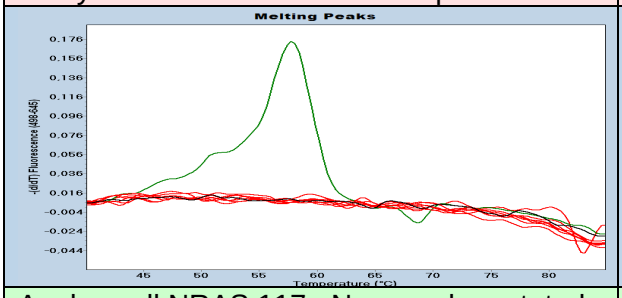
(3) Analyze all samples and the Positive Control **for each single mutation reaction**.



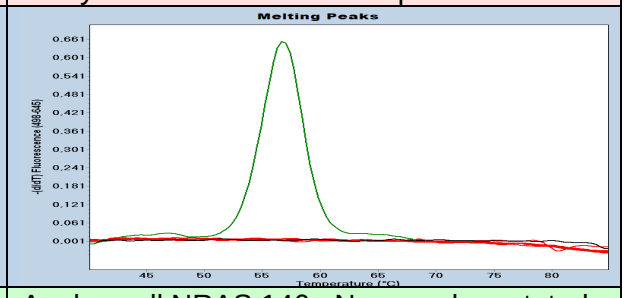
Analyze all NRAS 12/13 : 1 sample mutated



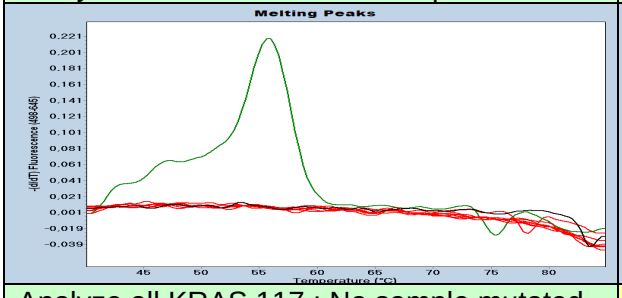
Analyze all NRAS 61 : 1 sample mutated



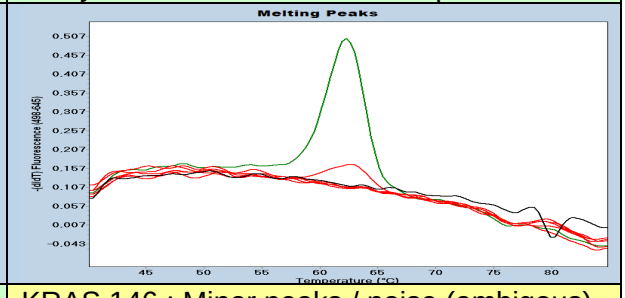
Analyze all NRAS 117 : No sample mutated



Analyze all NRAS 146 : No sample mutated



Analyze all KRAS 117 : No sample mutated

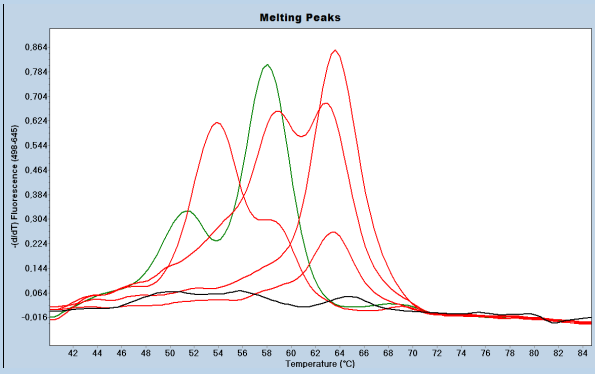


KRAS 146 : Minor peaks / noise (ambigous)

10. Sample Data and Interpretation of the Results

In this section we display melting curve results collected from clinical samples representing all discovered mutation types and hints for the interpretation of the results.

NRAS 12/13



Mutation

Green : PC = Variant c. 35G>Cm = 58°C
Red : Different Clinical Samples
Genotype not yet confirmed)
Black: No-Template-Control (NTC)

Characteristics:

- Single peak/double peak
- High signal intensity
- Melting temperature >46°C

Change in effect starting from Lot 26651403C and lots 2774 xxxx

The NRAS 12/13 reaction has been improved by moving the forward primer in order to exclude an additional sequence motif GGTGGT similar to the codons 12/13 sequence from the amplicon, which might have been a secondary binding site for the sensor probe causing minor melting curves (noise) and double peaks. The new version has been proofed to yield a flat baseline for wild type samples and has been tested to be able to detect mutated clinical samples.

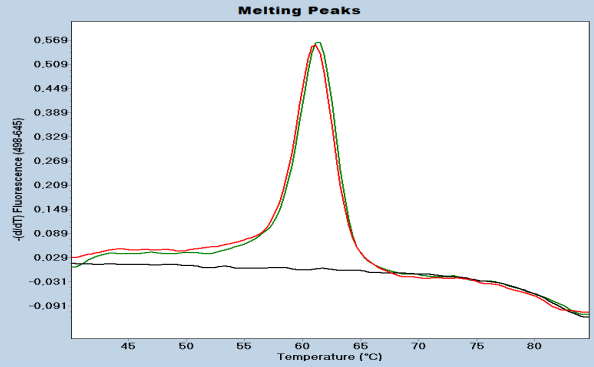
Change in effect starting from Lot 2992 xxx

The NRAS 12/13 sensor probe contains one mismatch base in order to prevent to the primer binding regions; the Tm values for NRAS 12/13 are reduced by about 5°C. See table 2.

Change in effect starting from Lot 33421501

The NRAS 12/13 sensor probe has been improved to prevent minor melting peaks.

NRAS 59-61

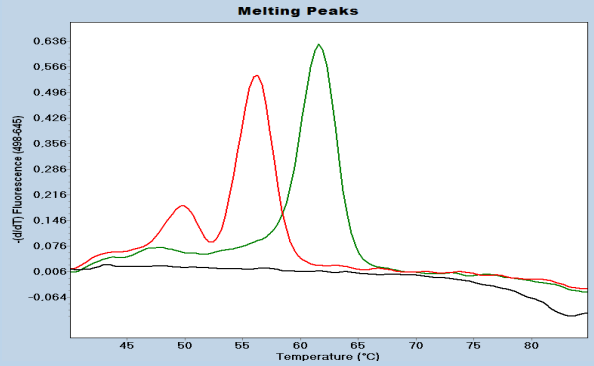


Unambiguous Result : Mutation

Green : PC = Variant c.182A>G Tm = 61.4℃
Red : Clinical Sample c.182A>G Tm = 61.3℃
Confirmed by Sanger sequencing
Black : No-Template-Control (NTC)

Characteristics:

- Sharp single peak
- High signal intensity
- Melting temperature >46℃

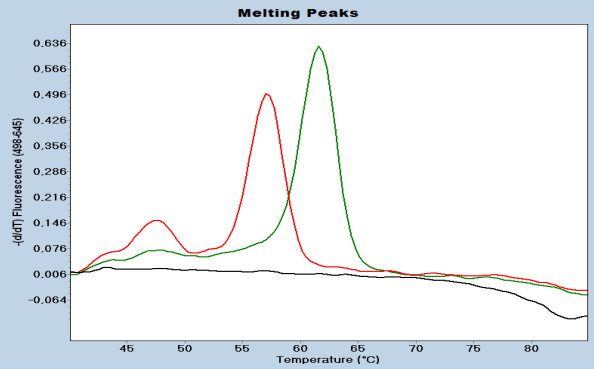


Unambiguous Result : Mutation

Green : PC = Variant c.182A>G Tm = 61.4℃
Red : Clinical Sample c.182A>T Tm = 56.2℃
Confirmed by Sanger sequencing
Black: No-Template-Control (NTC)

Characteristics:

- Sharp single peak
- High signal intensity
- Melting temperature >46℃

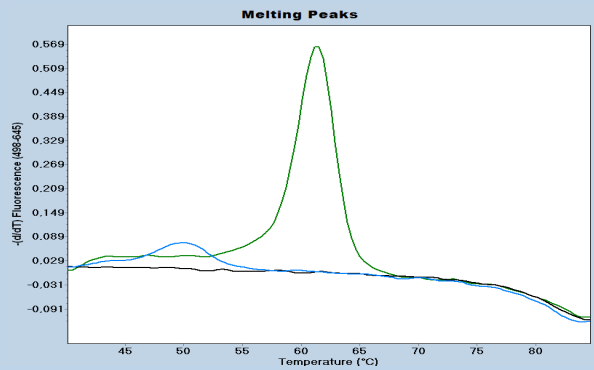


Unambiguous Result : Mutation

Green : PC = Variant c.182A>G Tm = 61.4℃
Red : Clinical Sample c.181C>A Tm = 57.2℃
Confirmed by Sanger sequencing
Black : No-Template-Control (NTC)

Characteristics:

- Sharp single peak
- High signal intensity
- Melting temperature >46℃



Ambiguous Result : No Mutation

Green : PC = Variant c.182A>G Tm = 61.4℃
Light blue : Wild type Tm = 50.0℃
Black : No-Template-Control (NTC)

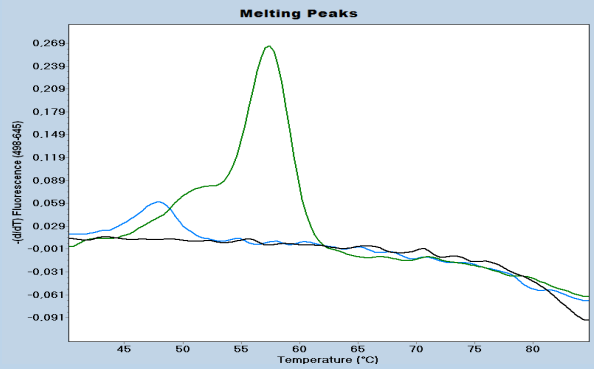
Characteristics:

- Broad peaks
- Double-peaks
- Low signal intensity (< 10%)
- Melting temperature <46℃

Change in effect starting from Lot 3100 xxx

The reaction mix contains sensor probes for variant 61R and in addition 61K, causing an upshift of the melting peak temperature for 61K and higher peaks. Variants 61K and 61L can be not dinstiguished

NRAS 117



Ambiguous Result : Wild type

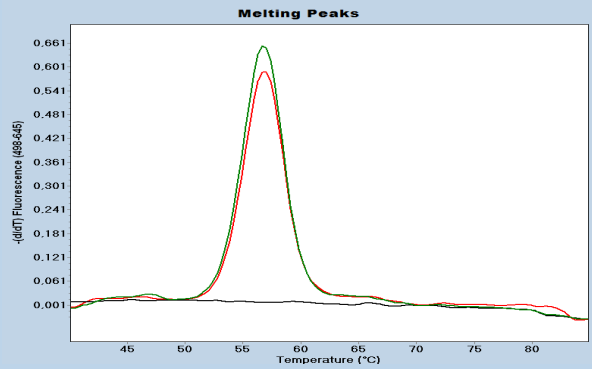
Green : PC = Variant c.349A>G Tm = 57,4℃
Light blue : Wild type Tm = 48.2℃
Confirmed by Sanger sequencing
Black : No-Template-Control (NTC)

Characteristics:

- Broad peaks
- Low / various signal intensities
- Melting temperature <50℃

Note : No NRAS 117 mutations in clinical samples observed so far (no mutations published).

NRAS 146



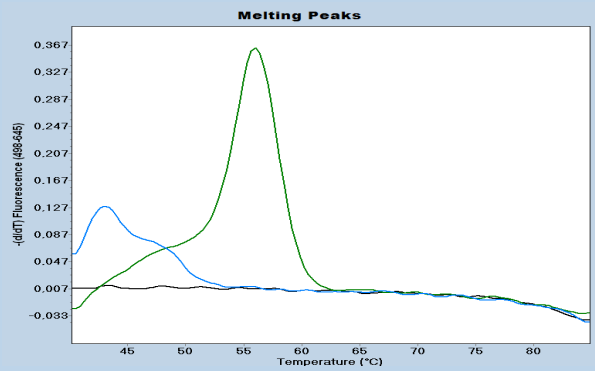
Unambiguous Result : Mutation

Green : PC = Variant c.436G>A Tm = 56.1℃
Red : Variant c.436G>A Tm = 56.3 ℃
Confirmed by Sanger sequencing
Black : No-Template-Control (NTC)

Characteristics:

- Sharp single peak
- High signal intensity
- Melting temperature >50℃

KRAS 117



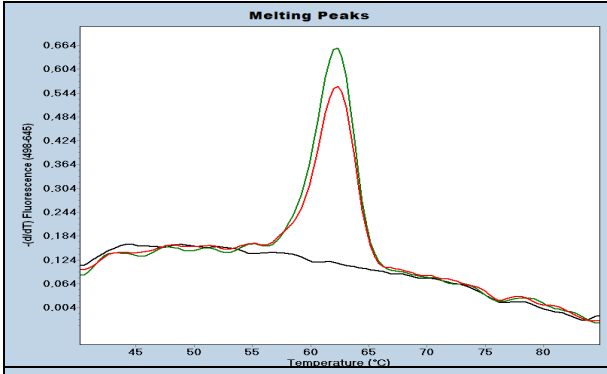
Ambiguous Result : Wild type

Green : PC = Variant c.351A>T Tm = 55.9℃
Light blue : Wild type Tm = 50.2℃
Black : No-Template-Control (NTC)

Characteristics:

- Broad peaks
- Various signal intensities
- Melting temperature ~50℃ and below

KRAS 146

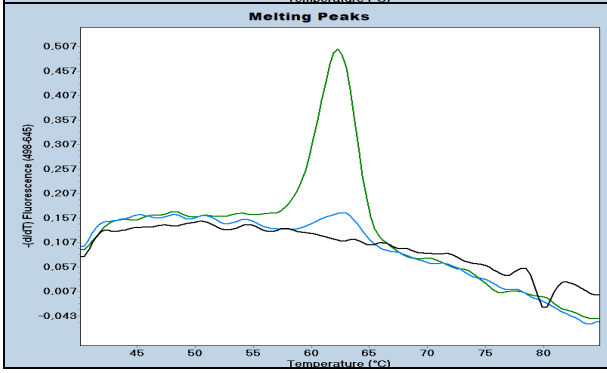


Unambiguous Result : Mutation

Green : PC Variant c.436G>A Tm = 62.9℃
Red : Variant c.436G>A Tm = 63.0℃
Confirmed by Sanger sequencing
Black : No-Template-Control (NTC)

Characteristics:

- Sharp peaks
- High signal intensities



Ambiguous Result : Wild type

Green : PC Variant c.436G>A Tm = 62.9℃
Light blue : Wild type
Confirmed by Sanger sequencing
Black : No-Template-Control (NTC)

Characteristics:

- Sharp peaks
- Low signal less than ~20% of Positive Control

11. Evaluation Status and Preliminary Laboratory Results

Sanger sequencing is the 'Gold Standard' for detection of somatic mutations. Wide-range testing of NRAS mutation has been started in 2013. The mutation frequencies for these mutations is not known and is estimated to be in the range of 10-15% for all KRAS codons 12-13 and 59-61 'wild type' tested samples (corresponding to 15-25% for all samples).

This *in-vitro* kit is currently tested in a clinical study (2013-2014).

12. Material Safety Data

According to OSHA 29CFR1910.1200, Australia [NOHSC:1005, 1008 (1999)] and the EU Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

13. Version History

Red notes mark events require changed procedures, blue mod. sequences

V131120	Release version
V131201	Number of positive control increased to 112 reactions
V131223	Amount of sample DNA to be used (section 6)
	Sensitivity and preliminary lab results - see section 9
V140204	UNG treatment of DNA included in the protocol
	Breakdown of z 480 runs : Cycle protocol changed
	Reaction volume 20 µl, amount of DNA limited to 100 ng
V140307	NRAS 12/13 reactions improved (primer exchanged)
	Section 10: Interpretation of Data
V140325	More Tm values in section 5 displayed, editorial changes
	Section 6.1 Volume allowed to be increased to 35 µl
	Stability in solution 21 days tested (before 10 days)
V140814	NRAS 12/13 specific probe changed > lower Tm
V141010	LightCycler 1.x/2.0 reaction volume reduced to 17 µl
	NRAS 61K-specific probe added to NRAS 59-61 reaction
V150420	NRAS 12/13 specific probe changed > higher Tm and peak
	N-Uracil-DNA Glycosylase (UNG) concentration reduced to 0,5U/rxn

Roche SAP order n° 07191766001

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