Instructions for use

MSI OncoKitDX

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Manufactured by:
Health in Code, S.L.
Agustín Escardino 9, 46980 Paterna (Valencia, España)
+34 963 212 340 – info@healthincode.com

healthincode.com

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Health in Code guarantees that all its products are free from defects, both in the materials used as well as in its manufacturing process. This guarantee extends through to the expiry date, so long as the storage conditions specified in this manual are observed.

Our products are intended for *in vitro diagnostic* use. Health in Code does not offer any other warranty, express or implied, that extends beyond the correct functioning of the components of this kit. Health in Code's only obligation, with respect to the aforementioned guarantees, will be to replace the products, or to return the purchase price thereof, at the customer's discretion, provided that the existence of a defect in the materials or in the development of the products, is proven.

Health in Code will not be responsible for any damage, direct or indirect, that results in economic losses or damages that may occur due to the use of this product by the buyer or user.

All products marketed by Health in Code are subjected to rigorous quality control.
MSI OncoKitDX has passed all internal validation tests, which guarantee the reliability and reproducibility of each test.

For any questions regarding the applications of this product or its protocols, please contact our Technical Department:

Telephone: +34 963 212 340
E-mail: tech.support@healthincode.com

Modifications to the Instructions for Use (IFU)

Version 02	Content review
Version 03	Change of manufacturer's identification from Imegen to Health in Code and format edition

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1. General Information

The term cancer makes reference to a wide and varied group of illness that are characterised by the uncontrollable growth of cells that can be dissected to tissues from other parts of the body. The causes that trigger the emergence of the cancer are very varied and, usually, are the result of the interaction of a high number of risk factors. These risk factors induce variations of the genes and the genome, this results in a loss of control of certain biological processes, that lead to uncontrolled cell growth.

The microsatellites instability (MSI) or error in replication is a manifestation of the genomic instability that emerges in a great variety of neoplasms, where the tumour cells have a global decrease in the capacity of faithfully DNA replication. The MSI is marker of the functional inactivation of some of the implicated genes in the repair of DNA, system MMR (mismatch repair); and it is associated with the elevated mutation ratios in the DNA coding.

The MSI has been studied mostly in colorectal cancer, sporadic and hereditary, but it has also been observed in other tissues, such as the endometrium and ovary. The MSI analysis gives the clinician valuable information about the diagnosis and prognostication of the tumor.

References

- *Pagin et al. British Journal of Cancer (2013). 108, 2079 – 2087.*
- *Karin D. Berg et al. Journal of Molecular Diagnostics (2000) Vol.2, No 1.*
- *John M. Carethers et al. Basic Science fundations in Colorectal Cancer (2017).*

2. Intended Use

Health in Code has developed **MSI OncoKitDX** for the analysis of the instability of microsatellites by conventional PCR and subsequently capillary electrophoresis.

This kit includes eight markers, of which, five are the recommended by the National Cancer Institute (NCI) since 1997 and are known as Bethesda panel (BAT25, BAT26, D2S123, D5S346 and D17S250). The sensibility and informativity of this panel in different tissues has been demonstrated in numerous studies. Besides the aforementioned 5 markers, the kit additionally contains other three: BAT40, NR21 and NR22.

These eight markers could be utilised for instability detection between the tumoral tissue and the healthy tissue of the patients. Furthermore, the markers D2S123, D5S346 and D17S250 are very polymorphic in the population and when they do not determine instability, they can also be utilised to guarantee the traceability of the samples.

The PCR products will be separated by capillary electrophoresis, and will be detected by different fluorophore labels, including 6-FAM™, NED™ y VIC®.

MSI OncoKitDX is only for *in vitro* diagnostic use and it is directed to professionals of the molecular biology sector.

Marker	Repetitive element	Size range (pb)	Size C+ (pb)	Fluorophore
BAT26	A	90 – 110	103	FAM
BAT25	T	130 – 150	145	FAM
BAT40	T	160 – 200	187	FAM
NR22	T	100 – 115	109	VIC
D2S123	CA	120 – 160	134,152	VIC
NR21	T	180 – 200	197	VIC
D5S346	CA	70 – 110	84,96	NED
D17S250	CA	170 – 200	176,180	NED

*Table 1. General information about the instability markers included in the **MSI OncoKitDX**.*

3. Technical characteristics

This kit has been validated using samples previously diagnosed by other laboratories, using different methodologies, as well as our own laboratories by the use of already validated systems. The kit detects specifically the defined targets in the section 2 of this document in a repetitive and reproductive way.

The necessary material for this study is genomic DNA from, both of the tumor tissue and of the healthy tissue of the same patient. This DNA can proceed from embedded tissues in paraffin, fresh or frozen tissue and peripherally blood. The total amount of necessary DNA is 10 ng.

Health in Code is certified, in accordance with the standard **UNE-EN ISO 13485:2018 Medical devices – Quality management systems - Requirements for regulatory purposes**, by THE AGENCIA ESPAÑOLA DE MEDICAMENTOS Y PRODUCTOS SANITARIOS for the Design, development and manufacturing of "in vitro" diagnostic medical devices:

- Kits for genetic testing
- Software for bioinformatic analysis of genetic data

4. Safety warnings and precautions

1. Strictly follow the instructions of this manual, especially regarding the handling and storage conditions.
2. Do not pipette by mouth.
3. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
4. You must properly protect any skin condition, as well as cuts, abrasions and other skin lesions.
5. Avoid discharge of reagents waste to the sink drinking water. Use waste containers established by the legislation and manage their treatment through an authorized waste manager.
6. In case of an accidental release of any of the reagents, avoid contact with skin, eyes and mucous membranes and clean with abundant water.
7. The materials safety data sheets (MSDS) of all hazardous components contained in this kit are available on request to Health in Code.
8. This product requires the handling of samples and materials of human and animal origin. You should consider all human and animal source materials as potentially infectious and handled in accordance with OSHA Biosafety Level 2 of bloodborne pathogens or must use other relevant biosafety practices for materials containing or suspect that they may contain infectious agents.
9. Reagents included in this kit are non-toxic, neither explosive, infectious, radioactive, magnetic, corrosive nor environmental polluters.
10. This kit has been validated with specific equipment under certain conditions, which could be different in other laboratories. It is recommended that each laboratory performs an internal validation when the kit is used for the first time.
11. The manufacturer is not responsible for the malfunction of the assay when one or more reagents included in the kit are replaced by other reagents not supplied by Health in Code.
12. The manufacturer does not guarantee the reproducibility of the assay when the user employs reagents not validated by Health in Code, considering them equivalent to those provided in the Kit.

5. Contents and storage conditions

This kit contains enough reagents to perform 48 determinations (24 cases of tumoral tissue and normal tissue from the same patient analysis). The kit includes the following reagents:

- **General Master Mix III:** Mix of PCR with the amounts of enzyme, nucleotides, and buffer necessary to carry out the amplification reactions.
- **MSI Buffer:** Contains MgCl2 to the necessary concentration to carry out the amplification of the target regions of the kit.
- **MSI Master Mix:** Contains the necessary oligonucleotides to carry on an amplification of the target regions of the kit.
- **Positive control:** Genomic DNA to the optimal concentration of the amplification in which normal alleles are present for all the targets included (See Section 8 of this document).

Reagents	Colour	Amount	Storage
General Master Mix III	White Disc	240 µL	-20°C
MSI Buffer	Green Disc	745 µL	-20°C
MSI Master Mix	Purple Disc	120 µL	-20°C
Positive Control	Purple Lid	24 µL	-20°C

*Table 2. Components of the **MSI OncoKitDX**.*

6. Equipment, reagents and material required but not supplied

Equipment:

- Conventional Thermocycler
- 10 µL, 20 µL, 200 µL and 1000 µL Micropipettes
- Vortex
- Centrifuge
- Capillary electrophoresis equipment

Reagents:

- GeneScan™ 500 LIZ® (Applied Biosystems cat. no. 4322682)
- Hi-Di™ formamide
- Nuclease-free water

Materials:

- Disposable micropipette filter tips (10 µL, 20 µL and 200 µL))
- 1.5 mL sterile tubes
- 0.2 mL tubes
- 93 plates compatible with capillary electrophoresis equipment
- Film for 96 plates
- Latex gloves

Note: This kit does not include the reagents and materials necessary to carry out capillary electrophoresis.

7. Assay protocol

7.1 PCR reaction preparation

MSI OncoKitDX is designed to perform a PCR reaction for each sample that is to be analysed, that is, two reactions for each case, a reaction with the sample, tumor tissue, and a reaction with the sample obtained from non-tumor tissue.

Here is shown the recommended protocol for the preparation of the reagents of PCR:

1. Thaw all the included reagents in the kit and the DNA from the samples. Vortex and spin each reagent and keep cold.
2. In a 1.5 mL tube add the following reagents per sample to prepare the PCR mix:

Reagents	Amount per reaction
General Master Mix III	5 µL
MSI Master Mix	2.5 µL
MSI Buffer	15.5 µL

Note: For the necessary amount estimation, the number of samples and the controls to be analyzed should be taking in count simultaneously. We recommend to do the calculations, considering one reaction more, or incrementing a 10% of the volume in each reagent.

3. Shake in the vortex.
4. In a 0.2mL tube dispense 23 µL of the mix of the PCR. Dispense as many 0.2 mL tubes as the number of samples or controls will be analyzed simultaneously.
5. In the corresponding 0.2mL tubes, add:
   - 2 µL of tumoral tissue
   - 2 µL of non-tumoral tissue.
   - 2 µL from the positive control included in the kit, to corroborate the well-functioning of the amplification systems.
   - 2 µL of water to corroborate that the reagents are not contaminated with DNA.
6. Place the tubes in the thermocycler and execute the next amplification programme:

Parameters	Stage 1	Stage 2			Stage 3	
No. of cycles	1x cycles	40x cycles			1x cycles	
	Enzymatic activation	Denaturation	Annealing	Extension	Final extension and conservation	
Temperature	95°C	95°C	62°C	72°C	60°C	10°C
Time	5 minutes	30 seconds	55 seconds	45 seconds	30 minutes	∞

Table 3. PCR optimum program for the SimpliAmp Thermal Cycler and GENEAMP® System 9700 of Applied Biosystems hardware

It is possible to stop the protocol at this point. The PCR products can be stored at 4°C if the protocol is continuing on the next 24 hours or at a -20°C for longer periods of time.

7.2 Fragment analysis preparation

To carry out the capillary electrophoresis, you have to prepare the products (fragments) in a 96-plate compatible with the capillary electrophoresis equipment, as indicated below:

1. In a 1.5 mL tube add the following reagents:

Reagents	Amount per reaction
Formamide	18 µL
GeneScan Marker™ 500 LIZ	0.5 µL

If there is more than one reaction, increase the volume of each reagent by 10%.

Note: the volume of the size marker can be increased or decreased to adjust the intensity of the peaks observed on the electropherogram.

2. Dispense 18.5 µL of the previous mix in each well.
3. Add 1 µL of the obtained DNA from the PCR reactions.

   Note: The volume of the sample can be increased or decreased (diluting the samples with nuclease-free water) to adjust the intensity of the observed peaks in an electropherogram.
4. Seal the plaque with the film, spin it and denaturalize with a thermocycler during 5 minutes at 98°C.
5. Keep the plate at 4°C until it is inserted into the capillary electrophoresis equipment.

7.3 Capillary electrophoresis

Once the fragment plaque is prepared, the reactions will be submitted to capillary electrophoresis. Depending on the sequencer model used. The electrophoresis conditions recommended by the manufacturer shall be used.

To set these conditions will be taken into account that the amplification range varies approximately between 100-200 pb, which primers labeled with 6-FAM,NED™ y VIC® are used and the molecular weight standard is labeled with GeneScan™ 500 LIZ.

Below is an image with the optimized conditions for the sequencer 3730xl DNA Analyzer (Thermo Fisher Scientific), using the polymer POP-7™.

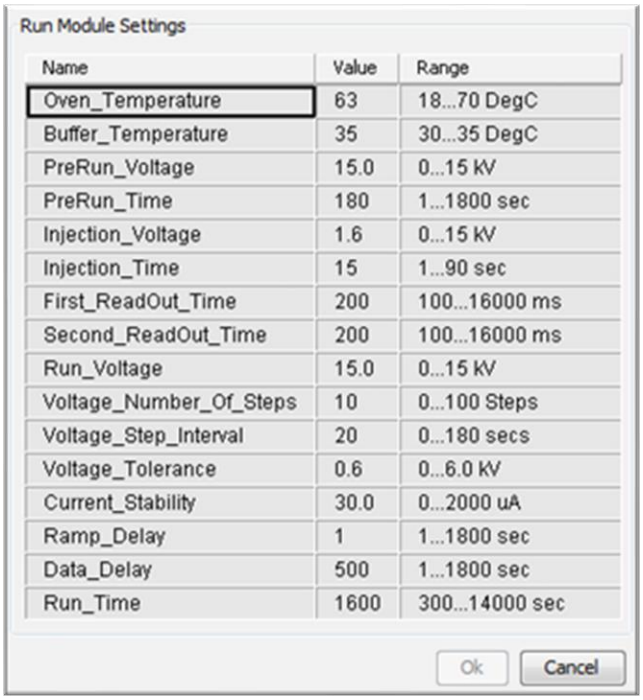

Figure 1. Optimised parameters for the sequencer 3730xl DNA.

The intensity in the detection can varies between different equipment, depending on the model, the state of the optical system and the time and the injection voltage. Therefore, it may be necessary to increase or decrease the amount of size marker or PCR product required to perform capillary electrophoresis.

8. Results analysis

The results analysis of the results is carried out with a specific programme for the analysis of fragments and the resulting .fsa file of the capillary electrophoresis. The result will be an electropherogram with peaks with a determinate intensity (height) and a distance directly proportional to the size of the fragment (figure 2).

For a correct analysis of the results it is recommended to follow the next indications:

- Check the absence of amplification in the negative controls (NTC). If amplification is detected, it is recommended to repeat the analysis to rule out accidental contamination.
- Check that the positive control has the amplification patron that is shown bellowed.
- For a good visualization of the results, it is recommended to analyze the markers by channels (FAM, VIC y NED), since markers of different channels can have the same size and not differentiate well when all the channels are analyzed simultaneously.

Figure 2. Electropherogram of positive control. The pink bar indicates the expected size range for each marker. Table 1 shows the base pair size of each marker for the positive control included in the kit, as the size range and the fluorophore of each marker.

- Analyse each case, observing simultaneously the sample of the tumor tissue and the sample of the non-tumoral tissue.

- The markers that have the same number of base pairs in both of the tissues, will be stable, meanwhile the ones that vary, in more or less degree, will be unstable markers.
- The D2S123, D5S346 and D17S250 markers, highly polymorphic in population, when they are not unstable, can be utilized for the traceability of the samples, being able to ensure that the sample of tumor tissue and the normal tissue sample come from the same patient if they have the same size in both tissues.
- It is possible, that in too degraded samples (DNAs from paraffinised tissue) not all of the markers can be amplified. Even so, the absence of up to two markers does not preclude the diagnosis of the case.
- Below is shown a scheme about the correlation between the number of unstable markers and the degree of instability, as well as two examples of the possible obtained results after the analysis of **MSI OncoKitDX**.

Degree of instability	Number of unstable markers
Stable (MSS)	0
unstable low (MSI-L)	1 - 3
unstable high (MSI-H)	> 3

Table 4. Correlation between the number of unstable markers and the degree of instability

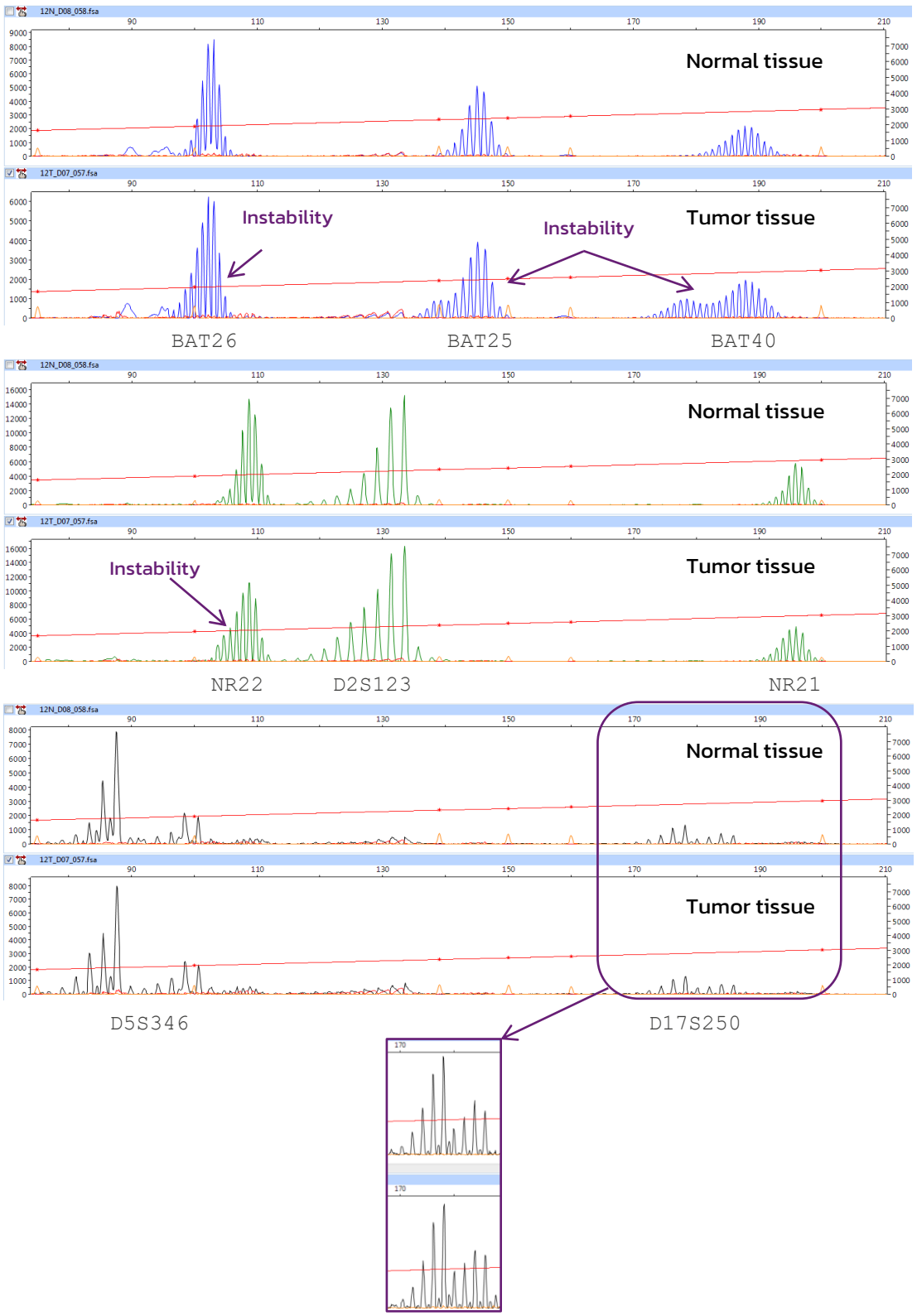

Figure 3. Electropherogram of an unstable high case, in which four of the eight markers are unstable.

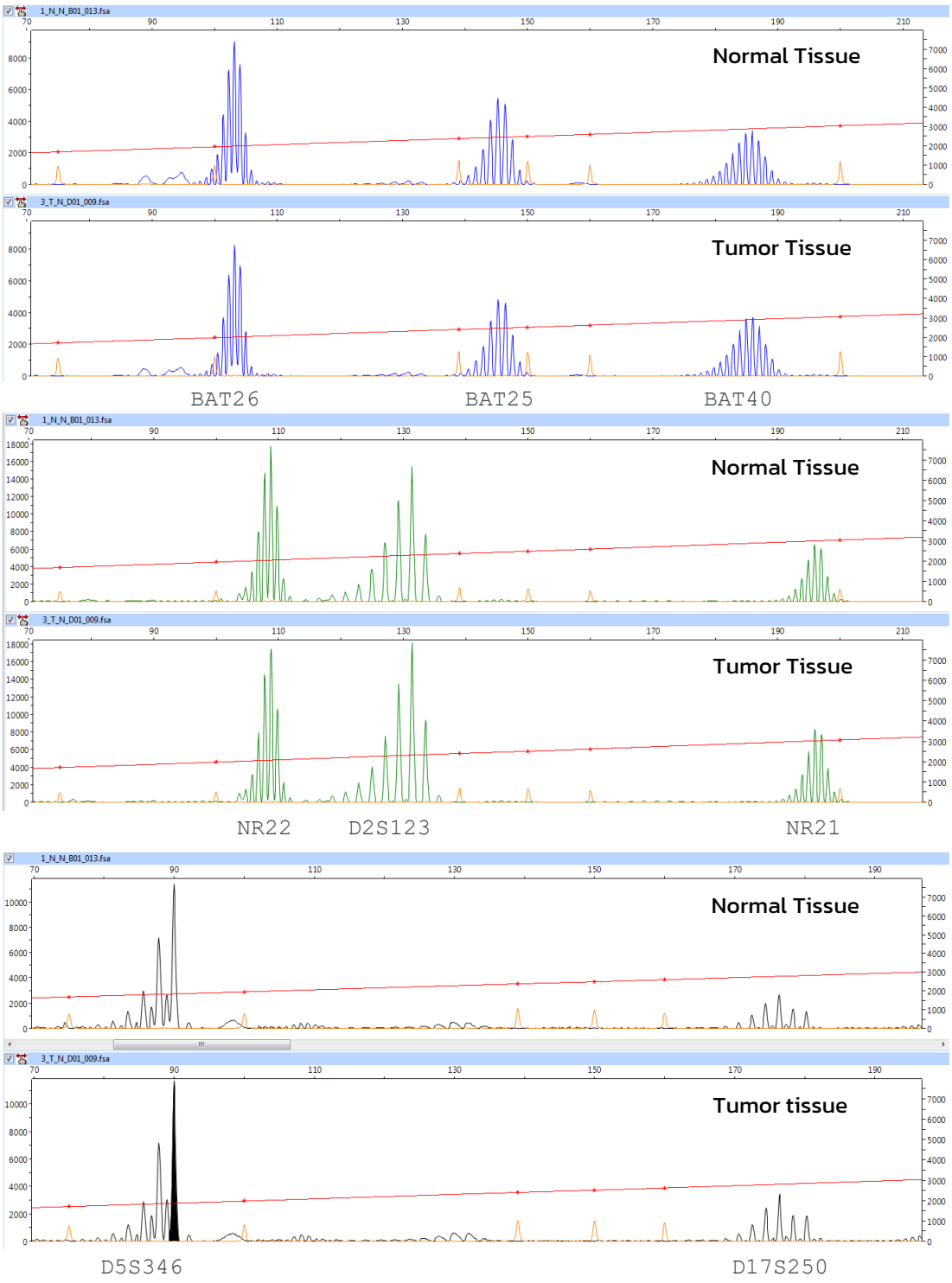

Figure 4. Electropherogram of a stable case, in which any of the markers present instability.

9. Troubleshooting

The following table resume problems or difficulties that can occur in the results of the samples, Positive control, the size marker and the negative control. In the eventuality of an unexpected result check for the probable cause in the table below.

Problem	Analyzed samples	Positive control	Size marker	Negative control	Possible causes
Week fluorescent signal for allele peaks				√	Expected result
	√			√	Insufficient/poor quality DNA template[1] Impure DNA template[2]
	√	√	√	√	Failure in the capillary electrophoresis[3] Failure in denaturalization [4]
	√	√		√	Failure in PCR[5]
Excessive fluorescent signal for allele peaks	√				Excessive amount of DNA [6]
	√	√			
Presence of more peaks than expected		√		√	Contamination[7]

*Table 5. Interpretation of possible results associated with the use of the **MSI OncoKitDx**.*

[1] **Insufficient/poor quality DNA template:** Make sure DNA has been accurately quantitated and use the recommended amount of template DNA. In the case of the DNA was correctly quantified, check for DNA integrity and eventually repeat DNA preparation.

[2] **Impure DNA template:** High salt concentration or altered pH can inhibit PCR amplification. If the DNA template is stored in TE buffer that is not pH 8.0 or contains a higher EDTA concentration, the DNA volume should not exceed 20% of the total reaction volume. Carryover from DNA sample of K+, Na+, Mg2+ or EDTA can negatively affect PCR. Changes in pH also may affect PCR. If it's so, clean the DNA sample or repeat DNA preparation.

[3] **Poor capillary electrophoresis injection:** Check if the instrument parameters are the specified ones and re-inject sample.

[4] **Samples were not properly denatured before loading:** Heat-denature samples for the recommended time (section 7 of this document) and cool on crushed ice or in an ice-water bath immediately prior to loading.

[5] **Thermal cycler or tube problems:** Check if PCR program is the specified one.

[6] **Too much template DNA:** Make sure DNA was accurately quantitated. If it is so, dilute the PCR product in sterile deionized water and repeat sample denaturation and loading.

[7] **Contamination:** It may be caused by another template DNA or a previously amplified DNA. Cross-contamination can lead to false positives or negative results, and consequently to problems in results interpretation. Use aerosol-resistant pipette tips, and change gloves regularly.

10. Limitations

10.1 Equipment

MSI OncoKitDX has been validated using the following PCR Thermal Cyclers:

- SimpliAmp Thermal Cycler (ThermoFisher Scientific)
- GeneAmp PCR System 2720 (ThermoFisher Scientific)

If you use another brand or model of thermal cycler, you may need to adjust the amplification programme. Please contact our technical support team for any query or clarification.

MSI OncoKitDX has been validated using the following sequencer:

- 3730xl DNA Analyzer (ThermoFisher Scientific)

This kit is valid only with the polymers compatible with 6-Carboxifluoresceina (6-FAM) labelling. If you use another brand or model of sequencer, follow the instructions and protocol recommendations of that instrument.

10.2 Reagents

MSI OncoKitDX has been validated using the reagents included in the kit and the ones recommended in the section 6 of this manual [Equipment and materials required but not supplied].

For the capillary electrophoresis it is advised to use the reagents recommended by the supplier of the sequencer: ThermoFisher Scientific.

Please contact our technical support team for any query or clarification.

10.3 Product Stability

The optimal analytical functioning of this product is confirmed as long as the recommended storage conditions are applied from the reception of the kit until the expiry date assigned to each production batch.