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Titano MSI

User manual – version 2017/03

The "**Titano MSI**" kit allows the determination of microsatellite instability in colorectal tumors by multiplex PCR and DNA fragment analysis.

For *in vitro* diagnostic use

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Changes made since the previous version 2016/09:

- Section “KIT CONTENTS”:
  - Substitution of separate amplification reagents with master mix **Taq Premix 700**.
  - Substitution of the four amp-mix Titano mix A, Titano mix B, Titano mix C, Titano mix D with the four amp-mix **Titano mix 1**, **Titano mix 2**, **Titano mix 3**, **Titano mix 4**.
  - Substitution of the positive control Titano Control DNA with **Titano pos ctrl**.

- Section “REQUIREDE EQUIPMENT”:
  - Addition of instrument “**Easy PGX® qPCR instrument**” (Diatech Pharmacogenetics); “**Labcycler Basic / Gradient**” (SensoQuest GmbH) as validated instrument for amplification.

- Section “ANALYTICAL PROCEDURE”: revision of the entire protocol.

- Upgrading of the section “PERFOMANCE VALIDATION”.

For further details contact the technical support of the Diatech Pharmacogenetics (support@diatechpharmacogenetics.com).

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INTENDED USE

The "**Titano MSI**" kit is an *in vitro* diagnostic assay for the determination of the microsatellite instability in colorectal carcinoma samples by multiplex PCR with fluorescent primers and DNA fragment analysis on automated sequencer. The test amplifies DNA extracted from normal and tumor tissue (fresh, frozen or formalin fixed and paraffin embebbed – FFPE) or from whole blood (if normal tussue is not available).

List of detectable markers:

BAT25 BAT26 D2S123 D17S250 D5S346	Bethesda panel
BAT40 D18S58 NR21 NR24 TGFβRII	
TPOX TH01	Control markers

The "**Titano MSI**" kit is validated to used with the following instrument:

- Amplification: "**Easy PGX® qPCR instrument**" (Diatech Pharmacogenetics); "**Labcycler Basic / Gradient**" (SensoQuest GmbH)
- Fragment analysis: "**3130/3130XL Genetic Analyzer**", "**ABI PRISM 310 Genetyc Analyzer**" (Applied Biosystems).

PRINCIPLE OF THE ASSAY

The "**Titano MSI**" kit allows the determination of the microsatellite instability in colorectal carcinoma samples.
For each patient, DNA extracted from tumor tissue and normal tissue (or from whoole blood) are tested in parallel.
Each sample is amplified by multiplex PCR with fluorescent primers using 4 oligos mixes to amplify all the markers. Amplification products are then analyzed with automated sequencer. The microsatellite instability is determined by comparison of the DNA fragments obtained from normal and tumor samples of the same patient.

The "**Titano MSI**" kit detects markers of the Bethesda panel (BAT25, BAT26, D2S123, D5S346, D17S250) and five other mono- and di-nucleotide markers for the determination of the microsatellite instability. The kit detects also two control markers (TPOX and TH01) for the identification of possible samples exchange or contaminations.

Makers	Mix	Dye	Filter
NR21	1	Atto550	Yellow
D18S58		HEX	Green
NR24		FAM	Blue
BAT40	2	HEX	Green
D2S123[1]		FAM	Blue
TGFβRII		FAM	Blue
BAT25[1]	3	HEX	Green
D17S250[1]		FAM	Blue
TPOX[2]		HEX	Green
D5S346[1]	4	FAM	Blue
BAT26[1]		Atto550	Yellow
TH01[2]		HEX	Green

1. Bethesda panel
2. Control marker

The "**Titano MSI**" kit includes the **Titano pos ctrl**, a genomic DNA to check the analytical process.

CLINICAL RELEVANCE

Microsatellites (or *Short Tandem Repeats - STR*) are short non coding DNA repeats of 1-6 nucleotides. Each microsatellite has a different number of repeats, so alleles with different length can be detected in the population.
Microsatellite instability (MSI) is caused by defects in the proteins involved in the *DNA Mismatch repair (MMR)* mechanism that are detectable in 85-90% of hereditary non polyposis colorectal cancer (HNPCC) cases and 15-20% of sporadic colorectal cancers (CRCs) [1, 2, 3, 4].
In HNPCC microsatellites instability is caused by germline mutations in the genes MLH1, MSH2, MSH6, PMS2 that codify for MMR proteins, while, in the majority of sporadic CRCs, is linked to MLH1 inactivation by promoter methylation [5, 6].
In 1997 and then in 2002, a reference panel of 5 microsatellites markers (BAT25, BAT26, D2S123, D5S346, D17S250), called Bethesda panel, has been proposed to classify a sample as stable (MSS), MSI-high (MSI-H), MSI-low (MSI-L) on the basis of the number of unstable markers.
For MSI-L samples, classified only on the basis of Bethesda panel, is recommended to test an additional panel of mononucleotide markers to obtain a more accurate tumor characterization[7, 8, 9].
MSI-H tumors show peculiar clinical, pathological, biological and genetic features, while MSI-L (5-10% of the cases) and MSS tumors share the same pathological and molecular profile.
Prognostic data indicate that MSI-H tumors have a better survival than MSI-L or MSS [10, 11].
Microsatellites instability can be a predictive factor of response to 5-fluorouracil (5-FU) therapy, in fact some studies suggest that patients with MSS/MSI-L have a better response to 5-FU therapy respect to MSI-H patients[10].
On the contrary, another prognostic study in patients with colorectal cancer at stage II or III treated with 5-FU indicates that patients with MSI-H stage II CRC have a better survival than MSS patients[12].
Moreover, recent studies report MMR status as a predictive factor for the response to the PD-1 antibody pembrolizumab: tumors with MMR deficit show a better response to immunotherapy than tumors without MMR deficit [13].
Therefore, evaluation of microsatellites instability is an important indirect method to detect MMR alterations linked to HNPCC tumors and can have a role as a predictive and prognostic factor in the sporadic CRC.

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KIT CONTENTS

The kit contains sufficient reagents to carry out 24 tests with a maximum of 3 independent runs (7 normal/tumor sample pairs and controls per run).

COMP		QUAN	Colour	
Titano mix 1		3 x 36 µl	GREEN	Mixture of specific fluorescent primers targeting NR21, NR24, D18S58 markers.
Titano mix 2		3 x 36 µl	RED	Mixture of specific fluorescent primers targeting BAT40, TGFβII, D2S123 markers.
Titano mix 3		3 x 36 µl	YELLOW	Mixture of specific fluorescent primers targeting BAT25, D17S250, TPOX markers.
Titano mix 4		3 x 36 µl	WHITE	Mixture of specific fluorescent primers targeting BAT26, D5S346, TH01 markers.
Taq Premix 700		3 x 700 µl	BLUE	Solution containing hot start Taq DNA polimerase, reaction buffer, Mg^{2+} and dNTP mixture.
Titano pos ctrl	CONTROL +	3 x 50 µl	VIOLET	Positive control containig genomic DNA.
WATER (small)	CONTROL -	3 x 0.5 ml		DNase-, RNase-free water to be used exclusively for the preparation of the PCR mix and as negative control.
WATER		3 x 1.5 ml		DNase-, RNase-free water: 1 aliquot to be used exclusively as diluent for input DNA, 2 aliquots to be used exclusively as diluent for amplicons during sequencng step.

DOCUMENTS AVAILABLE ON-LINE

"**Titano MSI**" User Manual and Material Safety Data Sheet (MSDS) are available at www.diatechpharmacogenetics.com/en/reserved-area.

MATERIALS REQUIRED BUT NOT PROVIDED

DNA extraction reagents

Recommended kits:

- Kit to be used with MagCore Automated Nucleic Acid Extractor (RBC Bioscience):
  - "**Genomic DNA Whole Blood Kit**" (code MGB-02, RBC)
  - "**Genomic DNA FFPE One-Step Kit**" (code MGF-03, RBC)
  - "**Genomic DNA Tissue Kit**" (code MGT-02, RBC)
- "**QIAamp® DNA Mini kit**" (code 51304, Qiagen)
- "**QIAamp® DNA FFPE Tissue kit**" (code 56404, Qiagen)
- In case you are using FFPE tessues, you will also need:
  - Xilene (eg. "Xylenes, histological grade" – code 534056, Sigma Aldrich)
  - Absolute ethanol (quality of analytical degree)

ⓘ In case you employ kits which are different from those recommended, it is the user's responsibility to use standardized samples (e.g,: VEQ – EQAS quality schemes, Horizon Diagnostics samples) to verify that this does not imply a reduction of the performance of the system under analysis.

Capillary electrophoresis reagent

INSTRUMENT	POLYMER	SPECTRAL CALIBRATION (fluorophore FAM-HEX-NED-ROX)	SIZE-STANDARD	RUNNING BUFFER
ABI310	**"POP-4™ Polymer for 310 Genetic Analyzers"** code 402838, Applied Biosystem by ThermoFisher Scientific	**"DS-30 Matrix Standard Kit (Dye Set D)"**, code 4345827, Applied Biosystem by ThermoFisher Scientific	**"GeneScan™ 500 ROX™ dye Size standard"** code 401734, Applied Biosystem by ThermoFisher Scientific	**"310 and 31xx Running Buffer, 10X"** code 402824, Applied Biosystem by ThermoFisher Scientific
ABI3130 / 3130XL	**"POP-7™ Polymer for 3130/3130xl Genetic Analyzers"** code 4352759, Applied Biosystem by ThermoFisher Scientific			

- Reagents suitable for the sequrencer in use and specifc for fragment analysis reported in the user manual, if different from the ones listed above.
- Deionized formamide (eg. **Hi-Di™ Formamide**, code 4311320, Applied Biosystem by ThermoFisher Scientific)
- Capillary suitable for the sequencer.
- Suitable tubes or plate and septa for the sequencer.

Materials
- Sterile filter tips DNase-, RNase-free (volumes from 1 to 1.000 µl).
- 1,5 ml polypropylene twist-lock tubes (DNase-, RNase-, DNA-, PCR inhibitor-free).
- Powder-free disposable gloves

For **Easy PGX® qPCR instrument** (Diatech Pharmacogenetics):

a. 8-well strip tube without caps "*low profile*" (volume 0.15 ml) (eg. **FrameStar® Low Profile Break-A-Way PCR Plate, 96 clear wells** code 4ti-1200, 4titude®)
   8-well strip flat caps suitable for 8-well strip tubes (eg. **Strips of 8 flat optical caps** code 4ti-0751, 4titude®)

For **Labcycler Basic / Gradient** (SensoQuest GmbH), possible alternatives:

a. 96-well plate (0.2 ml volume) (eg. **Thermo-Fast 96 Skirted PCR Plate with Black Lettering natural 25 plates** code AB-0800-L, ThermoFisher Scientific)
   Adeshive foils suitable for 96-well plate (eg. **Adhesive Sealing Sheets Natural 100 Sheets** code AB-0558, ThermoFisher Scientific)

b. 0,2 ml PCR tubes with thin wall DNase- and RNase free (eg. **PCR tubes 0.2 (1000)** code DIA-PL2, Diatech Pharmacogenetics)

c. 8-well strip tubes without caps "*low profile*" (0.15 ml volume) (eg. **FrameStar® Low Profile Break-A-Way PCR Plate, 96 clear wells** code 4ti-1200, 4titude®)
   8-well strip flat caps suitable for 8-well strip tube (eg. **Strips of 8 flat optical caps** code 4ti-0751, 4titude®)

REQUIRED EQUIPMENT

DNA extraction equipment
- specific equipment recommended by the user manual of the kit employed
- spectrophotometer suitable for reading small sample volumes (eg. "**Dropsense 16**", Trinean)

Amplification equipment
- "**Easy PGX® qPCR instrument**" (code RT800, Diatech Pharmacogenetics) or
- "**Labcycler Basic / Gradient**" (code 011-103 / 011-101, SensoQuest GmbH) with 96-well thermoblock ("**Labcycler Thermoblock 96**", code 012103 – SensoQuest GmbH) and related accessories (sealing pad and touchscreen pen).
- Micropipettes (volumes from 1 to 1.000 µl).
- Vortex and spinner.

Capillary electrophoresis equipment:
- Sequencer "**ABI 3130/3130XL Genetic Analyzer**" or "**ABI 310 Genetic Analyzer**" (Applied Biosystems by ThermoFisher Scentifics).
  Environmental condition required to guarantee the correct functionality of the system: temperature: 15-30°C; relative humidity 20-80%
- Data analysis software (eg. Gene Mapper - Applied Biosystems, Peak Scanner - Applied Biosystems).
- Thermalcycler or thermoblock for amplification product denaturation (required temperature 95°C).
- Micropipettes (volumes from 1 to 1.000 µl).
- Chemical hood.

ⓘ If using tools other than those recommended, it is the responsibility of the user to validate the procedure and verify with standard samples that this does not result in a reduction in system performance.

STABILITY AND STORAGE

Store all the reagents according to the instructions on the packages, in particular:

- Store all the reagents at -35/-20°C in the original package immediately upon receipt.
- Avoid thawing and re-freezing the reagents more than twice, as this could lead to poor performance.
- After using immediately store the **Taq Premix 700** at -35/-20°C
- Protect all mixes containing fluorescent primers from light to avoid degradation of the fluorescent dyes.
- If properly stored, the reagents remain stable until the expiration date displayed on the individual label.

SYMBOLS

REF	Catalog number (product code)	CONTROL -	Negative control
LOT	Lot number		Consult the instruction
	Content sufficient for <n> tests		User manual (handbook)
IVD	For *in vitro* diagnostic use		Expiration date
CONT	Content		Storage conditions
COMP	Components		Manufactured by
NUM	Number of aliquots		Important Note
QUAN	Quantity per aliquot	GTIN	Global Trade Item Number
CONTROL +	Positive control		

PRODUCT USE LIMITATIONS

- The "**Titano MSI**" kit can only be used by specialized personnel, properly instructed and trained.
- It is necessary to operate in compliance with the general guidelines of Good Laboratory Practice (GLP) and the instructions contained in this manual.
- Do not use expired or incorrectly stored reagents.
- The "**Titano MSI**" kit is designed to be use with "**Labcycler Basic / Gradient**" (SensoQuest GmbH) and "**Easy PGX® qPCR instrument**" (code RT800, Diatech Pharmacogenetics) amplification instruments and with the sequencer "**ABI 3130/3130XL**", "**ABI 310 Genetic Analyzer**" (Applied Biosystems).
- The use of "**Titano MSI**" kit with instrument that differs from the ones recommended above is out of the manifactured CE Mark for *in vitro* diagnostic use.
- The reliability of the results also depends on the procedures carried out in the pre-amplification stages, including the selection of starting biological speciments, the preservation of the samples and DNA extraction.
- Any diagnostic results generated by this procedure must be interpreted with reference to other clinical or laboratory findings.

QUALITY CONTROL

- The "**Titano MSI**" kit was designed, developed and validated in accordance with D.Lgs n. 332 of 08/09/2000 ("Implementation of Directive 98/79 / EC on in vitro diagnostic medical devices") and subsequent legislative changes.
- In accordance with the company system of full quality assurance (certified according to European standards EN ISO 9001 and ISO 13485), to ensure consistent product quality, each batch of "**Titano MSI**" is subjected to functional quality control according to technical specifications and is released only if it is compliant with the quality control criteria.

WARNINGS AND PRECAUTIONS

- The kit may only be used by specialist personnel, properly instructed and trained to perform *in vitro* laboratory techniques.
- Handle all samples as potentially infectious material inside a laminar flow hood (class II biological safety cabinet or higher).
- It is recommended to carry out all steps involving the use of formamide under a chemical hood.
- Follow the laboratory safety procedures described in "Biosafety in Microbiological and Biomedical Laboratories" (Richmond, JY and McKinney, RW (eds) - 5th edition (2009) and in the NCCLS (National Committee for Clinical Laboratory Standards) Document M29-T. Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids and Tissue. Tentative guidelines. – Villanova, PA:NCCLS, 1989).
- Do not eat, drink or smoke in the laboratory. When handling biological samples, disposable gloves, gowns and goggles or face masks should be worn to protect against biological agents.
- Constantly check that the gloves are free from contamination by the biological material being treated. If not, replace them immediately to avoid the possibility of cross-contamination between samples and contamination of the workplace. Wash hands thoroughly after handling samples and reagents.
- The Material Safety Data Sheet (MSDS) is available in the reserved area of the web-site Diatech Pharmacogenetics www.diatechpharmacogenetics.com.
- Perform the procedure in accordance with Good Laboratory Practice (GLP) general guidelines.
- It is recommended to ensure that the laboratory work flow proceeds in a unidirectional manner, preparing, if possible, three separate work areas for:
  - extraction of nucleic acids;
  - amplification reaction;
  - capillary electrophoresis
- Organize the laboratory so that dedicated pipettes, tips and materials are used for each activity.
- Use sterile filter tips. Avoid aerosols.
- Use tubes with twist-lock caps during the extraction of nucleic acids in order to avoid the leakage of the samples and potential contamination.
- During the procedures for nucleic acid extraction and amplification, avoid contamination of reagents with airborne microbes by only opening the reagents within the hood.
- Change the pipette tip before each extraction of reagents and every time you move from one sample to another in any stage of the procedure.
- The precision pipettes used should have an accuracy of within 3% of the set volume.
- Periodically check the calibration status of the dispensing instruments.
- Do not use reagents after the expiration date shown on each container.
- All reagents supplied in the "**Titano MSI**" kit are intended to be used solely with the other reagents in the same "**Titano MSI**" kit. Do not substitute or mix reagents in the kit from different batches, in order to maintain optimal performance.
- Use only the **Taq Premix 700** supplied in the kit. Do not replace it with **Taq Premix 700** from other kits or similar reagents from other suppliers.
- Place **Taq Premix 700** at -35 / -20 ° C immediately after use, do not store it at + 2 / + 8 ° C.
- Discard unused reagents and the expired kit and waste in accordance with current national laws and local regulations.
- Extraction area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products (e.g. **FD 322**, Dürr Dental, Germany) and UV irradiate the work surface of the biological cabinet where the pipettes should be carefully placed after decontamination.
- Amplification area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products to eliminate nucleic acids and amplicons (e.g. "**DNA Cleaner**" - code DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
- Post-amplification area: at the end of the procedure, decontaminate the pipettes and the laboratory surfaces on which work has been carried out, by cleaning with appropriate products to eliminate nucleic acids and amplicons (e.g. "**DNA Cleaner**" - code DC001, Diatech Pharmacogenetics) and subsequent UV irradiation, if available.
- Avoid contamination of samples and reagents.
- Store reagents and samples separately.
- Before use all reagents need to be thawed at room temperature, mixed by inverting 10 times and centrifuged briefly.
- All reagents contained in the kit are ready-to-use and don't need to be diluted. The reagent dilution may result in a loss of performance.
- Include in each run at least 1 negative control (**WATER small**) and 1 positive control (**Titano pos ctrl**).
- In order to avoid any mixing up of samples pay particular attention to samples dispensation, placement of tubes into the instrument, editing the sample name in the software.
- The right to contest the kit before the expiration date becomes void if the product is used in violation of GLP guidelines and the manufacturer's recommendations.
- The registered names and trademarks indicated in this document are to be considered protected by law, even when not explicitly stated.

ANALYTICAL PROCEDURE

ⓘ Carefully read this User Manual.
ⓘ Check that the version of this User Manual corresponds to the one described on the "**Titano MSI**" kit box label.

Analytical procedure includes the following steps:
1. Biological samples selection
2. DNA extraction
3. Amplification
4. DNA fragment analysis (capillary electrophoresis)
5. Results analysis

1. BIOLOGICAL SAMPLES SELECTION

- The biological samples for the DNA employed in the analysis of microsatellites instability must consist of tissue (fresh, frozen or formalin fixed and formalin embebbed FFPE).
- For each patient, DNA must be extracted both from colorectal carcinoma tissue and normal tissue.
- Tumor tissue must contain at least 50% of neoplastic cells.
- Normal tissue must be selected as far as possible from the tumor area. Normal tissue must contain less than 5% of neoplastic cells.
- If no normal tissue is available, DNA can be extracted from whole blood of the same patient.
- It is sufficient to have 3-5 medium-size sections (10-20 µm) from colorectal tissue, on their respective non-silanized slides or in 2 ml tubes for the analysis. However, it is advisable to consult the instructions of the kit that will be used for the DNA extraction and Diatech Pharmacogenetics information note "Tumor samples: protocol of the preliminary stages", available upon request to the Customer Service support@diatechpharmacogenetics.com.
- Sections of paraffin embedded tissues must be deparaffined with an appropriate solvent, rinsed in alcohol and dried before proceeding with the DNA extraction. For further details, please consult the information note "Tumor samples: protocol of the preliminary stages".

2. DNA EXTRACTION

ⓘ Perform this step in the area dedicated to DNA isolation and dilution, using dedicated materials and instruments.

- The "**Titano MSI**" kit does not include the reagents for DNA extraction.
- The quantity of biological material required for the DNA extraction depends on protocols.
- Refer also to the extraction kit manual for selection and treatment of FFPE slides.
- Do not use samples formalin fixed for more than 24 hours or treated with fixatives different from neutral buffered formalin.
- Extract DNA from EDTA whole blood. Do not use other anticoagulant, for example heparin, that could inhibit amplification reaction.
- Commercial kits working with silica filters or magnetic beads are recommended. Avoid methods that use phenol or boiling in basic solution without purification.
- Perform the DNA extraction following the instructions of the extraction kit in use.
- If the extraction protocol involves the use of wash buffers containing ethanol, it is advisable to perform a further centrifugation before final elution to remove any possible traces of ethanol. This will prevent inhibition of the reaction by the ethanol.
- After the extraction, proceed immediately with the quali-quantitative evaluation of the DNA and the amplification reaction, or store the extracted DNA at ≤-20°C, divided into aliquots in order to maintain the experimental conditions constant in case of repetition.
- It is recommended to proceed with the amplification reaction starting from DNA samples at a concentration of 5-20 ng/µl, consider a concentration of at least quadruple for DNA samples extracted from FFPE.

ⓘ Proceed with the dilution of DNA extracted using **WATER** supplied with the kit.

ⓘ For the correct comparison between tumor and Normal DNA from the same patient, it is recommended that the initial quantity of DNA used in the amplification reactions is comparable.

ⓘ As absorbance reading cannot distinguish between fragmented and not fragmented DNA and therefore it can overestimate the concentration of template FFPE DNA.

ⓘ All assays in the "**Titano MSI**" kit amplify short DNA sequences. However heavily fragmented DNA can generate no amplification product.

3. AMPLIFICATION

ⓘ Perform this step in the area dedicated to amplification mixes preparation, using dedicated materials and instruments. Before starting, decontaminate pipettes, benches and hood in order to degrade any trace of DNA and possibly radiate with UV light for at least 30 minutes.

- The following conditions have been optimized on "**Labcycler Basic / Gradient**" (SensoQuest GmbH) and "**Easy PGX® qPCR instrument**" (Diatech Pharmacogenetics) using the amplifier reagents contained in the "**Titan MSI**" kit.
- Switch on the instrument and the software at least 20 minutes before starting the reaction.
- Thaw all necessary reagents before use .
- Thoroughly mix the reagents in a vortex, or inverting each tube ten times, and spin them briefly before use.
- Prepare and mark an appropriate number of tubes or wells of the plate to use cosidering that each run must include at least one amplification negative control (**WATER small**) and one amplification positive control (**Titano pos ctrl**) for each mix.

ⓘ Each sample and control must be amplified in parallel with 4 different oligomixes **Titano mix 1**, **Titano mix 2**, **Titano mix 3**, **Titano mix 4**.

ⓘ For a correct comparison between the normal and tumor sample of the same patient, it is recommended to use a comparable amount of tumor and normal DNA for amplification.

ⓘ The analysis of 7 pairs of clinical samples (tumor/normal) and 2 controls (**Titano pos ctrl** and **WATER (small)**) for each session allows the best use of the reagents contained in the kit.

FA001 PCR sample grid

	1	2	3	4	5	6	7	8	9	10	11	12
A	DNA1 Normal	DNA2 Normal	DNA3 Normal	DNA4 Normal	DNA5 Normal	DNA6 Normal	DNA7 Normal	Titano pos ctrl				
B	DNA1 Normal	DNA2 Normal	DNA3 Normal	DNA4 Normal	DNA5 Normal	DNA6 Normal	DNA7 Normal	Titano pos ctrl				
C	DNA1 Normal	DNA2 Normal	DNA3 Normal	DNA4 Normal	DNA5 Normal	DNA6 Normal	DNA7 Normal	Titano pos ctrl				
D	DNA1 Normal	DNA2 Normal	DNA3 Normal	DNA4 Normal	DNA5 Normal	DNA6 Normal	DNA7 Normal	Titano pos ctrl				
E	DNA1 Tumor	DNA2 Tumor	DNA3 Tumor	DNA4 Tumor	DNA5 Tumor	DNA6 Tumor	DNA7 Tumor	WATER (small)				
F	DNA1 Tumor	DNA1 Tumor	DNA1 Tumor	DNA1 Tumor	DNA1 Tumor	DNA1 Tumor	DNA1 Tumor	WATER (small)				
G	DNA1 Tumor	DNA2 Tumor	DNA3 Tumor	DNA4 Tumor	DNA5 Tumor	DNA6 Tumor	DNA7 Tumor	WATER (small)				
H	DNA1 Tumor	DNA2 Tumor	DNA3 Tumor	DNA4 Tumor	DNA5 Tumor	DNA6 Tumor	DNA7 Tumor	WATER (small)				

Titano mix 1
Titano mix 2
Titano mix 3
Titano mix 4

- Prepare, for each sample and control, 4 different amplification mixtures (Amp-Mix), one for each assay, as indicated in the following scheme, where N is the numer of samples and controls to be tested for each mix.

Amp-Mix	Reagent Volume for 1 reaction (µl)	Reagent Volume for Nreaction +1 (µl)
Taq Premix 700	10	
Titano mix 1 or **Titano mix 2** or **Titano mix 3** or **Titano mix 4**	2	
WATER (small)	3	
Total Volume	15	

- Mix the four **Amp-Mixes** thoroughly by repeated pipetting or rapid vortexing, then centrifuge briefly.
- Pipette 15 µl of each **Amp-Mix** in all the wells or tubes previously marked.
- Add to the respective wells or tubes, for each of the four assays:

negative control	5 µl **WATER (small)**	CONTROL -
sample	5 µl DNA	
positive control	5 µl **Titano pos ctrl**	CONTROL +

- Reaction volume: 20 µl.
- When using the plate, seal it with a suitable foil, ensuring that it adheres perfectly.
- When using 8 well strips, place a flat cap strip, making sure that the wells are well sealed.
- Briefly centrifuge.
- Place the plate or tubes/strips in the thermocycler. If using the plate on **Labcycler** cover with the sealing pad.
  ⓘ If you are using the "**Easy PGX® qPCR instrument**" tool, fill any unused positions with empty strips.

- Follow the thermalcycler instruction to set the following thermal profile or check that the thermal profile is setted up correctly and start the run.

Easy PGX® qPCR instrument (Diatech Pharmacogenetics)

Hold 1		95°C for 120 seconds (ramp °C/s 5)
3-Step Touchdown [T.D.] Amplification	**10 cycles**	94°C for 30 seconds (ramp °C/s 5) 55°C for 45 seconds (ramp °C/s 4) √ T.D. 72°C for 30 seconds (ramp °C/s 5) - Touchdown setting: First temp. 5°C higher, drop 0.5°C for cycle
3-Step Amplification	**22 cycles**	92°C for 30 seconds (ramp °C/s 5) 55°C for 45 seconds (ramp °C/s 4) 72°C for 30 seconds (ramp °C/s 5)
Hold 2		72°C for 600 seconds (ramp °C/s 4)

Labcycler Basic/Gradient (SensoQuest GmbH)

Hold 1	95°C for 2 minutes
10 cycles	94°C for 30 seconds 60°C for 45 seconds [dT]°C 0.5 (Touchdown) 72°C for 30 seconds
22 cycles	92°C for 30 seconds 55°C for 45 seconds 72°C for 30 seconds
Hold 2	72°C for 10 minutes
	☒ Heated lid T [°C] 105 ☒ Preheating Pressure [N] 120 (for plate); 60 (for tubes/strops) Block temperature preheating [°C] 25 ☐ Fast block control

ⓘ Protect amplification products from direct light to avoid degradation of fluorophores.

ⓘ If no capillary electrophoresis is carried out immediately, store the amplification products at +2/+8 ° C for up to 24 hours. For longer times keep at -35/-20 °C.

4. DNA FRAGMENT ANALYSIS

ⓘ Perform the procedure in the post-PCR area using the dedicated materials and instruments. Before preparing the mixture of reaction, decontaminate the pipettes and the laboratory surfaces to degrade any traces of DNA and, if possible, irradiate them with UV light for 30 minutes.

ⓘ Dilute amplification products using **WATER** supplied with the kit. After use DO NOT re-store the **WATER** transferred to the post-amplification environment in the original box stored in the amplification environment to avoid carry-over problems.

ⓘ Perform all steps involving the use of deionised formamide under a chemical hood.

ⓘ Before capillary electrophoresis, check that spectral calibration with dye set D (DS-30) has been performed.

- Follow the automated sequencer instruction to set the optimal run parameters.
- For each normal and tumor sample is necessary to perform two capillary electrophoresis. A total of 4 capillary electrophoresis for each patien are required.

Marker	PCR mix	Capillary electrophoresis	Dye	Filter
NR21	1	A	Atto550	Yellow
D18S58			HEX	Green
NR24			FAM	Blue
BAT40	2		HEX	Green
D2S123			FAM	Blue
TGFβRII			FAM	Blue
BAT25	3	B	HEX	Green
D17S250			FAM	Blue
TPOX			HEX	Green
D5S346	4		FAM	Blu
BAT26			Atto550	Yellow
TH01			HEX	Green

1. For each sample:
   - Mix 2 µl of **Titano Mix 1** amplification product with 2 µl of **Titano Mix 2** amplification product.
   - Mix 2 µl of the **Titano Mix 3** amplification product with 2 µl of the **Titano mix 4** amplification product.

2. Dilute 1:10 each pool of **Titano mix 1** + **Titano mix 2** and **Titano mix 3** + **Titano mix 4** adding 36 µl of **WATER**.

   ⓘ Given the possible instrument variability if some signals were to be saturated or too low during the electrophorogram analysis, repeat the capillary electrophoresis, respectively, diluting further or testing for higher concentrations of the amplification products to determine the Optimum concentration for your instrument system.

3. Prepare, for each sample to be sequenced, a common mixture of HiDi ™ Deionized Formamide and GeneScan™ 500 ROX™ dye Size standard.

Sequencing-Mix	**Reagent volume for 1 reaction (µl)**	**Reagent Volume for N reaction (µl)**
Deionized Formamide HiDi™	10	
Size standard	0.5	
Total Volume	10.5	

4. Dispense 9 µl of Sequencing-Mix in each sequencing position.

5. Add to each sequencing position containing the Sequencing Mix 1 µl of **Titano Mix 1 + Titano Mix 2** or **Titano Mix 3** + **Titano Mix 4** for each sample to be sequenced.

6. Close the plate with the appropriate septa.

FA001 SEQUENCING sample grid

	1	2	3	4	5	6	7	8	9	10	11	12
A	**DNA1 Normal 1+2**	**DNA3 Normal 1+2**	**DNA5 Normal 1+2**	**DNA7 Normal 1+2**								
B	**DNA1 Normal 3+4**	**DNA3 Normal 3+4**	**DNA5 Normal 3+4**	**DNA7 Normal 3+4**								
C	**DNA1 Tumor 1+2**	**DNA3 Tumor 1+2**	**DNA5 Tumor 1+2**	**DNA7 Tumor 1+2**								
D	**DNA1 Tumor 3+4**	**DNA3 Tumor 3+4**	**DNA5 Tumor 3+4**	**DNA7 Tumor 3+4**								
E	**DNA2 Normal 1+2**	**DNA4 Normal 1+2**	**DNA6 Normal 1+2**	**Titano pos ctrl 1+2**								
F	**DNA2 Normal 3+4**	**DNA4 Normal 3+4**	**DNA6 Normal 3+4**	**Titano pos ctrl 3+4**								
G	**DNA2 Tumor 1+2**	**DNA4 Tumor 1+2**	**DNA6 Tumor 1+2**	**WATER (small) 1+2**								
H	**DNA2 Tumor 3+4**	**DNA4 Tumor 3+4**	**DNA6 Tumor 3+4**	**WATER (small) 3+4**								

Titano mix 1 + Titano mix 2
Titano mix 3 + Titano mix 4

7. Denaturate amplification products on a thermocycler (leave the lid open) or thermoblock by performing the following thermal profile:

Hold 1	95°C for 2 minutes
Hold 2	4°C for 2 minues

8. Load the denatured amplification products on the sequencer by selecting the dye set D and following the instructions of the instrument used for setting and starting the run.

5. DATA ANALYSIS

- Retrieve the .fsa of individual electrophoretic run by following the instrument user manual, and import them into the dedicated analysis software.
- Such software allows the display of row data as peaks within the electrophoresis plot. The software associates the corresponding bp length to each peak (for all details, refer to your software user manual).
- The evaluation of microsatellite instability is based on comparison between the fragments obtained for the normal and tumor samples of the same individual for each of the markers analyzed. The appearance of additional fragments in the tumor sample, relative to the corresponding normal sample, is index of microsatellite instability.
  - ⓘ Analyze a marker at a time. Do not overlap the spectra of different fluorophores.
  - ⓘ If the height of the signal in the electropherogram is inhomogeneus between markers, use the "Zoom" function of the analysis software to evaluate each marker under the best condition.
- Following the amplification, for each marker, one or two peaks will be obtained depending on whether the individual is homozygous or heterozygous respectively.
- In addition to the peak of the allele there may be additional peacks due to amplification artifacts. For a proper analysis, in addition to comparing the lengths in bp of the obtained fragments, a visual evaluation of peaks by the operator is indispensable to exclude from the analysis possible peaks due to artifacts (see Appendix A).
- In a stable marker the tumor sample and the corresponding normal have the same pattern of peaks. Small differences in height between the same peak between tumor and normal samples do not indicate microsatellite instability.
- Take advantage of the simultaneous display of multiple sample electropherograms to easily compare the pattern of the tumor and normal samples of the same individual.
- The absolute height of individual peaks depends on the quality and quantity of amplified DNA. Thus, the generalized variation of the height of all the peaks between tumor and normal samples is not a sign of microsatellite instability.

- Refer to the table below for the details of markers analyzed by the "**Titano MSI**" kit:

Marker	PCR Mix	Capillary electrophoresis	Repetitions	Lenght (bp)[3]	Dye	Filter
NR21	1	A	$(T)_{21}$	96-112	Atto550	Yellow
D18S58			$(CA)_n$	145-165	HEX	Green
NR24			$(T)_{24}$	120-140	FAM	Blue
BAT40	2		$(A)_{40}$	97-125	HEX	Green
D2S123[1]			$(CA)_n$	190-215	FAM	Blue
TGFβRII			$(A)_n$	78-86	FAM	Blue
BAT25[1]	3	B	$(T)_{25}$	110-125	HEX	Green
D17S250[1]			$(CA)_n$	140-185	FAM	Blue
TPOX[2]			$(TGAA)_n$	225-250	HEX	Green
D5S346[1]	4		$(CA)_n$	100-120	FAM	Blue
BAT26[1]			$(A)_{26}$	105-120	Atto550	Yellow
TH01[2]			$(TCAT)_n$	155-165	HEX	Green

1. Bethesda panel
2. Control markers
3. The lenght of the fragments is purely indicative and is based on the most frequent alleles. There exist rare variants that can have external values.

- Evaluate the general quality of the electropherograms by verifying for each sample and positive control that the signal relative to one or more markers:
  - It is not too high to reach saturation (analysis software does not allow full peak visibility).
  - Do not be too weak (<200 RFUs, *Relative Fluorescent Units*), so that the marker's profile is not clearer.

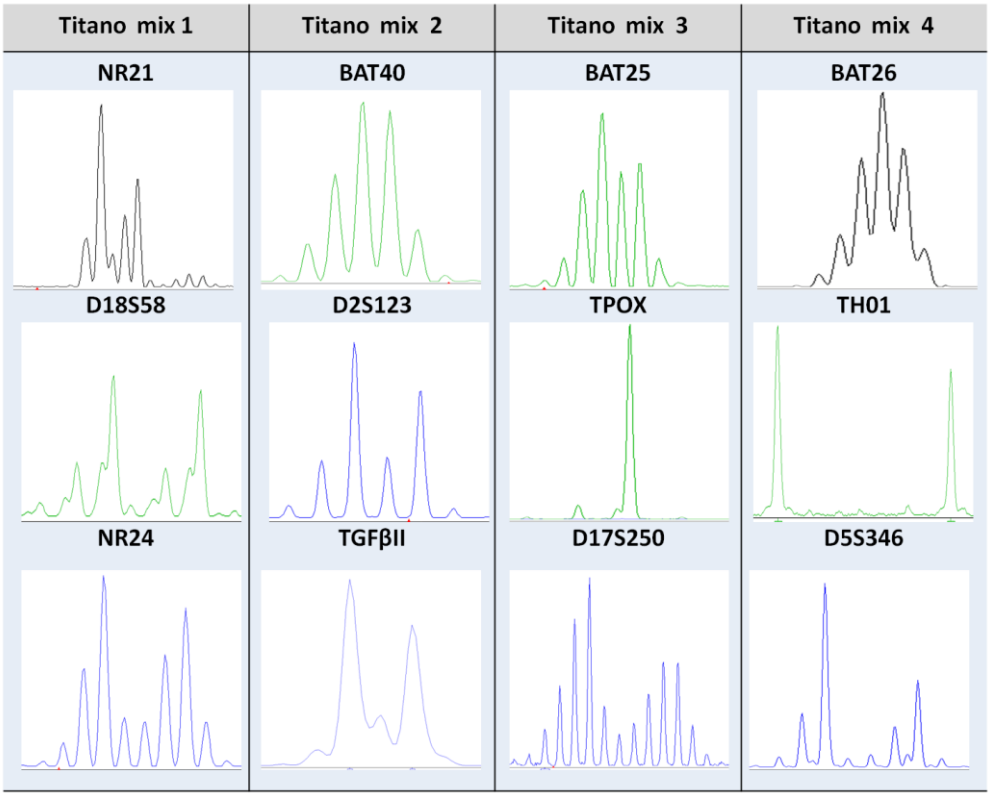
  In both cases, due to the inability to correctly interpret the electropherograms, it is necessary to repeat the electrophoretic run. See Troubleshooting section for details.

- Make sure the negative control **WATER (small)** does not generate any specific signal at the target.

- Make sure the positive control **Titano pos ctrl** generates a peak pattern such as the one shown in Figure A.

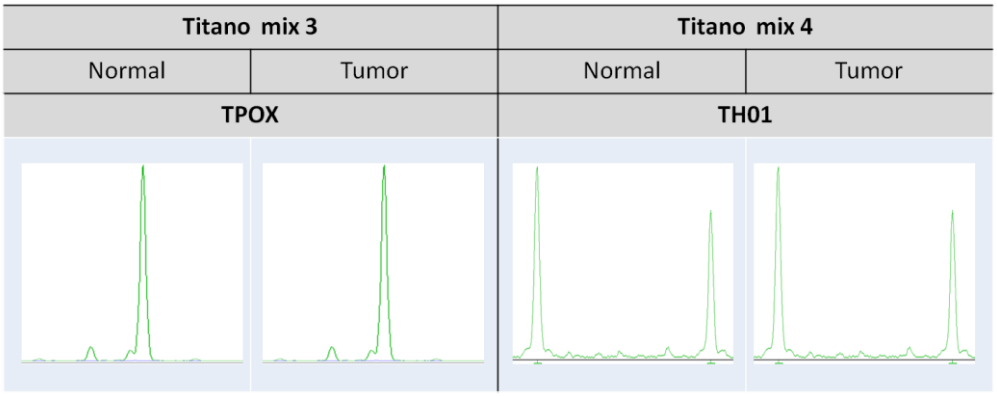
  ⓘ Only if the positive control **Titano pos ctrl** and negative control **WATER (small)** generated the expected results, it is possible to proceed with sample analysis.

Fig. A: Electropherograms obtained for **Titano pos ctrl** for the twelve markers analyzed with the "**Titano MSI**" kit.



- Make sure the two normal and tumor DNA compared belong to the same individual by comparing the fragments obtained for the TH01 and TPOX control markers. They must present a similar peaks profile between normal and tumor DNA of the same individual. These markers, also used in forensic analysis, are in fact highly polymorphic in the population, so it is highly unlikely that two different individuals will have the same profile.

  ⓘ In the presence of a discordance between the normal and tumor tissue control markers results, a sample exchange could have occurred. If this is the case, repeat the procedure. However, in MSI-H samples, high microsatellite instability can also involve TH01 and TPOX markers.



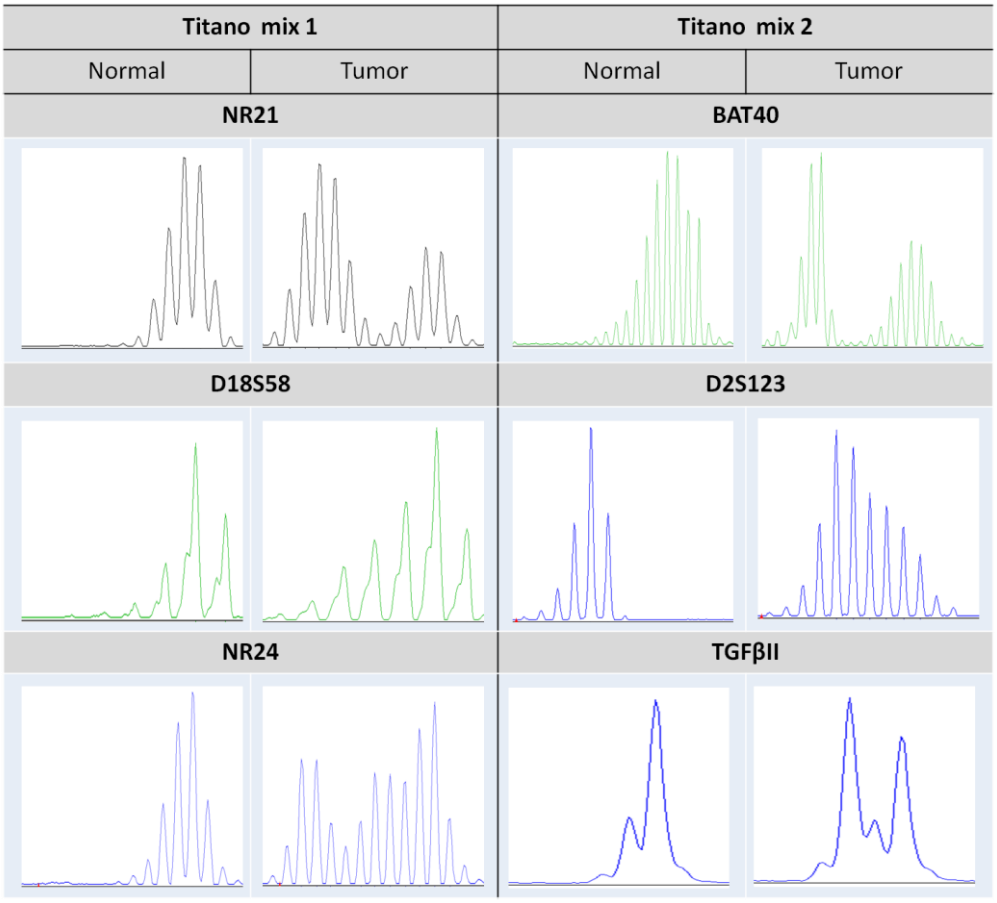
- Assess the instability of the sample considering the table below. Generally, a sample is considered unstable if ≥40% of unstable microsatellites are present:

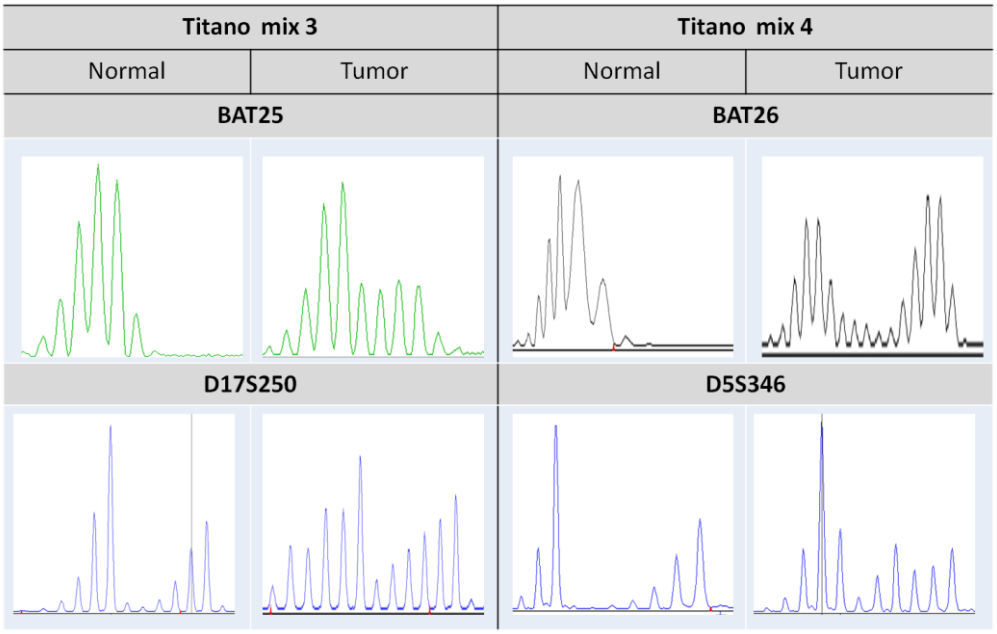
Unstable markers	Microsatellite instability	
≥4	High instability	(MSI-H)
1-3	Low instability	(MSI-L)
0	Stability	(MSS)

ⓘ If one or more markers fail, and all those that can be analyzed stable, it is recommended to repeat the analysis to detect the possible low instability (MSI-L) of the sample

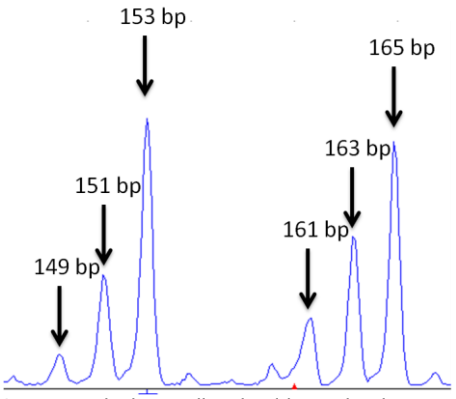
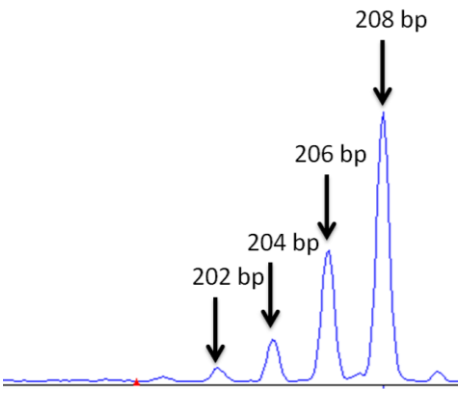
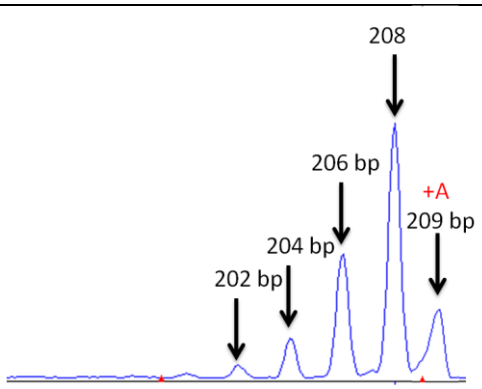
ⓘ Control markers TH01 and TPOX should not be considered in the analysis of microsatellite instability.

Here are some examples of microsatellite instability:





APPENDIX A – Amplification artifacts

Artifact	Description	Example
Stutter peaks	Peaks with intensity usually smaller than the peak of the main allele from which they differ in length of a multiple of the repetition in question. Stuttering is caused by slipping of the polymerase during the elongation phase and their number and intensity are proportional to the length of repetition (eg mononucleotide repetitions generate more stutter than dinucleotide ones).	 Stutter peaks into a dinucleotide marker in a heterozygous individual (153bp, 165bp)  Stutter peaks into a dinucleotide marker in a homozygous individual (208 bp)
A-plus band	The addition of a terminal nucleotide, generally adenine, by the polymerase during PCR, generates a longer fragment of a nucleotide than the expected allele for the marker in question. In the electropherogram, the peaks of the amplicon with an additional nucleotide lie at 1 base distance from the real allele.	

TROUBLESHOOTING

Problem	Possible reason	Recommendation
Wrong genotype of **Titano pos ctrl**	Sample excenge of during analytical procedure.	▪ Repeat all procedure starting from amplification step.
	Wrong sample identification during analysis step.	▪ Verify that, in the software, the correct name is associated to each sample during the analysis step.
Detection of signal in negatif control **WATER (small)**	Contamination	▪ Decontaminate carefully all laboratory surfaces. ▪ Repeat all procedure starting from amplification step. If possible, use a seal reagent vial.
	Sample excenge of during analytical procedure.	▪ Repeat all procedure starting from amplification step. If possible, use a seal reagent vial. ▪ Decontaminate carefully all laboratory surfaces to exclude enviroment contamination.
	Wrong sample identification during analysis step.	▪ Verify that, in the software, the correct name is associated to each sample during the analysis step.
One or more than one microsatellites are not amplified	Insufficient amount of DNA input.	▪ Repeat the amplification using the DNA quantity recommended in this user manual.
	Fragmented DNA or low quality DNA	▪ All assays in the "**Titano MSI**" kit amplify short DNA sequences. However in case of heavily fragmented DNA can be necessary to repeat the extraction.
	Presence of PCR inhibitors in the sample	▪ Repeat the amplification diluting DNA 1:5 in **WATER**.
	No dispensation of the sample during amplification step	▪ Repeat the amplification using the DNA quantity recommended in this user manual.
	Incorrect setting of the thermal-profile.	▪ Check the temperature profile and repeat amplification with the settings described in this manual.
	Incorrect dispensation or manipulation of the oligomix.	▪ Mix reagents by vortexing or inverting the tubes ten times and briefly spin before use. ▪ Verify that all 4 Amp-Mix show a fluorescent color proving that the oligomix is added.
	Reagents improperly stored or expired.	▪ Protect all mixes from light. ▪ Store all reagents at -35/-20°C and avoid more than two thawing. ▪ Pack away **Taq Premix 700** at -35/-20°C immediatly after use, do not stock at +2/+8°C. ▪ Do not stock the oligomixes at +2/+8°C for more than five hours. ▪ Check the expiry date of the kit reported on the label, eventulay use not expired reagents.
	Slow migration of the sampe becaouse of presence of bubbles into the elettroforetic capillary	▪ If selecting an area in which a signal of a marker is expected and instead no peaks are detected, verify if the signal has bled into adjacent regions because of aberrant electrophoretic migration .
Absence of signal for all markers of a specific oligomix	No addiction of one of amplicon mix during sample preparation in the sequencing step	▪ At first, repeat the sequencing step mixing carefully all 4 pcr multiplex products in the following order: Titano mix 1 + Titano mix 2; Titano mix 3 + Titano mix 4. ▪ If the signal is not visible yet, repat the amplification using the DNA quantity recommended in this user manual.
	No dispensation of the sample for the specific oligomix during amplification step	▪ Repat the amplification using the DNA quantity recommended in this user manual.
Difficult results interpretation because of sovrapposition of signal of different dyes.	Wrong spectrl calibration	▪ Before capillary electrophoresis, verify that the spectrl calibration was made using dye set D (DS-30) reagents.
	Wrong pcr product mix during sample preparation in the sequencing step	▪ Repeat the sequencing step mixing carefully all 4 pcr multiplex products: Titano mix 1 + Titano mix 2; Titano mix 3 + Titano mix 4.
Weak fluorescent signal (<200 RFU), difficult interpretation.	Insufficient amount of starting DNA	▪ Repeat the amplification using the DNA quantity recommended in this user manual.
	Fragmented DNA or low quality DNA	▪ All assays in the "**Titano MSI**" kit amplify short DNA sequences. However in case of heavily fragmented DNA can be necessary to repeat the extraction.
	Presence of PCR inhibitors in the sample	▪ Repeat the amplification diluting DNA 1:5 in **WATER**.

	The amplification product is too diluted	▪ Repeat the sequencing step using more concentrated amplification product. If necessary load not diluted pcr product.
	Use of low quality formamide	▪ Use only HiDi formamide (eg. Hi-Di™ Formamide, code 4311320, Applied Biosystem by ThermoFisher Scientific). ▪ Store formamide according to the manufacturer instructions.
	Low injection time	▪ Increase injection time and repeat the electrophoresis.
	High salt concentration in the sample	▪ Use extraction methods recommended in this user manual. ▪ Repeat amplification diluting DNA 1:5 in **WATER**.
Too high fluorescent signal (saturated), difficult interpreatation.	Excess of amplification product	▪ Perform capillary electrophoresis as recommended in this user manual. ▪ If the intensity of the signal is still too high, dilute amplification product 1:50 and repeat the electrophoresis.
Tumor and normal sample of the same patient do not have the same TPOX and TH01 pattern.	Samples exchange	▪ To exclude a sample exchange repeat all the protocol starting form DNA extraction. ▪ If even after the repetition control markers have a different peaks pattern, consider that in tumor sample with MSI-H instability, also control marker gene could be involved in instability.
If the problems persist despite the implementation of the recommendations given and for any other questions or problems, please contact technical support of Diatech Pharmacogenetics: ▪ e-mail: support@diatechpharmacogenetics.com ▪ telephone: +39 0731 213243 ▪ fax: +39 0731 213239		

PERFORMANCE VALIDATION

Kit performance using DNA extracted from tissue samples

The performance validation has been performed with all the reagents supplied with the kit.

The experiments have been performed according to the instructions reported in this user manual on the following instruments:
AMPLIFICATION

- **"EasyPGX® qPCR instrument"** (code RT800, Diatech Pharmacogenetcis)
- **"Labcycler Basic / Gradient"** (code 011-103 / 011-101, SensoQuest GmbH) complete with 96 thermoblock (**"Labcycler Thermoblock 96"**), code 012103- SensoQuest GmbH)

FRAGMENT ANALYSIS

- **"ABI PRISM 3130/3130XL Genetic Analyzer"**, Applied Biosystems™ (software Data Collection v.4 e v.3.1.1)
- **"ABI PRISM 310 Genetic Analyzer"**, Applied Biosystems™ (con software Data Collection v.4)

Analytical sensitivity
Analytical sensitivity has been evaluated tested standardized samples, positive control **Titano pos ctrl** included, and clinical samples at different concentration within a range from 0.4 to 100 ng/µl in independent experiments.

Clinical specificity
In order to evaluate the kit specificity 18 pairs of tumor / normal DNA samples isolated from FFPE tumor tissue have been tested. These samples were suitable for DNA input quantity and for presence of instability detectable by the kit. Samples were already characterized for microsatellite instability through alternative methods ("GENEQUALITY CC-MSI" code 04-61R, AB Analitica"

In table below is shown the concordance % of results comparing the two methods.
Samples were divided based on the level of their instability: high instability (MSI-H), low instability (MSI-L), stable (MSS).

	MSI-H	MSI-L	MSS
% samples per instability level	22%	6%	72%
Concordance %	100%	100%	100%

Detected instability are subdivided in the population of tested sample as shown below.

	Bethesda Panel									
	BAT25	BAT26	D2S123	D17S250	D5S346	BAT40	NR21	NR24	D18S58	TGFβRII
% unstable samples	22%	22%	16%	11%	22%	11%	17%	17%	22%	6%
% Concordance	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Reproducibility
System reproducibility (*inter-assay* variability) has been evaluated analyzing the data deriving from three independent runs with standard DNA samples. Results were100% reproducible in terms of genotyping for all markers and samples analyzed.

Repeatability
System repeatability (*intra-assay* variability) has been evaluated analyzing the data deriving from three independent runs with standard DNA samples. Results were reproducible in terms of genotyping for all markers and samples analyzed.

Robustness
Lot to lot consistency
3 different batches of Taq PreMix and 3 different batches of oligo (from 2 different suppliers) have been tested with the same DNA samples. Results from the different lots are comparable in terms of genotyping for all markers and samples analyzed.

Instrument Variability
The same batch of standardized DNA samples have been tested using 5 different amplification instruments:

- **Labcycler"** (SensoQuest GmbH), 3 different serial numbers
- **EasyPGX® qPCR instrument"** (code RT800, Diatech Pharmacogenetcis), 2 different serial numbers

The same batch of standardized DNA samples have been tested using 2 different sequencer model:

- **"ABI PRISM 3130/3130XL Genetic Analyzer", Applied BiosystemsTM**
- **"ABI PRISM 310 Genetic Analyzer", Applied BiosystemsTM**

Obtained results were 100% reproducible in terms of genotyping for all the markers and the samples analyzed.

diatech pharmacogenetics

FA001 PCR sample grid – Titano MSI

DATE		RUN NAME	

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Titano mix 1
Titano mix 2
Titano mix 3
Titano mix 4

INSTRUMENT n.				USER MANUAL VERSION	
CODE		LOT		EXPIRY	
NOTE					
OPERATOR				SIGNATURE	

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FA001 SEQUENCING sample grid – Titano MSI

DATE		RUN NAME	

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Titano mix 1 + Titano mix 2
Titano mix 3 + Titano mix 4

INSTRUMENT n.				USER MANUAL VERSION	
CODE		LOT		CODE	
NOTE					
OPERATOR				SIGNATURE	

OPERATOR NOTES

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