



IFU505

v150904

Research Use Only

Instructions For Use

TP53 MASTR™ with MID for Illumina MiSeq

MANUFACTURER:

Multiplicom N.V.
Galileilaan 18
2845 Niel
Belgium



TABLE OF CONTENTS

1. KITS	4
2. INTENDED USE	4
3. TEST PRINCIPLE	4
4. MASTR KIT COMPOSITION	6
5. MID FOR ILLUMINA MISEQ KIT COMPOSITION	7
6. MATERIALS AND EQUIPMENT REQUIRED	7
7. STORAGE AND STABILITY	8
8. PRECAUTIONS.....	9
9. SPECIMEN	9
9.1. HIGH MOLECULAR WEIGHT DNA.....	10
9.2. FFPE-DERIVED DNA	10
10. TEST PROTOCOL.....	11
10.1. PREPARATION OF MULTIPLEX MASTER REACTION MIX(ES).....	11
10.2. PCR REACTION SETUP.....	12
10.3. PCR CYCLING PROFILE.....	12
10.4. QUALITY CONTROL OF MULTIPLEX PCR BY AGAROSE GEL OR ALTERNATIVE METHOD	12
10.5. TAGGING OF AMPLICONS ORIGINATING FROM A SINGLE SPECIMEN WITH MIDS	13
10.5.1. PREPARATION OF UNIVERSAL MASTER REACTION MIX.....	13
10.5.2. PCR REACTION SETUP.....	14
10.5.3. PCR CYCLING PROFILE	14
10.6. QUALITY CONTROL OF UNIVERSAL PCR BY AGAROSE GEL OR ALTERNATIVE METHOD	15
10.7. QUALITY CONTROL OF UNIVERSAL PCR BY FLUORESCENT LABELING AND FRAGMENT ANALYSIS .	15
10.7.1. PREPARATION OF MASTER REACTION MIX.....	15
10.7.2. PCR REACTION SETUP.....	16
10.7.3. PCR CYCLING PROFILE	16
10.7.4. FRAGMENT ANALYSIS.....	16
10.8. MIXING OF THE MULTIPLEX REACTIONS FROM A SINGLE DNA SAMPLE.....	17
10.9. PURIFICATION OF THE AMPLICON LIBRARIES.....	17
10.10. POOLING OF DIFFERENT AMPLICON LIBRARIES	18
11. BRIDGE AMPLIFICATION & SEQUENCING	19
12. DATA ANALYSIS	21
12.1. FILES PROVIDED FOR DATA ANALYSIS	21

12.2. GENERAL CONSIDERATIONS22

 12.2.1. DATA FILES22

 12.2.2. STRUCTURE OF THE SEQUENCING READS22

 12.2.3. TRIMMING OF THE SEQUENCING READS22

 12.2.4. ALIGNMENT TO THE REFERENCE SEQUENCE23

 12.2.5. VARIANT CALLING23

 12.2.6. MINIMAL COVERAGE23

 12.2.7. QUALITY SCORES24

12.3. SPECIFIC INSTRUCTIONS24

 12.3.1. MISEQ REPORTER24

 12.3.2. SEQNEXT MODULE OF THE SEQUENCE PILOT24

 12.3.2.1. DEFINITION FILE FOR SEQNEXT24

 12.3.2.2. GUIDELINES SEQNEXT V4.1.1 OR 4.1.2 WITH SEQNEXT .BED FILE24

 12.3.3. SOPHIA DDM INSTRUCTIONS26

13. LIMITATIONS OF THE PROCEDURE26

14. DISCLAIMER26

15. ATTACHMENT: SEQNEXT SETTINGS (RUO)27

 15.1. SEQNEXT SETTINGS FOR DETECTION OF VARIANTS AT 50% ALLELE FREQUENCY27

 15.2. OTHER SEQNEXT SETTINGS28

 15.3. EXAMPLE OF SEQNEXT SETTINGS FOR DETECTION OF VARIANTS AT < 50% ALLELE FREQUENCY ..31

16. LIST OF ABBREVIATIONS31

1. KITS

The MASTR (Multiplex Amplification of Specific Targets for Resequencing) kits are to be used in combination with one or more of Multiplicom's "Complementary MASTR products", such as the molecular identifier (MID) kit(s). In general, our workflow enables sequence analysis of genes of interest on massively parallel sequencing (MPS) instruments.

A list of available MASTRs and "Complementary MASTR products" can be found on Multiplicom's website (<http://www.multiplicom.com>) under the Products section.

Multiplicom's MASTR products are divided into two groups: germline and somatic MASTRs, which can be found on our website under the sections Human Genetics and OncoGenetics respectively.

Remark: To guarantee always working with the most accurate IFUs, it is critical to check the KEY-CODE of each newly opened Multiplicom kit. A new KEY-CODE (higher MTP number) indicates the availability of new instructions and/or files and scripts on the website.

2. INTENDED USE

The MASTR assays kits are required for the PCR-based amplification of a specific set of genomic targets (hereafter referred to as "MASTR-derived amplicons"), followed by the incorporation of MID(s) or barcodes and the MPS-specific sequencing adaptors using Multiplicom's MID kits. The TP53 MASTR assay is Research Use Only and enables the identification or confirmation of the presence or absence of single nucleotide variations (SNV) in the TP53 gene.

3. TEST PRINCIPLE

Multiplicom's MASTR assays enable multiplex PCR amplification of all specified target regions of the gene(s) of interest in a limited number of PCR reactions. The recommended amount of DNA for each multiplex PCR reaction is (i) 20 to 50 ng for high molecular weight DNA (extracted for blood or fresh frozen tissue, FFT) OR (ii) according to Multiplicom's QC plex for DNA isolated from formaline-fixed paraffine-embedded tumor tissue. Next, the resulting amplicons are barcoded, and mixed per sample. Finally, the resulting amplicon libraries of different samples are pooled and sequenced using a MPS instrument according to the manufacturer's instructions. The resulting sequence reads are subsequently analyzed by comparison with the reference sequence of the targeted gene(s) to identify variant positions, which can be confirmed by consulting various public and/or private databases and analyzing the predicted change on the protein level.

MASTR assays serve as front-end amplification for sequence analysis on all commercially available bench top MPS instruments. The technology is based on "target amplification". The principle of the MASTR assays relies on two key technologies: multiplex PCR amplification and Massively Parallel Sequencing (the detection method).

In the first step, all target regions of the gene of interest are amplified in separate multiplex PCR amplification reactions (number of multiplex reactions is defined per MASTR assay) per individual, using a hot-start DNA polymerase (Figure 1). The resulting amplicons of each multiplex are diluted.

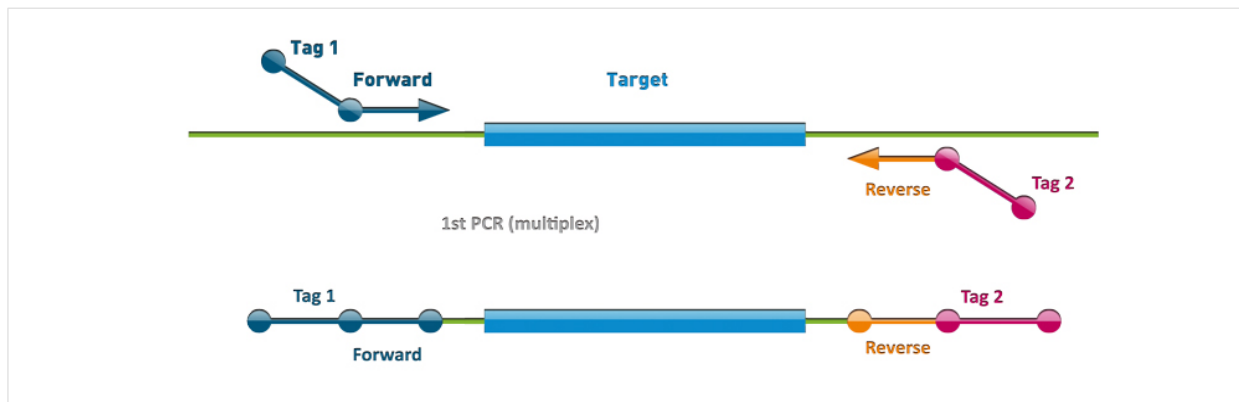


Figure 1. First step: multiplex PCR using a Multiplicom MASTR assay

In the second step, a Universal PCR is performed enabling tagging of the amplicons with specific MID and adaptors required for sequencing using MPS Instruments (Figures 2). The sequences of Multiplicom’s Universal Tags (added in the multiplex PCR and used for primer annealing in the Universal PCR) are AAGACTCGGCAGCATCTCCA (Tag1) and GCGATCGTCACTGTTCTCCA (Tag2).

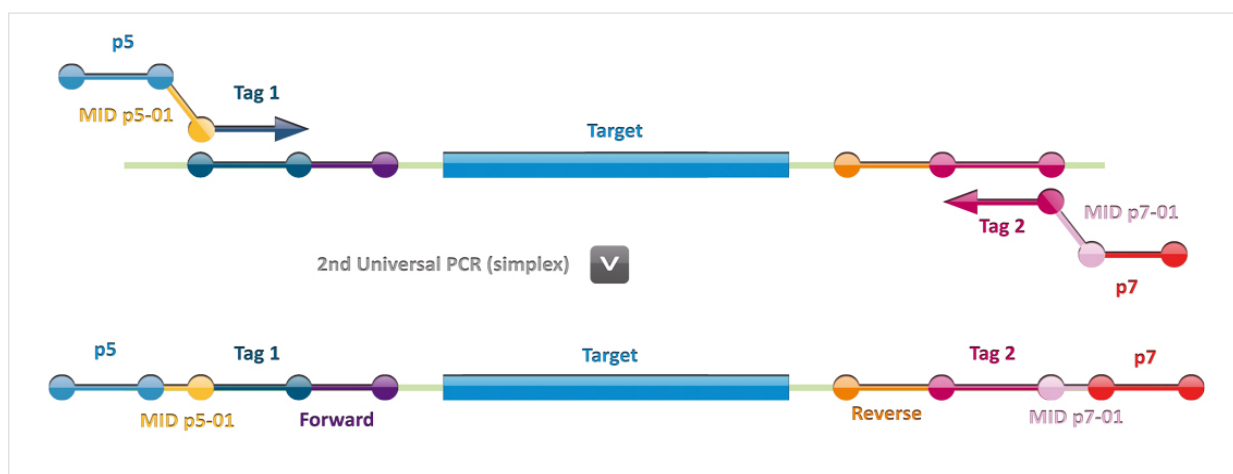


Figure 2. Second step: Universal PCR using a Multiplicom MID for Illumina MiSeq

The resulting tagged amplicons are mixed per individual, applying a predefined assay-specific mixing scheme. Each amplicon library is subsequently purified to remove small residual DNA fragments and the DNA concentration determined.

Next, these purified and individually tagged amplicon libraries are pooled equimolarly, resulting in an amplicon pool or sequencing sample, which is then further processed using custom sequencing primers and one of the Reagents kits provided by Illumina Inc., containing all necessary consumables and Reagent Cartridge for sequencing the purified amplicon library on the Illumina MiSeq.

During the sequencing run, different sequence reads are generated on the same cluster. The positions of the three custom sequencing primers supplied by Multiplicom (Read1, Read2 and the Index Read1), as well as the Illumina Index Read2 primer are indicated and clarified in Figure 3.

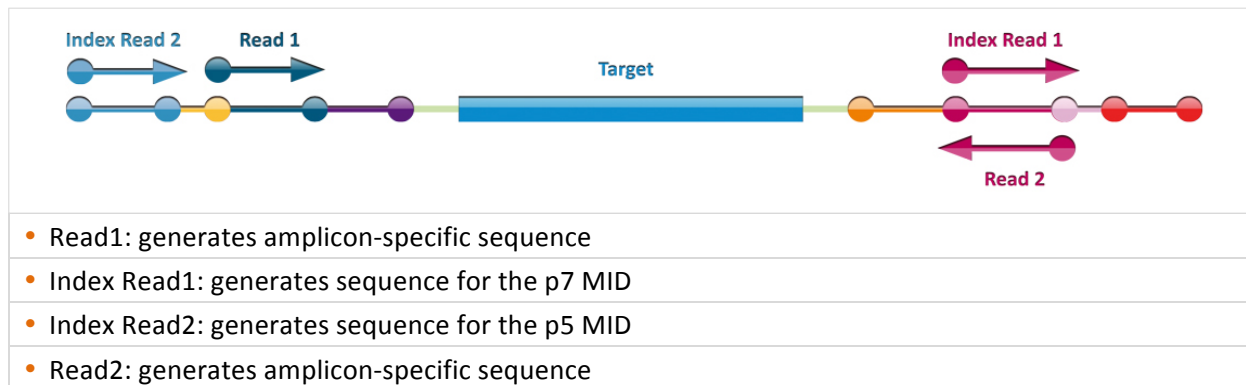


Figure 3. Illumina MiSeq Sequencing

4. MASTR KIT COMPOSITION

Each MASTR kit contains:

Components	Description
PCR Mix Plex 1	Each PCR mix contains specific oligonucleotide primers in a Tricine buffer (pH 8.0) and dNTPs, BSA, KCl and MgCl ₂
PCR Mix Plex 2	
Taq DNA polymerase	
Amplification Reagent (AR)	Amplification reagent to be used in the Universal PCR

For each MASTR assay, the kit insert lists the exact content and a short protocol. Assay specific information, as used in the test protocol described below, is listed in a separate MASTR Assay Specification Sheet.

The MASTR Assay Specification Sheet contains the following information:

- Number of PCR Mixes (number of plexes)
- Number of amplicons
- Amplification Reagent
- Dilution of MASTR-derived amplicons for Universal PCR
- Assay-specific mixing scheme
- Ratio Amplicon versus Agencourt® AMPure® XP beads
- Number of purifications
- Average amplicon size after Universal PCR
- Minimal coverage per sample
- Additional information for MiSeq Sequencing
- References

The MASTR Assay Specification Sheet can be downloaded from <http://www.multiplicom.com> under the Products and/or Key-code section (using the KEY-CODE printed on the box label of the used MASTR kit).

5. MID FOR ILLUMINA MISEQ KIT COMPOSITION

Each MID for Illumina MiSeq kit for MiSeq Sequencing contains:

Components		Description
Box 1	MID p7 and p5 Primer Mixes	- 8 specific p7 MID oligonucleotide primers - 6 specific p5 MID primers (enabling 48 possible p7-p5 MID combinations)
Box 2	Universal PCR Mix (UPM)	The PCR mix contains dNTPs in a Tricine buffer (pH 8.0, supplemented with BSA, KCl and MgCl ₂)
	Taq DNA polymerase	
	GS-labeling PCR Mix	To perform an optional quality control step
	Read1 Primer Mix	Custom sequencing primer mix for Read 1
	Read2 Primer Mix	Custom sequencing primer mix for Read 2
	Index Primer Mix	Custom sequencing primer mix for Index Read 1

For each MID kit, the kit insert lists the exact content and a short protocol. Assay specific information, as used in the test protocol described below, is listed in a separate MASTR Assay Specification Sheet.

6. MATERIALS AND EQUIPMENT REQUIRED

Equipment	Recommendations/ Comments
TP53 MASTR kit	A complete list of available kits can be found on the Multiplicom website (http://www.multiplicom.com) under the Products section.
MID kit	
QC plex	
DNA purification reagents	For more details, see Section 9 for DNA quality.
TE Buffer	10 mM Tris, 1 mM EDTA, pH 8.0
TE ⁻⁴ Buffer*	10 mM Tris, 0.1 mM EDTA, pH 8.0
Molecular biology grade water	
Spectrophotometer	Trinean, DropSense® 96 or equivalent
Fluorometer*	Life Technologies, Qubit®2.0 or equivalent
QC plex	Multiplicom cat. no. QC-0521.100
Agarose gel or digital alternative	Genetic analysis grade, 1x TBE buffer, intercalating dye Microfluidic-based method (e.g. Agilent Technologies 2100 Bioanalyzer and 2200 TapeStation, Perkin Elmer's LabChip® GX)
Power supply, and horizontal electrophoresis system	
Thermal cycler	With a ramp rate between 1 and 2°C/s, temperature accuracy: ± 0.25°C (35-99.9°C), temperature non-uniformity across all zones < 0.5°C (10 s after reaching 95°C)

Thermal cycling reaction tubes for PCR and accessories	Tubes, 96-well plates, caps, plate covers
Single/multichannel pipettes and disposable tips	Sterile-packed, aerosol-resistant disposable pipette tips
Vortex	
Centrifuge(s)	1.5 ml tubes at 12,000 x g; 96-well plates at $\geq 2,000$ x g
Ice and proper ice recipient	
Freezer	-15°C > x > -25°C for storage kits and reagents
MPS Instrument and associated reagents for sequencing	Illumina MiSeq
Analysis software for read counts and variant calling of the generated MPS data	Several software packages are commercially available.
Hi-Di™ Formamide	Applied Biosystems™ cat. no. 4311320
GS600 size standard	Applied Biosystems (e.g. GS600 LIZ®, cat. no. 4408293)
Fragment analyzer	ABI® capillary sequencer with GeneScan module
MAQ-S analysis software	Download from http://www.multiplicom.com/keycode
Agencourt AMPure XP beads	Beckman Coulter Genomics cat. no. A63880, A63881 or A63882
Magnetic Bead Separator	
70% ethanol in water	Freshly made

* Additionally required for analysis of FFPE-derived DNA

7. STORAGE AND STABILITY

- Do not use reagents beyond the expiration date.
- Store all kit reagents in a freezer between -15°C and -25°C.
- All components of the kit (open and unopened) will remain stable until the expiration date if properly closed and stored.
- Reagents may undergo five freeze-thaw cycles without affecting the performance, provided that they are kept on ice while thawed. If more than five freeze-thaw cycles are required, the reagents should be aliquoted appropriately in labeled tubes when first completely thawed and stored accordingly for a maximum of four freeze-thaw cycles.
- Immediately after use, all remaining reagents should be stored accordingly in a freezer at the recommended temperature.

8. PRECAUTIONS

- All recipients should be labeled properly to avoid mix-ups.
- Do not reuse disposable lab material.
- Do not mix reagents from different kits unless the components have identical lot numbers.
- Use a new DNA/DNase free pipette tip for each pipetting step. Sterile-packed, aerosol-resistant, disposable pipette tips are recommended.
- DNA sample contamination can be a major source of error. Therefore, it is highly recommended that pre- and post-PCR activities are performed in separate rooms and that good laboratory practices and procedures are followed at all times.
- Correctly extracted and stored high quality DNA sample should be used. DNA concentration has to be determined accurately (preferably at least two matching measurements per DNA sample). For details on DNA quality, see Section 9 of this IFU.
- All equipment should be properly validated, calibrated and maintained.
- Incorrect pipetting handling can be a major source of errors and can be avoided using the following strict guidelines: (1) only open lid(s) of recipient(s) prior to pipetting; (2) close lid(s) immediately after pipetting (in case lid(s) are not physically attached to the recipient use new lids each time closing the recipient(s)).
- To avoid cross contamination, the following strict guidelines are highly recommended at each step: (1) make sure that the lab bench contains only those reagents required for the next step; (2) open only recipients just prior to pipetting and close immediately after use (in case lid(s) are not physically attached to the recipient use new lids each time closing the recipient(s)); (3) remove recipients from the lab bench when no longer required.
- Keep reagents always on ice when in use, unless otherwise specified. After use, immediately return reagents to the freezer at the recommended temperature.
- Include a negative control sample (no DNA template) during the entire procedure to allow detection of cross contamination.
- Take care not to include samples labeled with the same MID in one MPS sequencing run since this will render them indistinguishable from each other.
- To avoid issues with low nucleotide diversity in the Illumina MiSeq run, use the Support tool for MID selection that can be downloaded from <http://www.multiplicom.com/keycode>.
- To avoid differences in amplification efficiency between the different plexes of a given sample, make sure that for each individual DNA sample all multiplex PCR reactions are included in the same PCR run using the same materials and equipment.
- Take care not to include samples labeled with the same p5-p7 MID combination in one MPS sequencing run since this will render them indistinguishable from each other.
- To prevent run-to-run sample carryover, it is recommended to avoid sequencing the same MASTR Plus assay with the same MID combination in two consecutive runs.

9. SPECIMEN

Human DNA should be either freshly prepared or stored in a freezer. Preferred buffers for storage are TE (10 mM Tris, 1 mM EDTA; pH8) or TE⁻⁴ (10 mM Tris, 0.1 mM EDTA; pH8); please review Tables 3 and 4 for further specifications.

TP53 MASTR assay has been designed to enable amplification of high molecular weight (HMW) DNA or DNA derived from formalin-fixed paraffin-embedded (FFPE) DNA. Table 2 shows the amplicon length and the compatibility with DNA from different sources.

Table 2: Amplicon length and DNA sources for TP53 MASTR assays

	Amplicon length	Type of DNA	DNA sources
Somatic MASTR	135 - 255 bp	HMW DNA FFPE-derived DNA	Blood Fresh frozen tissue FFPE tissue

9.1. High molecular weight DNA

The HMW DNA should comply with the parameters listed in Table 3. Prior to use, dilute DNA to a concentration between 4 and 10 ng/μl in molecular biology grade water and foresee 5 μl for each plex.

Table 3. Requirements for high molecular weight DNA

	Parameters and boundaries	Detection method
Concentration	≥ 4 ng/μl	Spectrophotometry Fluorometry
Purity	OD260/280 ≥ 1.8 OD260/230 ≥ 1.8	Spectrophotometry
Integrity	No visible degradation	1% agarose gel using ~50ng of each DNA sample together with (an) appropriate lane standard(s) or alternative method
Buffer	TE or TE ⁻⁴	

9.2. FFPE-derived DNA

Successful amplification of FFPE-derived DNA is highly dependent on the DNA quality. Therefore, Multiplicom's QC plex assay should be used to determine the optimal input of FFPE-derived DNA for a somatic MASTR assay (see IFU384 for detailed QC plex assay guidelines). The DQC value determines the volume of FFPE-derived DNA that is needed for successful amplification. The required input volume per plex is 5 μl in each somatic MASTR assay. When input DNA volumes are lower, adjust to 5 μl with molecular biology grade water as indicated in Table 4.

Table 4. DNA quality-based matrix FFPE-derived DNA input

DQC value	DNA quality	Volume input DNA per plex	Molecular biology grade water
> 5	Excellent DNA quality	1 μl	4 μl
5.0-1.1	Good DNA quality	2 μl	3 μl
1.0-0.1	Acceptable DNA quality	5 μl	-
< 0.1	Poor DNA quality	It is advised not to use the sample in a MASTR assay	

If the QC plex is not performed, the FFPE-derived DNA should comply with the parameters listed in Table 5. A minimum input of at least 20 ng per plex is recommended in a volume of 5 μl.

Table 5. Requirements DNA extracted from FFPE tissue in absence of DQC values

	Parameters and boundaries	Detection method
Concentration	≥ 4 ng/μl	Fluorometry
Purity	OD260/280 ≥ 1.8	Spectrophotometry
Integrity	Average DNA size ≥ 200bp	Alternative method for agarose gel
Buffer	TE ⁻⁴ or equivalent low EDTA buffer	

10. TEST PROTOCOL

Before starting the Test Protocol, make sure your specimen meets the requirements as specified in Section 9. The following procedure should be strictly followed and performed by qualified personnel; any deviation from the prescribed protocol can lead to erroneous results.

10.1. Preparation of multiplex master reaction mix(es)

For each PCR Mix or Plex, a fresh master reaction mix needs to be prepared prior to use. When preparing the master reaction mixes for the required number of samples, keep in mind that for each preparation a negative control sample must be included (i.e. # of specimens + one negative control).

The number of PCR Mixes is specified in the “MASTR Assay Specification Sheet”: Section 1.

For the list of amplicons that will be amplified using a certain PCR Mix (Plex x), review the GS Reference Pattern of the specific MASTR, which can be obtained from <http://www.multiplicom.com/keycode> using the KEY-CODE printed on the box label of the used MASTR kit.

- Remove the PCR Mix tubes of all Plexes from the freezer and allow complete thawing on ice. After thawing, vortex each vial thoroughly and centrifuge at 12,000 x g for 10 s before use.
- Remove the Taq DNA polymerase from the freezer and put on ice. Centrifuge at 12,000 x g for 10 s before use.
- To minimize the chance of cross contamination of the different PCR Mixes, prepare the master mixes one by one. For each of the PCR Mixes, label a new PCR tube and mix the PCR Mix of that Plex with Taq DNA polymerase according to the scheme exemplified in the table below (volumes as required for the number of samples indicated):

# of Samples	PCR Mix Plex x	Taq DNA polymerase	Taq DNA polymerase 1/10 diluted*
2	20 μl	-----	1.5 μl
8	80 μl	0.6 μl	-----
24	240 μl	1.8 μl	-----
40	400 μl	3 μl	-----

* Do not pipette less than 0.5 μl of Taq DNA polymerase because the solution is viscous. If less than 0.5 μl of undiluted Taq DNA polymerase is required (< 7 samples), prepare a 1/10 dilution by adding a minimum of 0.5 μl Taq DNA polymerase in molecular biology grade water, followed by vortexing (2-3 s) and centrifuging 10 s at 12,000 x g. Add the required amount to the master reaction mix.

- Vortex briefly (2-3 s) and centrifuge each master reaction mix vial at 12,000 x g for 10 s. Vortexing at this step is essential for successful amplification.

10.2. PCR reaction setup

- Each multiplex master reaction mix requires the addition of input DNA in a volume of 5 µl (see Sections 9.1 and 9.2 for input DNA requirements).
- Combine 5 µl of the input with 10 µl of each master reaction mix (prepared under Section 10.1), and close the vial or well immediately.
- For each preparation a negative control should be included by adding 5 µl molecular biology grade water to 10 µl of each of the master reaction mixes.
- Vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.

10.3. PCR cycling profile

- Run the following thermal cycling profile:

# of cycles*	Temperature	Time
1	98°C	10 min
20	95°C	45 s
	60°C	45 s
	68°C	2 min
1	72°C	10 min
1	4°C	< 1 h

* Set the ramp rate of the PCR cycler between 1 and 2°C/s for all heating and cooling steps.

- Do not store the amplified products for more than 1 h at 4°C (since this would induce the formation of primer dimers): continue immediately with the Universal PCR step, otherwise immediately store amplified products between -15°C and -25°C.

10.4. Quality control of multiplex PCR by agarose gel or alternative method

This section is optional but highly recommended.

Remark for FFPE-derived DNA: no or hardly any bands or signal will be visible due to the low concentrations of MASTR-derived amplicons. Proceed with the Universal PCR and the subsequent quality control step.

- Following thermal cycling, a quality control step can be performed:
 - Agarose gel: run 5 µl of each PCR product on a 2% agarose gel containing 0.1 µg/ml ethidium bromide or equivalent (carefully follow all safety instructions applicable in your laboratory) to check for the presence of amplicons.
 - Digital alternative (microfluidic-based method): check for the presence of amplicons according to manufacturer's instructions.
- Successful amplification is indicated by a clearly visible, but dispersed band or equivalent digital signal that ranges between 150 and 300.
- The negative control should not display any amplification product for the run to be valid. Amplification in the negative control indicates cross-contamination. The complete PCR run should be discarded and the source of contamination should be investigated before attempting a new PCR run. Suitable measures should be taken to avoid reoccurrence of this problem.

10.5. Tagging of amplicons originating from a single specimen with MIDs

The MASTR-derived amplicons are tagged resulting in the incorporation of MIDs and the p7 and p5 adaptors required for sequencing on Illumina MiSeq run. The MID kit reagents are required for tagging the MASTR-derived amplicons and are provided as separate MID kits for sequencing on the Illumina MiSeq, each containing unique MID sequences.

For the complete list of available MID for Illumina MiSeq kits, visit the section “Complementary MASTR products” on our website: <http://www.multiplicom.com>.

The precise sequences of the MIDs for Illumina MiSeq (IFU243) can be obtained from the section “Complementary MASTR products” on our website: <http://www.multiplicom.com>.

10.5.1. Preparation of Universal master reaction mix

For each sample (including the MASTR negative control sample) to be sequenced in the same Illumina MiSeq run, a fresh master reaction mix using a unique combination of MID p7-p5 Primer Mixes should be freshly prepared as specified below.

Remarks:

- (1) To avoid issues with low nucleotide diversity in the Illumina MiSeq run, use the Support tools for MID selection.
- (2) If you want to detect variants with an allele frequency of 10% or lower, run-to-run sample carryover can be a confounding factor. To prevent this, avoid sequencing the same MASTR assay with the same MID combination in two consecutive runs.

The Support tools for MID selection (IFU142, IFU143 and IFU240) can be obtained from <http://www.multiplicom.com/keycode> using the KEY-CODE printed on the box label of the used MID kit.

- Remove the required Universal PCR Mix, Amplification Reagent (AR) (supplied with the MASTR kit) and MID p7 and p5 Primer Mixes from the freezer and allow complete thawing on ice. The obligatory AR is indicated in Section 1 of the MASTR Assay Specification Sheet.
- After thawing, vortex each vial thoroughly and centrifuge at 12,000 x g for 10 s before use.
- Remove the Taq DNA polymerase from the freezer and put on ice. Centrifuge at 12,000 x g for 10 s before use.
- To minimize the risk of cross-contamination, prepare a Universal master reaction mix for each MID combination to be used. Label a new PCR tube and mix the necessary reagents according to the scheme in the table below:

# plexes MASTR	Universal PCR Mix	AR	MID p7 Primer Mix	MID p5 Primer Mix	Taq DNA polymerase 1/10 diluted
2	20 µl	20 µl	4 µl	4 µl	1.25 µl

The number of PCR Mixes is specified in the “MASTR Assay Specification Sheet”: Section 1.

- Vortex briefly (2-3 s) and centrifuge each Universal master reaction mix vial at 12,000 x g for 10 s. Vortexing at this step is essential for successful amplification.

10.5.2. PCR reaction setup

- When the Universal PCR does not follow immediately on the execution of the specific MASTR (and amplified products were stored between -15°C and -25°C), remove the MASTR-derived amplicons from the freezer, allow complete thawing on ice and proceed as soon as possible.
- Dilute the purified amplicon library from each sample (including the MASTR negative control sample).

The specific dilution factor is provided in the “MASTR Assay Specification Sheet”: Section 2.

- Add 1 µl of this dilution to 24 µl of the Universal master reaction mix prepared under Section 10.5.1, and close the vial or well immediately.
- Vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.

10.5.3. PCR cycling profile

- Run the following thermal cycling profile:

# of cycles*	Temperature	Time
1	98°C	10 min
20	95°C	45 s
	64°C	45 s
	68°C	2 min
	72°C	10 min
1	4°C	Max. 12 h

* Set the ramp rate of the PCR machine between 1 and 2°C/s for all heating and cooling steps.

- Do not store the amplified products for more than 12 h at 4°C: continue immediately with the quality control step, otherwise immediately store amplified products between -15°C and -25°C.

The sets of tagged MASTR-derived amplicons per individual can be controlled for their quality as described below under Sections 10.6 and 10.7. Multiplicom recommends performing at least one of these quality control steps (preferentially Section 10.7).

10.6. Quality control of Universal PCR by agarose gel or alternative method

This section is optional but highly recommended

Remark for FFPE-derived DNA: no or hardly any bands or signal will be visible due to the low concentrations of MASTR-derived amplicons. Quality control of Universal PCR by fluorescent labeling and fragment analysis is recommended.

- Following thermal cycling, a quality control step can be performed:
 - Agarose gel: run 5 µl of each PCR product on a 2% agarose gel containing 0.1 µg/ml ethidium bromide or equivalent (carefully follow all safety instructions applicable in your laboratory) to check for the presence of amplicons.
 - Digital alternative (microfluidic-based method): check for the presence of amplicons according to manufacturer's instructions.
- Successful amplification is indicated by a clearly visible, but dispersed band or equivalent digital signal that ranges between 250 and 400.
- The negative control should not display any amplification product for the run to be valid. Amplification in the negative control indicates cross-contamination. The complete PCR run should be discarded and the source of contamination should be investigated before attempting a new PCR run. Suitable measures should be taken to avoid reoccurrence of this problem.

10.7. Quality control of Universal PCR by fluorescent labeling and fragment analysis

This section is optional but highly recommended

The quality of the Universal PCR can be reviewed in detail, based on the pattern of the amplicons of a given plex for a specific MASTR. The required chromatograms can be obtained using an ABI capillary sequencer with GeneScan (GS) module.

10.7.1. Preparation of master reaction mix

- Remove the GS-labeling PCR mix from the freezer and allow complete thawing on ice.
- After thawing, vortex each vial thoroughly and centrifuge the vials at 12,000 x g for 10 s before use.
- Remove the Taq DNA polymerase from the freezer and put on ice. Centrifuge at 12,000 x g for 10 s before use.
- Prepare a GS labeling master reaction mix for all Universal PCR reactions that will be labeled with following volumes per reaction:

Reagents	Volume
GS-labeling PCR Mix	13 µl
Taq DNA Polymerase*	0.075 µl

* Do not pipette less than 0.5 µl of Taq DNA polymerase because the solution is viscous. If less than 0.5 µl of undiluted Taq DNA polymerase is required (< 7 reactions), prepare a 1/10 dilution by adding a minimum of 0.5 µl of Taq DNA polymerase in molecular biology grade water, followed by vortexing and centrifuging 10 s at 12,000 x g. Add the required amount to the GS-labeling PCR Mix.

- Vortex briefly (2-3 s) and centrifuge the vial at 12,000 x g for 10 s.

10.7.2. PCR reaction setup

- For each sample, combine 2 µl of Universal PCR product obtained after completion of Section 10.5.3. with 13 µl of the freshly prepared GS labeling master reaction mix.
- Vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.

10.7.3. PCR cycling profile

# of cycles*	Temperature	Time
1	98°C	10 min
5**	95°C	30 s
	65°C	30 s
	68°C	30 s
1	72°C	10 min
1	4°C	∞

* Set the ramp rate of the PCR cycler between 1 and 2°C/s for all heating and cooling steps.

** Especially in case of FFPE-derived DNA, if the signal detected by your fragment analyzer is too low, increase the number of cycles to 7.

10.7.4. Fragment analysis

- Prepare a size standard mix for all samples that will be analyzed by using the following volumes per reaction:

Reagents	Volume (x no. of samples)
Hi-Di™ Formamide	10 µl
GS600 size standard	0.4 µl

- For each reaction, dispense 10 µl of size standard mix per well into a 96-well plate.
- Add 2 µl of the GS labeling PCR-product to a well containing the “Size standard mix”.
- Centrifuge the plate at 1,000 x g for 10 s.
- Denature samples at 95°C for 3 minutes and immediately put on ice.
- Run samples on the fragment analyzer according to the manufacturer’s instructions.
- Review the generated data (.fsa files) with MAQ-S software using required Assay Description file (.enc) and GS Reference Pattern. The Universal PCR amplification is valid if the analysis pattern is consistent with the GS Reference Pattern.

The MAQ-S software, Assay Description file and GS Reference Pattern can be obtained from <http://www.multiplicom.com/keycode> using the KEY-CODE printed on the box label of the used MASTR kit.

If a valid analysis pattern is obtained, proceed with Section 10.8.: “Mixing of the multiplex reactions from a single DNA sample” to mix all amplicons, and subsequently purify and pool the obtained amplicon libraries.

If the analysis is not valid, carefully review the assay protocol. If the analysis is persistently non-valid, please contact customer service: customerservice@multiplicom.com.

10.8. Mixing of the multiplex reactions from a single DNA sample

- The tagged MASTR-derived amplicons, obtained after completion of Section 10.5.3., are mixed per individual DNA sample, applying a MASTR specific mixing scheme.
- Vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.

The specific fixed mixing scheme is provided in the “MASTR Assay Specification Sheet”: Section 3.

Each amplicon library is subsequently purified to remove small residual DNA fragments using magnetic bead based DNA purification as specified below in Section 10.9.

Proceed immediately, otherwise store the amplicon libraries in a freezer between -15°C and -25°C.

10.9. Purification of the amplicon libraries

Purify each pooled amplicon library using Agencourt AMPure XP beads using the protocol below.

The volumes of amplicon library versus Agencourt AMPure XP and the number of required purification rounds are specified in the “MASTR Assay Specification Sheet”: Section 4.

- (1) Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.
- (2) Combine xx µl amplicon library with xx µl Agencourt AMPure XP.
- (3) Mix reagent and amplicon library thoroughly by pipette mixing 10 times. Let this mixture incubate for 5 minutes at room temperature for maximum recovery.
- (4) Place the tubes on a magnetic bead separator for 2 minutes to separate beads from the solution. Aspirate the cleared solution from the reaction plate and discard.
- (5) Dispense 200 µl of freshly made 70% ethanol to each tube, and incubate for 30 s at room temperature while moving the tubes around on the magnetic bead separator. Aspirate the ethanol and discard. Repeat this wash step once more.
- (6) Proceed with the appropriate steps below.

Second round of purification:

- (7) Remove tubes from the magnetic bead separator. Add 42 µl of molecular biology grade water to each tube. Pipette mix 10 times.
- (8) Place the tubes on the magnetic bead separator for 1 minute to separate beads from the solution.
- (9) Transfer the eluent to a new appropriately labeled tube.
- (10) Add xx µl Agencourt AMPure XP beads (if necessary, recalculate the recommended amount of beads specified in the Assay Specification Sheet for a sample volume of 40 µl to ensure the optimal ratio of beads per volume).
- (11) Repeat steps 3-6.

Final purification round:

- (7) Allow the pellet to dry.
- (8) Remove tubes from the magnetic bead separator. Add 20 µl of molecular biology grade water to each tube. Pipette mix 10 times.
- (9) Place the tubes on the magnetic bead separator for 1 minute to separate beads from the solution.
- (10) Transfer the eluent to a new appropriately labeled tube.

Proceed immediately with Section 10.10, otherwise immediately store the purified amplicon libraries in a freezer between -15°C and -25°C.

10.10. Pooling of different amplicon libraries

- Determine the concentration of the obtained purified amplicon libraries by either:
 - a spectrophotometric method,
 - a fluorometric method,
 - qPCR (e.g. KAPA Library Quant Kits, KAPA Biosystems).
- Calculate the concentration of each amplicon library in nM, using the following equation:

$$\text{Sample conc [nM]} = \frac{\text{Sample conc [ng/}\mu\text{l]} \times 10^6}{656.6 \times \text{average amplicon size}}$$

The average amplicon size after the Universal PCR is specified in Section 5 of the appropriate MASTR Assay Specification Sheet.

- Dilute each amplicon library to 4 nM in 1x TE buffer, vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.
- If multiple amplicon libraries need to be sequenced on an MPS system, pool equal volumes for samples requiring the same minimal coverage per sample.

For more details on the influence of tumor tissue content (TTC) and minimal required variant allele frequency ($\text{VAF}_{\text{sample}}$) on the minimal coverage per sample, see the Sequencing Calculator Technical Note at <http://www.multiplicom.com/calculator>.

- Vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.
- In case of combining multiple amplicon pools, use the volumes mentioned in the Sequencing Calculator report which describes the exact run setup.

Samples belong to a different amplicon pool when derived from a different MASTR assay and/or have a different TTC, $\text{VAF}_{\text{sample}}$ within one MASTR assay.

- Vortex briefly (2-3 s) and centrifuge at 12,000 x g for 10 s.
- Proceed immediately with Section 11, otherwise immediately store the amplicon pool in a freezer between -15°C and -25°C.

Remarks:

- (1) Take care to pool only purified tagged amplicon libraries that contain different MID combinations (incorporated in the Universal PCR step of the MID for Illumina MiSeq kit).
- (2) The Sequencing Calculator takes into account the PhiX control library that needs to be spiked in when defining the capacity of the run.

The minimal number of read pairs per sample (for detection 50% VAF_{sample}) is specified in the "MASTR Assay Specification Sheet": Section 5.*

The required percentage of the PhiX control library is specified in the "MASTR Assay Specification Sheet": Section 6.

For the calculation of the number of samples per run and combining multiple amplicon pools use the Sequencing Calculator at <http://www.multiplicom.com/calculator>.

* The coverage of an Illumina MiSeq run is indicated as "number of read pairs", in which a read pair is defined by Read1 and Read2 of the same cluster.

11. BRIDGE AMPLIFICATION & SEQUENCING

For the detection of variants with an allele frequency of 10% or lower, run-to-run sample carryover can be a confounding factor. To reduce carryover, perform a maintenance wash prior to sequencing. The wash tray should be thoroughly cleaned after each use to eliminate any risk of template molecules remaining in the tray. When still suffering from carryover effects, please contact Illumina Technical Support for further assistance.

The amplicon pool is further processed using one of the Reagents kits provided by Illumina Inc., containing all necessary consumables and Reagent Cartridge for sequencing the purified amplicon library on the Illumina MiSeq following the guidelines described below.

A detailed description on how to set up a MiSeq run can be found in the MiSeq system user guide. Always use the latest version of the user guide.

Remark: When not proceeding immediately with Section 11 after completing Sections 10.8 and/or 10.9 re-measure the concentration of your sequencing sample using the equation above.

- Choose the applicable Illumina Reagent Kit for the specific MASTR used, based on the required/desired number of cycles for Read1 and Read2: Kit v2 or Nano Kit v2 (500 cycles, 2 x 251 bp; MS-102-2003 or MS-103-1003 respectively), or Kit v3 (600 cycles, 2 x 301 bp; MS-102-3003).

The theoretical percentage of bases sequenced bidirectional when using the maximal number of cycles enabled by the v2 and v3 Reagent kits is specified in the "MASTR Assay Specification Sheet": Section 6.

- Create a Sample Sheet with the Illumina Experiment Manager (IEM) software by following the guidelines below:

Remark: Always use the latest version of IEM and MiSeq Reporter software.

- (1) On a Windows PC, extract the files from 'IFU336 MASTR SamplePrepKit for IEM1.9 v150405.zip', which can be obtained from <http://www.multiplicom.com/keycode> where you will be requested to enter the product KEY-CODE found on the box label of the MID for Illumina MiSeq kit.
- (2) Save the files at the following directories:
 - 'GenerateFASTQ.txt' and 'PCRAmplicon.txt' at ...\Illumina\Illumina Experiment Manager\Applications; overwrite the existing files.
 - 'Multiplicom MASTR (MID 1-48).txt', 'Multiplicom MASTR (MID 49-96).txt' and 'Multiplicom MASTR (MID 1-192).txt' at ...\Illumina\Illumina Experiment Manager\SamplePrepKits.
- (3) Open the IEM software.
- (4) For the Instrument Selection, choose 'MiSeq', click 'Next'.
- (5) For the MiSeq Application Selection:
 - PCR Amplicon: choose 'Targeted Resequencing' (Category) and 'PCR Amplicon' (Application), click 'Next'.
 - FASTQ Only: choose 'Other' (Category) and 'FASTQ Only' (Application), click 'Next'.
- (6) Fill out the Workflow Parameters, with these specific settings:
 - Reagent Cartridge Barcode: to be found on the Illumina reagent cartridge
 - Sample Prep Kit: select a 'Multiplicom MASTR'
 - Index Reads: select '2'
 - Read Type: select 'Paired End'
 - Cycles Read 1: specified in the Assay Specification Sheet, Section 6
 - Cycles Read 2: specified in the Assay Specification Sheet, Section 6
 - Select the 3 'Custom Primers' (Read1, Index Read and Read2)
 - Select both 'Use Adapter Trimming' boxes (selected by default)
 - Click 'Next'
- (7) Add rows to the Sample Sheet. Select Indexes from the list. Ignore eventual warning on diversity of index cycles. In case the PCR Amplicon application was chosen, select the correct Manifest file (can be downloaded from <http://www.multiplicom.com/keycode> using the KEY-CODE on the box label of the used MASTR kit). Click 'Finish' and save the Sample Sheet.

The IFU336 and IFU266 can be obtained from <http://www.multiplicom.com/keycode> using the KEY-CODE on the box label of the used MID for Illumina MiSeq and MASTR kit respectively.

- Denature and dilute the prepared amplicon pool to the desired concentration as described in the MiSeq system user guide. Start with 8 pM for MiSeq Reagent kit v2, or 13 pM for MiSeq Reagent kit v3. If necessary, optimize to reach the optimal cluster density for your system (cfr. Illumina Technical Support). Note that sequencing samples with a concentration > 10 pM should be denatured starting with a 4 nM library as described in Illumina's manual on preparing libraries for sequencing on the MiSeq.
- Spike-in PhiX Control v3 (Catalog No. FC-110-3001, Illumina) as described in the MiSeq system user guide under 'Performing a run' to obtain the Final Sample.

The targeted percentage of the PhiX control library is specified in the 'MASTR Assay Specification Sheet': Section 6.

- Pipette 600 µl of the Final Sample into the Load Samples reservoir. Take care to avoid touching the foil seal as you dispense your sample.
- Custom sequencing primers and a custom index primer are required for MiSeq based sequencing. These 3 primers are provided as 100 µM solutions and can be found in the amplification box of the MID kit for Illumina MiSeq Sequencing. The 3 Primer Mixes are termed:
 - Read1 Primer Mix
 - Index Primer Mix
 - Read2 Primer Mix
- From each Primer Mix, prepare 600 µl of custom primer at 0.5 µM by adding 3 µl of the Primer Mix to 597 µl of HT1 (Hybridization Buffer). Mix well by vortexing 2-3 s.
- Add the diluted Primer Mixes to the reagent cartridge:
 - (1) With a clean pipette tip, pierce the foil seal covering the port associated with the primer you are loading.
 - (2) Load 600 µl of custom primer in the appropriate port on the cartridge:
 - Read1 custom primer to cartridge position 18
 - Index custom primer to cartridge position 19
 - Read2 custom primer to cartridge position 20

Take care to avoid touching the foil seal as you dispense the primer.

 - (3) Inspect the bottom of the cartridge to make sure that no bubbles are present. Remove bubbles by tapping the cartridge.
- Proceed directly with the run setup steps using the MiSeq Control Software (MCS) interface.

12. DATA ANALYSIS

12.1. Files provided for data analysis

All files listed here can be downloaded from <http://www.multiplicom.com/keycode> where you will be requested to enter the product KEY-CODE found on the box label of the specific MASTR assay.

File description	Type and content
Support tools selection MID* (IFU142, IFU143 and IFU240)	General .xls file enabling the selection of unique and compatible combinations of p7-p5 MID Primers to guarantee good nucleotide diversity. These tools can be consulted for sequences of the MIDs present in the MID for Illumina MiSeq kits.
PCR specific primers	MASTR-specific .txt file listing the primers used for amplification to enable sequence trimming (Section 12.2.3).
Manifest file	MASTR-specific .AmpliconManifest file specifying the reference genome (<i>Homo sapiens</i> hg19), amplicon positions (MASTR-specific primers are not trimmed off) and primer sizes to enable alignment of read pairs to a reference genome and variant calling using Illumina's MiSeq Reporter (Section Error! Reference source not

	ound.12.3.1).
Definition file for SeqNext	MASTR-specific .zip file containing target info for data analysis in format specific for JSI SeqNext (Section 12.3.2): SeqNext .bed file with the amplicon positions on hg19.
BED-file	MASTR-specific .txt file listing the amplicon positions in <i>Homo sapiens</i> hg19 (MASTR-specific primers are trimmed off) to target info for data analysis in general format.

* Can be downloaded from <http://www.multiplicom.com/keycode> using the KEY-CODE located on the box label of your MID kit.

12.2. General considerations

12.2.1. Data files

The Illumina MPS instruments generate for each cluster a forward and a reverse read, Read1 and Read2, together defining a read pair. Using both index reads, the data is automatically demultiplexed by MiSeq Reporter resulting in the output of 2 FASTQ files per sample defined in the sample sheet. These FASTQ files contain all filter passed sequencing reads generated during the run.

12.2.2. Structure of the sequencing reads

The structure of the sequencing reads is depicted in Figure 2 and 3: the Read1 reads start with the PCR specific Forward primer and continue with the amplified region. Depending on the size of the amplified region and the length of the read, this sequence of the amplified region is further followed by the reverse complement of the Reverse PCR specific primer, universal Tag2, the p7 MID sequence and p7 adaptor. In the same way, the Read2 reads start with the PCR specific Reverse primer and continue with the amplified region. Depending on the size of the amplified region and the length of the read, this sequence of the amplified region is further followed by the reverse complement of the Forward PCR specific primer, universal Tag1, the p5 MID sequence and p5 adaptor. The universal tag sequences are AAGACTCGGCAGCATCTCCA (Tag1) and GCGATCGTCACTGTTCTCCA (Tag2).

12.2.3. Trimming of the sequencing reads

The PCR specific primer part in the sequencing reads is by definition equal to the genomic reference sequence and thus independent of the individual sample that is sequenced. As depicted in Figure 4, when 2 amplicons overlap, failure to trim the PCR primer sequences from the reads can result in skewed variant allele frequencies. Since virtually all MASTR assays contain overlapping amplicons, primer trimming is a mandatory step in the data analysis.

The sequences of PCR primers (Figure 4a – Forward2 and Reverse2) should be removed from those reads generated directly with them (Figure 4a – Amplicon2 reads), and should not be removed from reads generated with other PCR primers (i.e. from overlapping amplicons; Figure 4a – Amplicon1 reads). This discrimination can be made based on the fact that the start of the Read 1 and Read 2 sequences consist of respectively the Forward and Reverse PCR primer (Figure 2 and 3). Depending on the size of the amplified region and the length of the read, the Read1 and Read2 sequences may contain also the reverse complement of respectively the Reverse PCR primer, flanked by Tag2, and Forward PCR primer, flanked by Tag1.

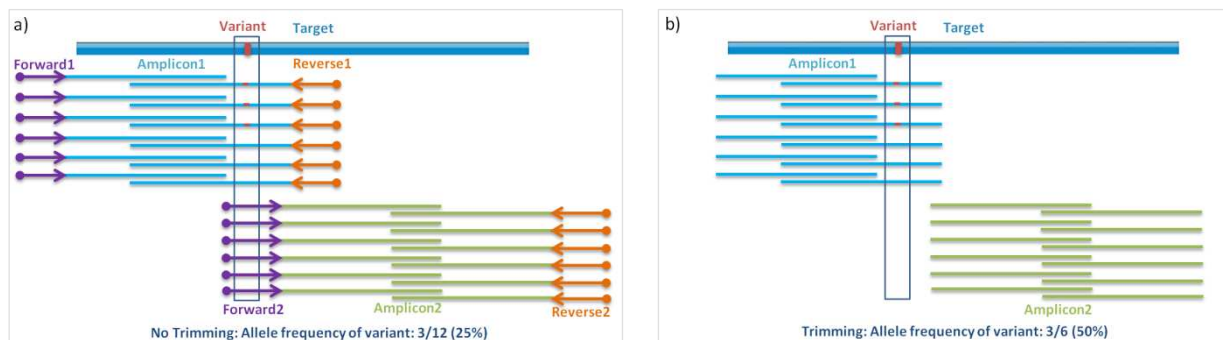


Figure 4. PCR Primer trimming. a) Illustration before PCR primer trimming: alignment of Amplicon1 and Amplicon2 reads with Forward and Reverse primers. b) Illustration after PCR primer trimming.

Remark: During design, great care was taken to select primer binding sites avoiding regions with variants. In addition, a periodic review is performed to identify newly reported variants in those regions and to test their impact on amplification. It can, however, not be excluded that a variant in a binding site of a primer may be present in a sample, which may lead to the amplification of only one of the alleles, masking the presence of a clinically relevant mutation in the amplicon. If such a case is suspected, calculation of the dosage quotient of each amplicon can be used for confirmation. For further support, contact customer services at customerservice@multiplicom.com.

12.2.4. Alignment to the reference sequence

The sequence reads can be aligned to the targeted regions or to the entire human genomic sequence. To facilitate the transfer of assay specific information to the different analysis software packages, a BED file with the amplicon positions on hg19 is available for download at <http://www.multiplicom.com/keycode> where you will be requested to enter the product KEY-CODE found on the box label of the specific MASTR assay.

12.2.5. Variant calling

Different parameters can be analyzed to discriminate true positive variants from false positive or background signals. Below, you find a non-exhaustive list of parameters whose effect on the sensitivity and specificity of variant calling might be evaluated:

12.2.6. Minimal coverage

The coverage, or number of aligned read pairs, at the site of the variant has to reach a given threshold for confident variant detection. The minimal coverage recommended by Multiplicom for MASTRs in combination with an Illumina MiSeq Sequencing System is 50 read pairs per allele for SNV analysis. It is advised that target regions that do not reach this minimal coverage are eliminated from the list of analyzed target regions in the final variant calling report.

For amplicon libraries of tumor samples (FFPE tissue, FFT or blood), the variant allele frequency (VAF_{sample}) and/or tumor tissue content (TTC) need to be taken into account to determine the required coverage for somatic variant calling.

Remark: more background information on these guidelines can be found in the Technical Note at <http://www.multiplicom.com/calculator>.

12.2.7. Quality scores

The quality of the aligned bases at the position of the potential variant has an effect on the confidence in the variant call. This quality is generally influenced by the position in the read (i.e. the overall quality typically increases during the first 20 cycles, remains constant for a large part of the read and, depending on the length of the read, starts declining again) and the genomic context (e.g. homopolymer stretches have a negative impact on the quality of the following bases). This leads to two derived parameters:

- **Presence in forward and reverse reads**
Depending on the lengths of the sequencing reads and the sizes of the amplicons of the specific MASTR assay used, the outer parts of the amplicons will be sequenced unidirectionally and the central parts bidirectionally (see Figure 4a). This is an important concept as both parts should be treated differentially when assessing presence of a variant in forward and reverse reads: for the central parts of the amplicons, presence in both directions is a good discriminator between true and false positives variant calls. For the outer parts, this discriminator can't be used but Q-scores are generally higher in these parts and hence provide higher confidence in correct variant calling.
- **Changes in/around homopolymeric stretches**
In view of the inherent difficulties of the PCR technology to accurately amplify homopolymer stretches, special care has to be taken when calling variants in or flanking a homopolymeric stretch. Due to DNA polymerase stuttering, homopolymer stretches with a length of 10 bp or more require special care at a VAF_{sample} of 50%. However, when calling variants at lower VAF_{sample} , the length of homopolymer stretches that can be accurately called decreases.

12.3. Specific instructions

Data analysis can be performed using a variety of analysis software packages. Below, specific instructions are provided for the use of the MiSeq Reporter (Section 12.3.1), the SeqNext module of the Sequence Pilot software of JSI medical systems (Section 12.3.2), and the *Sophia DDM* (Data Driven Medicine) application of the Integrated Clinical NGS Dry Lab Service of Sophia Genetics (Section 12.3.3).

12.3.1. MiSeq Reporter

Illumina's MiSeq Reporter software aligns reads to a reference genome and calls variants, resulting in .bam files and .vcf files respectively. To be able to do this, the MiSeq Reporter sample sheet needs to contain a manifest file specifying the reference genome (*Homo sapiens* hg19) and targeted regions. For more information on MiSeq Reporter, refer to Illumina Inc.

12.3.2. SeqNext module of the Sequence Pilot

12.3.2.1. Definition file for SeqNext

The SeqNext .bed file with the amplicon positions on genome build hg19 allows PCR primer trimming of the sequencing reads in the SeqNext module of the Sequence Pilot software (JSI Medical Systems).

12.3.2.2. Guidelines SeqNext v4.1.1 or 4.1.2 with SeqNext .bed file

- SeqPilot version 4.1.1 or 4.1.2 should be used following the guidelines in the manufacturer's manual, in combination with the instructions in this section.

- The reference sequence(s) of the targeted gene(s) should be defined using human genome data downloaded from Ensembl or UCSC database, as described in the manual of SeqNext.
 - Prior to defining the Regions of Interest (ROI), download the MASTR Plus-specific Definition file for SeqNext from <http://www.multiplicom.com>. Copy the .bed file from this zip-file.
 - Define the ROI in the Operation 'ROI', tab 'add Enrichment / Kit' as described in the SeqNext manual's section for Enrichment or csv-file containing ROI information:
 - Select the 'Enrichment' option
 - Browse and select the SeqNext .bed file for the field 'File'
 - Select 'Homo Sapiens' for the field 'Organism'
 - Fill out a name for the field 'Category' and for the field 'Suffix' (eg, MTP)
 - Optional: Select the number of 5' and 3' flanking bases and activate the 'auto cut' checkbox
 - Leave 'build amplicons' active
 - Click on button 'Columns' to define the content of the different columns from the .bed file
 - Activate 'Ignore Header'
 - Chr: '1'
 - Chr Start: '2'
 - Chr End: '3'
 - Gene: '4'
 - Leave the field for 'Transcript' blank
 - Comment: '6'
 - Activate 'Chr positions include primers'
 - Sense primer: '7'
 - Antisense primer: '8'
 - Click on button 'build'
 - Check the proposed transcript(s), and change them if they do not cover all amplicons of the specific MASTR Plus
 - Move the automatically build ROIs to the 'ROI list' with the button 'Add Act ->' or 'Add Sel ->'. Click the 'Save' button at the bottom, the regions of interest are now defined and can be used to define a ROI Group and when starting an analysis in the Operation 'Run'.
 - Define a 'ROI Group' with all defined ROIs in the Operation 'ROI Groups'.
 - Start an analysis in the Operation 'Run'. Some guidelines for SeqNext settings for calling of variants at 50% allele frequency are shown in the screenshots in Figures 5-10 of Section 15.1 and 15.2.
 - To enable reliable somatic variant detection at < 50% allele frequency, the parameters 'Min. % coverage' at '[4] Mutations' and 'Distinct / % Other coverage' at '[5] Mutations Sorting' have to be modified as shown in Figure 11 of Section 15.3. The other settings remain as shown in Figures 6-10 of Section 15.2.
- Remark:** optimized RUO SeqNext settings need to be properly validated.
- Connect to each subject sample the correct Read1 and Read2 .fastq files, add the correct ROIs and start the analysis.
 - After analysis, select the correct 'Order' in the Operation 'Joining' or 'Worklist', go to the Operation 'Sequence' and perform technical validation of the variants in the 'distinct' tab for the genes of interest. Change the condition to '1' to ignore variants that:
 - are purely changes in homopolymer lengths of 10 bp or more for a VAF_{sample} of 50%.
Remark: caution needs to be taken since the length of homopolymer regions that can be accurately called decreases at lower VAF_{sample} .
 - are in the other tabs ('other', 'homopolymer')
 - Verify the coverage of target positions, i.e. the number of read pairs obtained at a given position after the sequencing run. For SNV analysis, the minimal coverage advised for reliable variant calling is ≥ 100 read pairs per amplicon for heterozygous variants, i.e. a VAF_{sample} of 50%; see Technical

Note at <http://www.multiplicom.com/calculator> for more information. Only target positions with \geq 100 read pairs are advised for the final analysis. Those below 100 read pairs should be reported as excluded regions due to insufficient coverage.

- Print the sequencing report for the variants in the distinct category.

12.3.3. *Sophia DDM instructions*

The *Sophia DDM* application should be used according to manufacturer's instructions.

To access and use Sophia Genetics' service, laboratories should request an account on the *Sophia DDM* application by contacting Sophia Genetics directly: <http://www.sophiagenetics.com/contact>.

13. LIMITATIONS OF THE PROCEDURE

- The reduction or absence of sequence reads of a particular amplicon is not sufficient to conclude the presence of a deletion and needs to be confirmed by an alternative method. It is advised to contact the local sales representative in case the deletion cannot be confirmed.
- Homopolymer (HP) stretches longer or equal to 10 bp are difficult to unambiguously detect at a VAF_{sample} of 50% using an Illumina MiSeq System. When calling variants at lower VAF_{sample} , the length of homopolymer stretches that can be accurately called decreases.

14. DISCLAIMER

The purchase of this product enables the purchaser to use it for the amplification of nucleic acid sequences for human genes. Purchaser has to take into account that information obtained from amplicons generated using this product may not be used in procedures that are protected by valid claims owned and/or controlled by a third party, unless prior written approval of such party has been obtained.

IVD product performance claims apply only when combining MASTR Dx and MID Dx, and when both CE-labeled kits are used according to the specific CE-IVD Instructions For Use.

15. ATTACHMENT: SEQNEXT SETTINGS (RUO)

15.1. SeqNext settings for detection of variants at 50% allele frequency

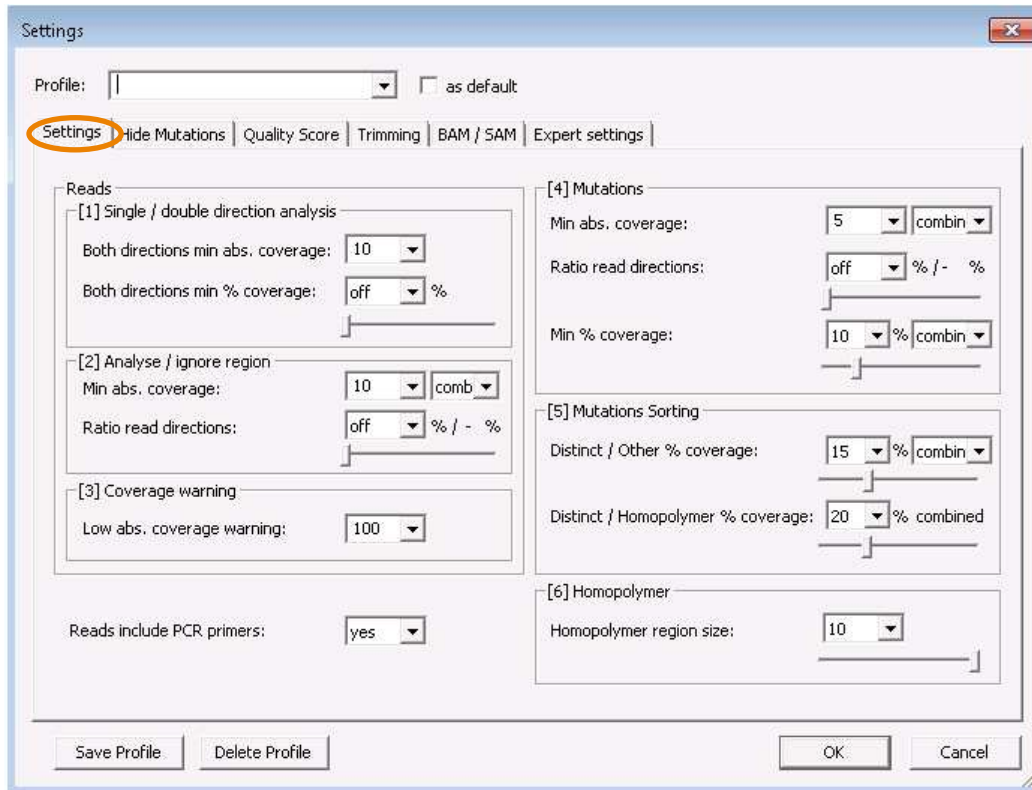


Figure 5. General settings for variants at 50% allele frequency

15.2. Other SeqNext Settings

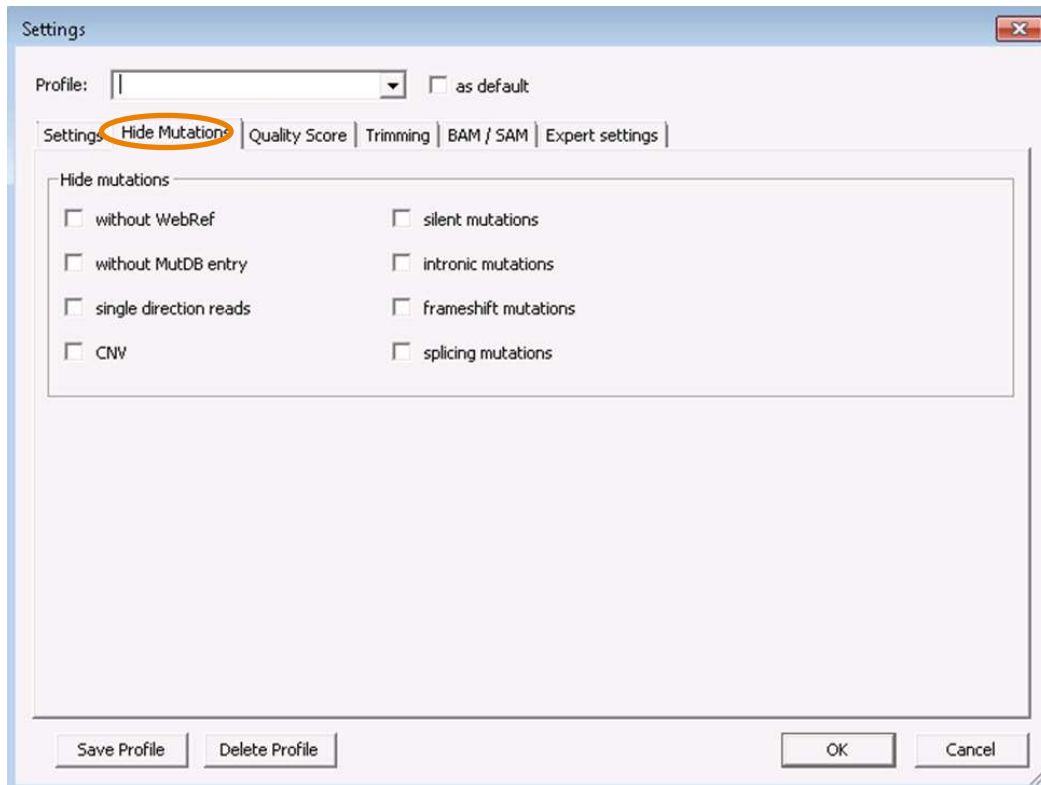


Figure 6. Hide Mutations settings

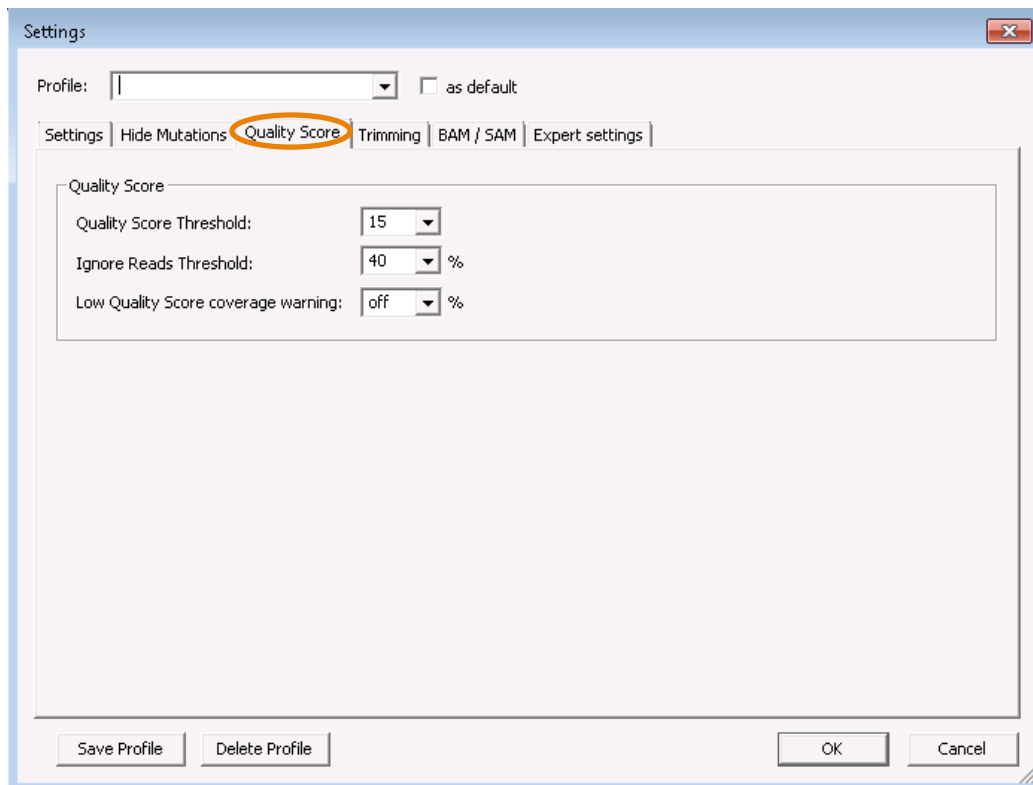


Figure 7. Quality Score settings

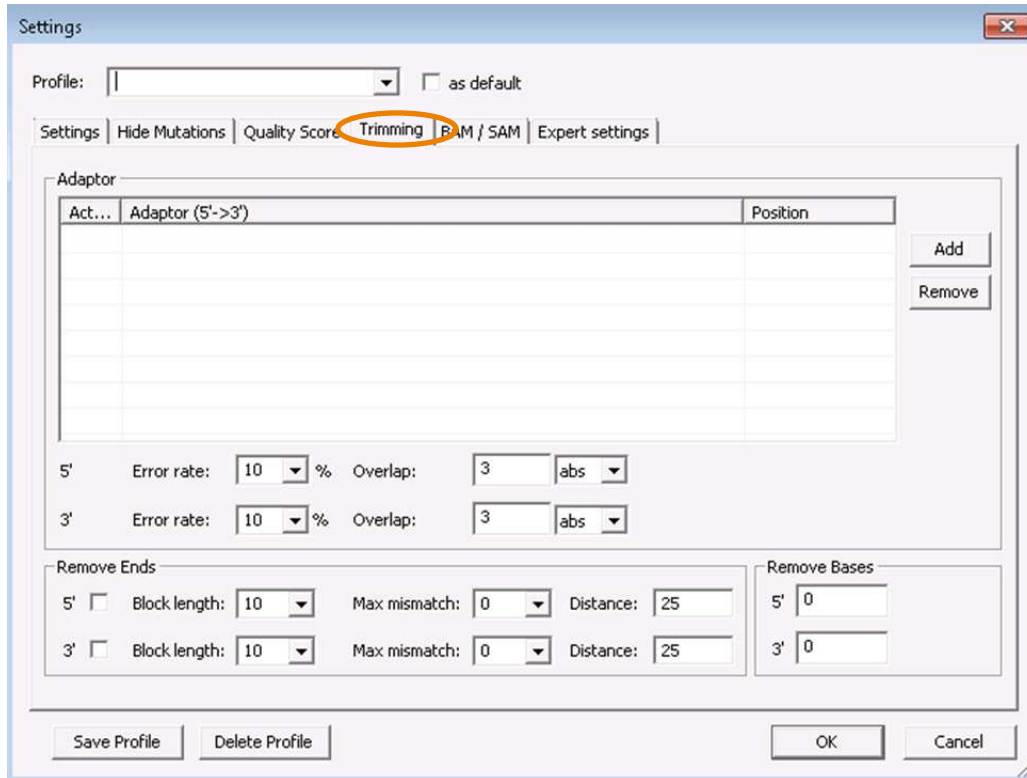


Figure 8. Trimming settings

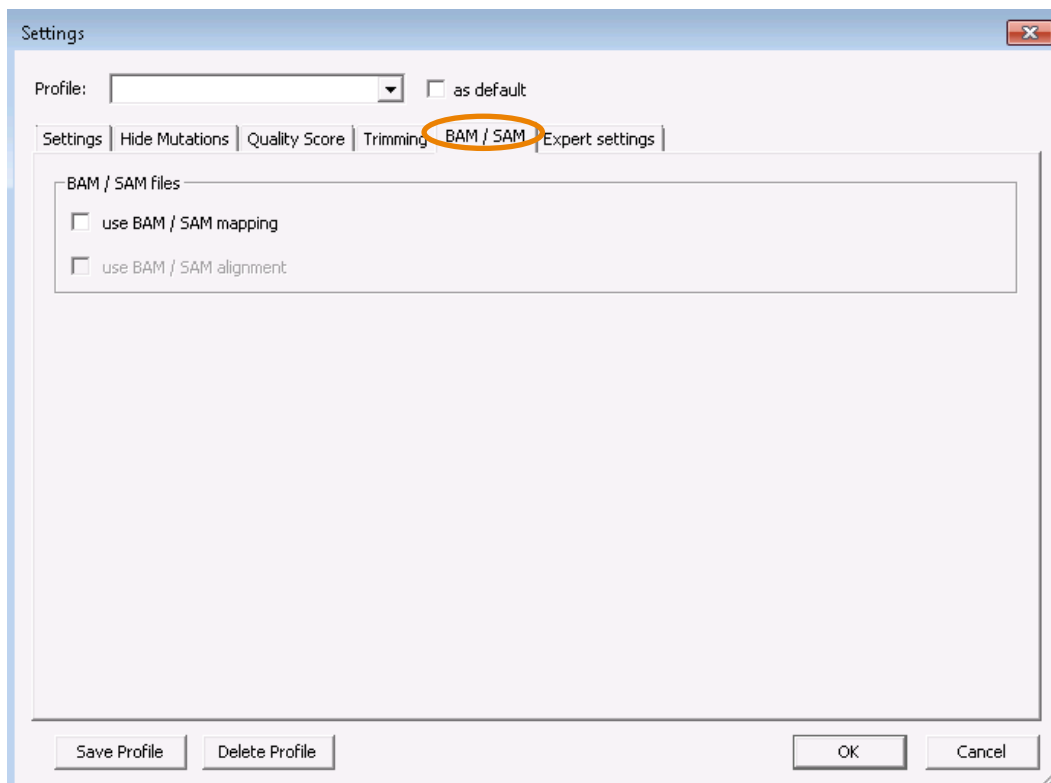


Figure 9. BAM/SAM settings

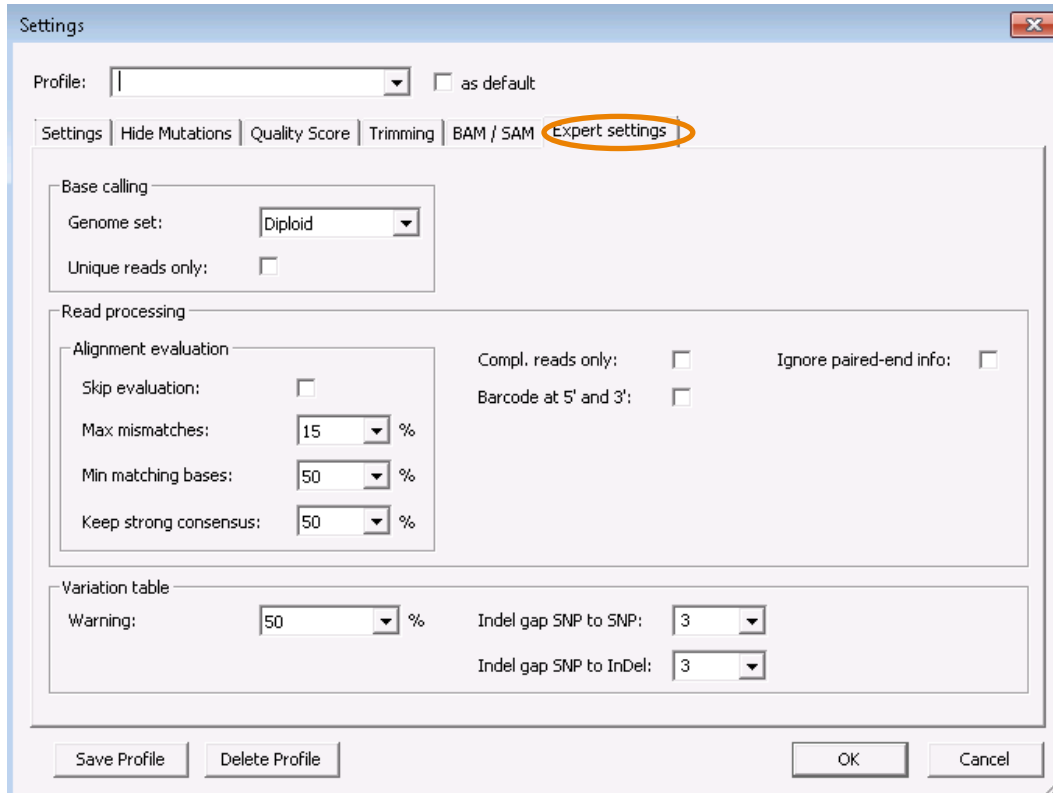


Figure 10. Expert settings

15.3. Example of SeqNext settings for detection of variants at < 50% allele frequency

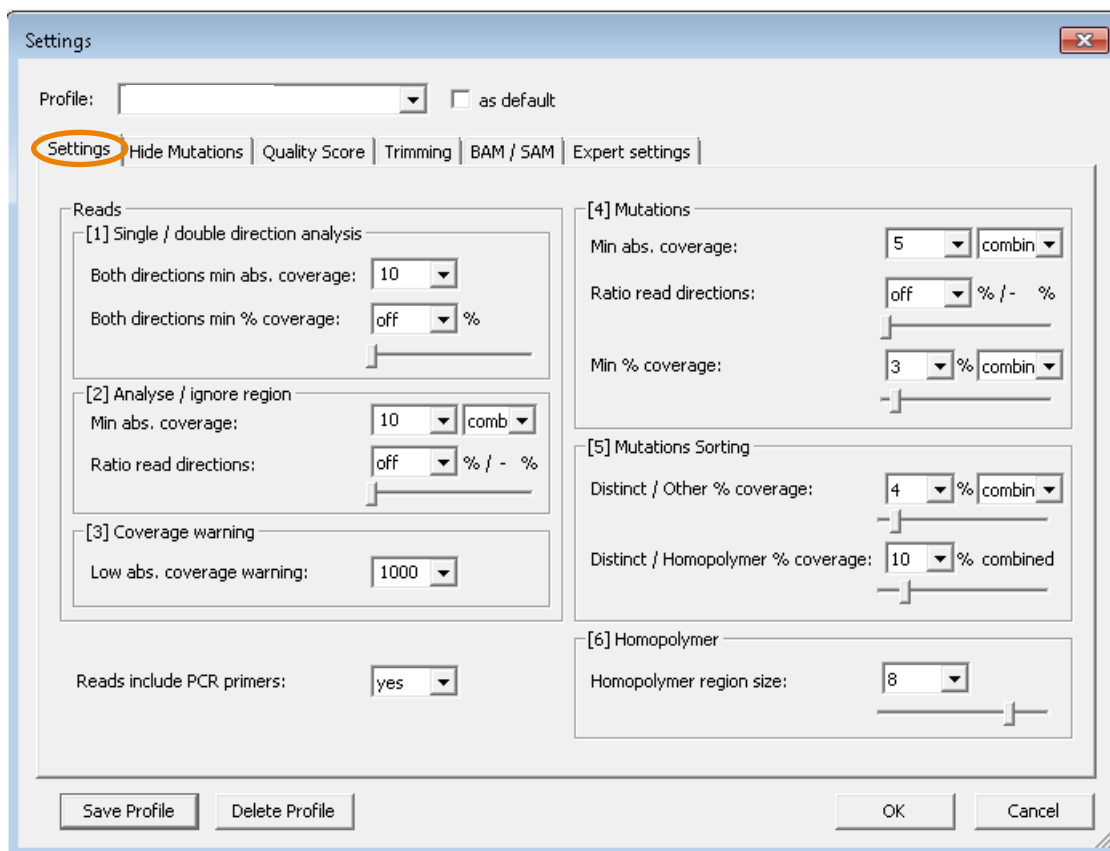


Figure 11. Example of general settings for variants at < 50% allele frequency

16. LIST OF ABBREVIATIONS

AR:	Amplification Reagent
CE:	The CE symbol certifies that a product complies with the European standards.
CNV:	Copy Number Variation
DDM:	Data Driven Medicine (Sophia Genetics' application)
DNA:	Deoxyribonucleic acid
dNTPs:	Deoxynucleotide Triphosphates
EDTA:	Ethylenediaminetetraacetic acid
FFPE:	Formalin-Fixed Paraffin-Embedded
FFT:	Fresh Frozen Tissue
HP	Homopolymer
IFU:	Instructions For Use
IVD:	<i>In Vitro</i> Diagnostics

MASTR:	Multiplex Amplification of Specific Target for Resequencing
MAQ	Multiplex Amplicon Quantification
MID:	Molecular Identifier
MPS:	Massively Parallel Sequencing
OD:	Optical Density
PCR:	Polymerase Chain Reaction
Plex:	Set of MASTR-derived amplicons
RUO:	Research Use Only
ROI:	Region of Interest
SNV:	Single Nucleotide Variation
TBE buffer:	Tris-Borate-EDTA buffer
TE buffer:	Tris-EDTA buffer
TTC:	Tumor Tissue Content
VAF:	Variant Allele Frequency