Novel diagnostic solutions for the detection of microsatellite instability in tumors using fastGEN technology

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Introduction

Tumor DNA testing of **microsatellite instability (MSI)**, which is a sensor of **MMR deficiency** is a prerequisite for personalized treatment in colorectal and endometrial carcinoma. Tumors with MSI can be targeted with immunotherapy using check-point molecules. The restart of the immune response against tumor using PD-1, PD-L1 or CTLA-4 inhibitors (ICI) leads to good therapeutic response in hypermutated MSI positive tumors.

Deep amplicon next-generation sequencing (NGS) has a potential to be a suitable method for simultaneous direct detection of several MS loci.

Aim of the study was to develop and verify a fast and robust NGS library preparation of tumor DNA samples for the detection of **changes in 13 clinically relevant MSI loci** (Tab. 1)

Tab. 1: Overview of clinically relevant MSI loci based on the consenus of information from several diagnostic kits, guidelines and MSI reference standards. NA – selected locus was not amplified and not analysed.

Bio-marker MSI loci	type of repetition	OncoMate MSI Dx PROMEGA	TrueMark MSI Assay THER. FISH.	Bethesda guidelines (Boland *et al.*, 1997)	Horizon FFPE Ref. Std.	Titano MSI Test Diatech	PentiPlex MSI PentaBase	Selected loci fastGEN MSI
BAT25	(T) 25							
BAT26	(A)27							
BAT40	(T) 37							
CAT25	(T) 25							
NR21	(A)21							
NR22	(T) 21							
NR24	(T) 23							
MONO27	(T) 27							
NR27	(A)26							
TGFBR2	(A)10							
D2S123	(AC) 21							
D5S346	(TG) 20							
D17S250	(GT) 20							NA
D18S58	(GT) 18							

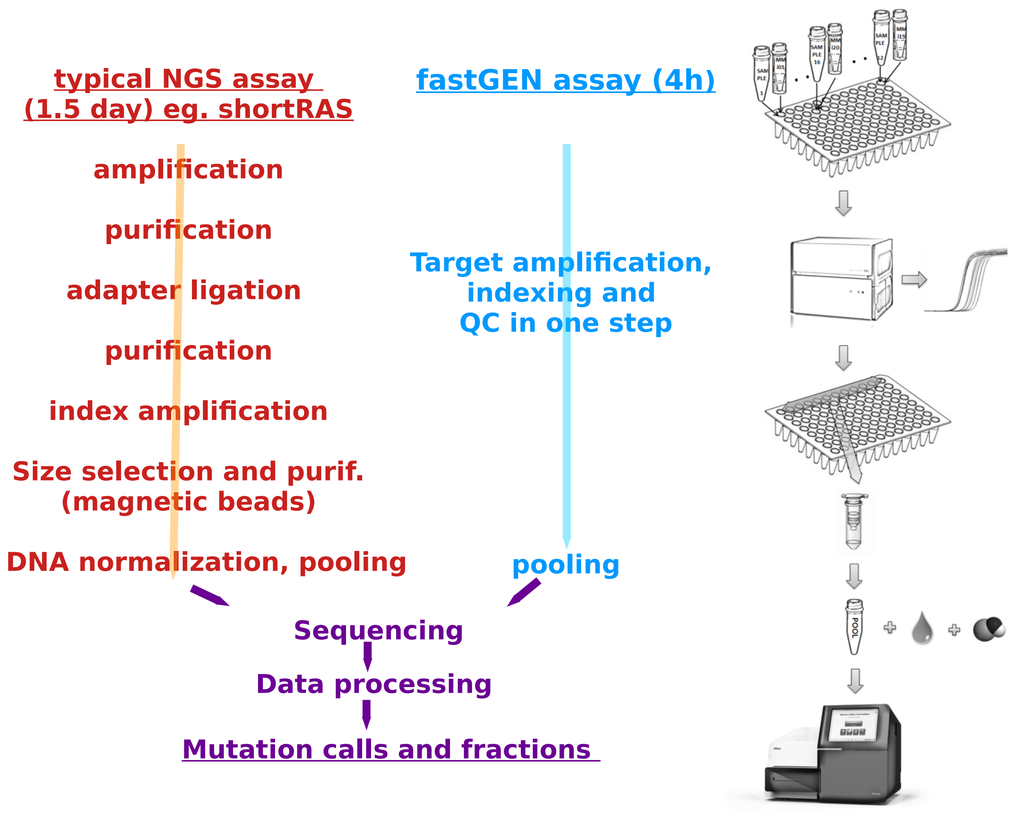

Fig.1: Scheme of procedures in **typical** and **fastGEN** NGS assays.
(typical assay is described for example in Slavkovsky 2022, Neoplasma Vol.69)

Materials and methods

Samples: DNA isolated from formalin fixed paraffin embedded (FFPE) block of colorectal carcinoma (CRC, n=24), endometrial cancer (EC, n=1) , bone cancer (BoC n=1) and MSI/MSS FFPE DNA Reference Standards (Horizon Discovery, n=2).

NGS library preparation: **NGS method based on proprietary amplicon fastGEN technology (a.k.a. fastGEN MSI Kit, BioVendor)** were introduced into laboratory as described at Fig.1. Usually 3 to 9 samples per run were pooled and run with other fastGEN libraries.

Sequencing: **Illumina MiSeq** using pair-end 2x150 bp reads.

Data analysis: Fastq files were analyzed using QIAGEN CLC Genomics Workbench 23 (CLC GxW) or MSIsensor-pro. Mapped reads of 15 MSS samples were used for generation of baseline using tool *Generate MSI Baseline (*settings Flanking signature length 15) or *baseline* command for MSIsensor-pro. MSI status was analyzed from mapped reads using tool *Detect MSI Status* (settings *: Earth mover's distance algorithm)* or command *pro* for MSIsensor-pro.

Results interpretation: Ratio of unstable to all analyzable markers was calculated and status was interpreted with these thresholds : ≥15 % MSS, 15-35% MSI-L, >35% MSI-H.

Sample		Ref. method	fastGEN MSI						fastGEN
id	origin	Expected MSI status	MSpro unstable loci	MSpro status	CLC GxW unstable loci	CLC GxW status	loci sites	base line	BRAF status
1	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
2	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
3	CRC	MSI-H (5/5)	10	MSI-H	12	MSI-H	13		V600E 18%
4	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
5	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
6	CRC	MSS (0/5)	0	MSS	2	MSI-L	13	yes	wt
7	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
8	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
9	CRC	MSI-H (3/5)	8	MSI-H	10	MSI-H	13		wt
10	CRC	MSI-L (1/5)	1	MSS	0	MSS	13		wt
11	CRC	MSI-H (3/6)	9	MSI-H	11	MSI-H	13		V600E 22 %
12	CRC	MSS (0/5)	1	MSS	0	MSS	13		wt
13	CRC	MSI-L (1/5)	1	MSS	3	MSI-L	13		wt
14	CRC	MSI-H (3/5)	8	MSI-H	11	MSI-H	13		wt
15	CRC	MSI-L (1/5)	0	MSS	0	MSS	13		wt
16	CRC	MSI-H (4/5)	9	MSI-H	12	MSI-H	13		wt
17	CRC	MSS (0/5)	0	MSS	2	MSI-L	13	yes	wt
18	CRC	MSS (0/5)	0	MSS	1	MSS	13	yes	V600E 39%
19	CRC	MSI-H (5/5)	9	MSI-H	12	MSI-H	13		V600E 9%
20	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
21	CRC	MSS (0/5)	0	MSS	1	MSS	13	yes	wt
22	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
23	CRC	MSS (0/5)	0	MSS	1	MSS	13	yes	wt
24	CRC	MSS (0/5)	0	MSS	0	MSS	13	yes	wt
25	std	MSI-H (13/13)	9	MSI-H	11	MSI-H	13		wt
26	std	MSS (0/13)	1	MSS	0	MSS	13	yes	wt
27	EC	MSI-H (6/9)	6	MSI-H	6	MSI-H	13		wt
28	BoC	MSS (0/8)	0	MSS	0	MSS	13		wt

Tab.3: Results of analytical comparison of 28 samples. Samples no. 25 and 26 were reference standards (Horizon Discovery). Discrepant results are highlighted in red.

Results

In a **pilot validation study** of 28 samples we observed **100 % concordance** between fastGEN MSI and an accredited method in detection of MSI-high presence or absence (Tab.2a), and 89 % concordance in detailed MS status assessment (Tab.2b).

Accredited method was done by PCR and fragmentation analysis of BAT25, BAT26, BAT40, TGFRBRII, D17S250 loci, by Idylla MSI assay (n=2), Qiaseq DNA Targeted MSI NGS panel (n=2) or references were used.

accredited method (expected)		fastGEN ICI- (MSS/MSI-L)	fastGEN ICI+ (MSI-H)
	ICI- (MSS/MSI-L)	20	0
	ICI+ (MSI-H)	0	8

Tab.2a: Summary of detection of absence or presence of MSI-H status relevant for ICI indication using MSIsensor-pro software and CLC GxW (both provided identical results)

accredited method (expected)		fastGEN MSS	fastGEN MSI-L	fastGEN MSI-H
	MSS	15	2	0
	MSI-L	1	2	0
	MSI-H	0	0	8

Tab.2b: Summary of detection of detailed MS status using MSIsensor-pro software.

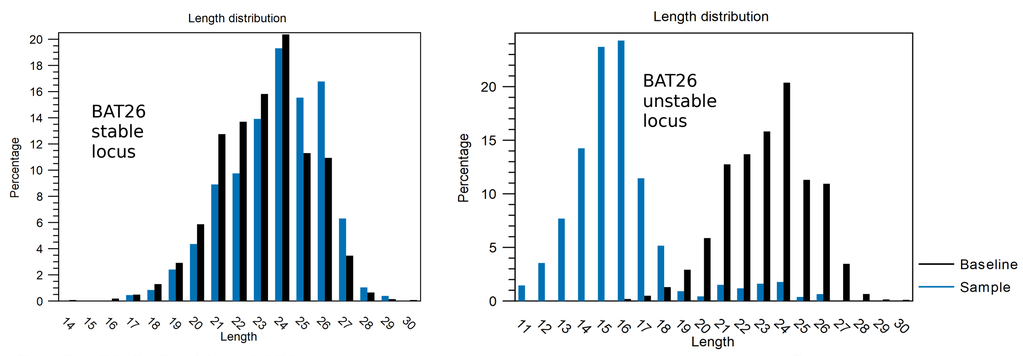

Fig.2: CLC GwX – MSI loci length distribution in seq. reads. Compared to MSS baseline. Left : MSS sample - stable locus, right : MSI-H sample. Percentage = length supporting reads/total reads.

Comparison of bioinformatic methods

When compared CLC GxW *Detect MSI* vs MSIsensor-pro workflows both correctly identified all MSI-H with minor discrepancies in MSS/MSI-L samples (Tab 3). Overall concordance of CLC method in MS status was slightly lower (86% vs 89%), however average number of detected unstable loci was higher for CLC (10.6 vs 8.5). Further optimization of parameters is possible for both approaches.

Visualization of MSI markers

An example of the distribution of lengths of MSI loci in supporting reads is shown in Fig.2 for CLC GxW and in Fig.3 for MSIsensor-pro. MSS sample show full overlap with reference baseline or ref. sample, whereas unstable samples show bimodal distribution with typical shift to lower lengths arising from the mixture of tumor cells and normal cells.

MSI vs BRAF in CRC samples

Interestingly, half (50%, 3/6) of the MSI-H CRC samples were *BRAF* V600E positive, whereas this was the case only in one sample (6%, 1/18) of MSS/MSI-L CRC samples. This supports previous findings of MSI-H, *BRAF* mut. and *MLH1* demethylation co-occurrence (Thiel A et al. 2013, PMID: 23963522).

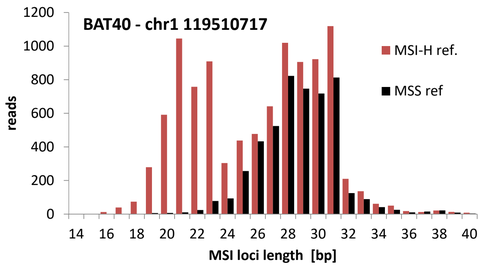

Fig.3: MSIsensor-pro - MSI loci length distribution in seq. reads. MSI-H ref. sample compared to MSS ref. sample.

Fig.4: Typical fastGEN kits contain 16 ready-to-use master mixes with sample indexes and sequencing primers.

Conclusion

We showed that **deep fastGEN NGS assay could be a suitable method for routine diagnostic of MSI biomarker** where several loci need to be analyzed especially in case of FFPE derived samples, direct single-step library preparation could be beneficial for high quality results. fastGEN kits are nowadays commercially available from BioVendor Group (Fig.4) and most likely novel MSI dedicated kit will be available soon.

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