# ABSTRACT #2806
# HIGH SENSITIVITY QPCR MICROSATELLITE INSTABILITY DETECTION IN FFPE TISSUE

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## INTRODUCTION

Microsatellite instability (MSI) refers to genetic instability in short nucleotide repeats (e.g., tandem repeats of 1-6bp segments), where a cell comprises a different number of repeats as compared to what was inherited from a progenitor cell. This genetic instability is often observed in tumor cells with impaired mismatch repair (MMR), especially in colorectal (CRC) and endometrial cancers. MSI can be used as a biomarker to predict response to treatment with immunotherapy. Colorectal cancer patients with MSI have a significantly better prognosis compared to those with intact mismatch repair. Capillary electrophoresis is often used to detect microsatellite instability; however, it has no better than 5% analytical limit of detection for any given microsatellite instability locus.

Here we present our MSI Assay, which enables high-sensitivity detection of microsatellite instability. The assay uses NuProbe's PCR-based Blocker Displacement Amplification (BDA) technology to enable the selective amplification of MSI unstable alleles with as low as 1% analytical limit of detection. In BDA, a blocker oligo is introduced into the PCR reaction that is designed to be complementary to a known template sequence and to contain an overlap region with the forward primer. This enables molecular competition between the primer and the blocker to suppress wildtype amplification. When the BDA blocker is bound to a variant template, the resulting mismatch bubble between the blocker and the template increases the favorability of the primer to displace the blocker, allowing amplification to proceed. This assay is intended for the qualitative detection of mutations in 5 MSI loci (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) without the need of matched normal, and reports MSI-high (MSI-H), MSI-low (MSI-L) and Microsatellite Stable (MSS) with an easy-to-use software.

We have validated our assay on synthetic samples formulated from plasmids containing MSI-H alleles and cell line-derived genomic DNA free of MSI related mutations. The effective DNA input range of the assay is 1.5-15ng/reaction (3-30ng/specimen), with an LOD of 1% with a 10ng or greater input and 5% With less than 10ng of input. The assay is also quite robust, with greater than 98% sensitivity and specificity and greater than 95% reproducibility.

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## MATERIALS AND METHODS

### DNA extraction from FFPE tissue

Extraction of DNA from FFPE tissue slides or curls was performed using the QIAamp DSP DNA FFPE Tissue Kit. The kit procedure follows six steps to provide high yield, highly purified DNA extract without long overnight incubations. 1. Remove paraffin with xylene. 2. Lyse cells by incubating with Protinase K. 3. Heat the sample to remove formalin cross-linking. 4. Bind DNA extract in a silica column. 5. Wash out unwanted cellular debris. 6. Elute DNA.

### qPCR amplification utilizing blocker displacement amplification

This MSI assay utilizes two independent reactions to amplify the 5 MSI loci and GAPDH as a genetic control. NR21, NR24, and BAT26 are amplified in the first reaction, with BAT25, MONO27, and GAPDH in the second reaction. Each reaction has its own oligo mix containing primers, probes, and blockers. Each marker utilizes 7 blockers targeting WT alleles and small indels not indicative of MSI in CRC. Oligo mix is combined with enzyme master mix and up to 5uL of sample for 1.5-15ng of DNA input per reaction. If necessary, nuclease-free water is used to reach a reaction volume of 20uL. This assay was designed for use on the BioRad CFX96 real-time PCR system using a simple two-step cycling protocol.

### Analyzing data with the MSI software

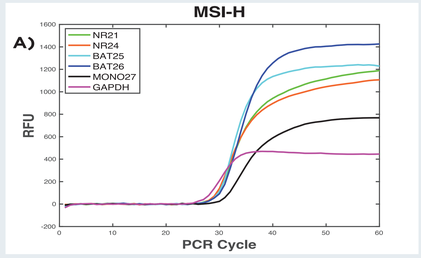
This system includes software that can quickly analyze data exported from the qPCR instrument and determine the MSI status of the sample. The software analyzes the raw fluorescence data from the instrument and applies custom normalization and calling algorithms. The MSI status of individual markers is determined by comparing the markers amplification to that of the positive control run with each plate, utilizing the delta-delta Ct method. If no markers show instability the sample is called MSS, with one marker showing instability the sample is called MSI-L, and if two or more markers show instability, the sample is called MSI-H. The software is also able to detect if an sample input amount outside the validated range is used and call the sample invalid. The results of each experiment can be exported in a simple, easy-to-read format.

**References**

**1.** Petrelli F, Ghidini M, Cabiddu M, Pezzica E, Corti D, Turati L, Costanzo A, Varricchio A, Ghidini A, Barni S, Tomasello G. Microsatellite Instability and Survival in Stage II Colorectal Cancer: A Systematic Review and Meta-analysis. Anticancer Res. 2019 Dec;39(12):6431-6441

**2.** Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol. 2005 Jan 20;23(3):609-18

## RESULTS



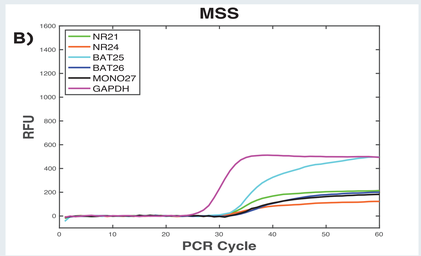




**Fig 1. A)** Amplification plot of synthetically created 5% VAF MSI-H sample with 5/5 markers showing instability. **B)** Amplification plot of cell line derived genomic DNA showing no microsatelite instability.

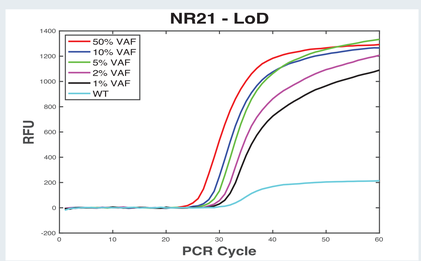



**Fig 2.** Amplification of NR21 in 5 samples with percentage of variant allele ranging from 1% to 50% compared to amplification of WT genomic DNA.

## RESULTS

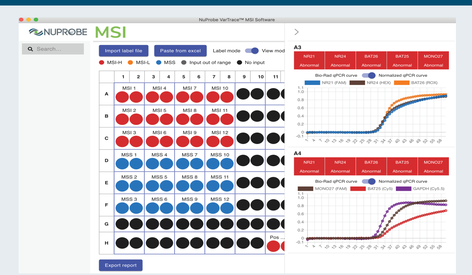



**Fig 3.** A screenshot taken from the MSI Software. The middle of the software shows a representation of the plate map for the samples analyzed. Each well is colored red, orange, blue, gray, or black to represent the samples status as MSI-H, MSI-L, MSS, input out of range, or no input respectively. Samples can be labeled manually in the software or by importing a simple excel label file. To see more information on samples, individual wells can be selected. This brings up the display seen on the right of the screen. Allowing the user to view the amplification curves and MSI status of each individual marker for the selected sample.

## CONCLUSIONS

This MSI assay utilizes NuProbe's patented BDA technology to effectively determine the MSI status of an individual. BDA allows for the targeted amplification of variant alleles of interest, greatly increasing the sensitivity of the assay compared to other methods currently on the market. The coupling of BDA with qPCR also allows for simultaneous analysis of the sample. Creating an assay that is simple and easy-to-use with a low barrier to entry. The development work completed for this research use assay can be used to inform development of future products, possibly including those specifically intended for diagnostic use. These future products could allow for fast and cost-effective determination of MSI status, potentially leading to improved treatment and better outcomes for patients.

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