

# Development and Validation of ColoScape™ – A New Non-Invasive Colorectal Cancer Mutation Detection Assay

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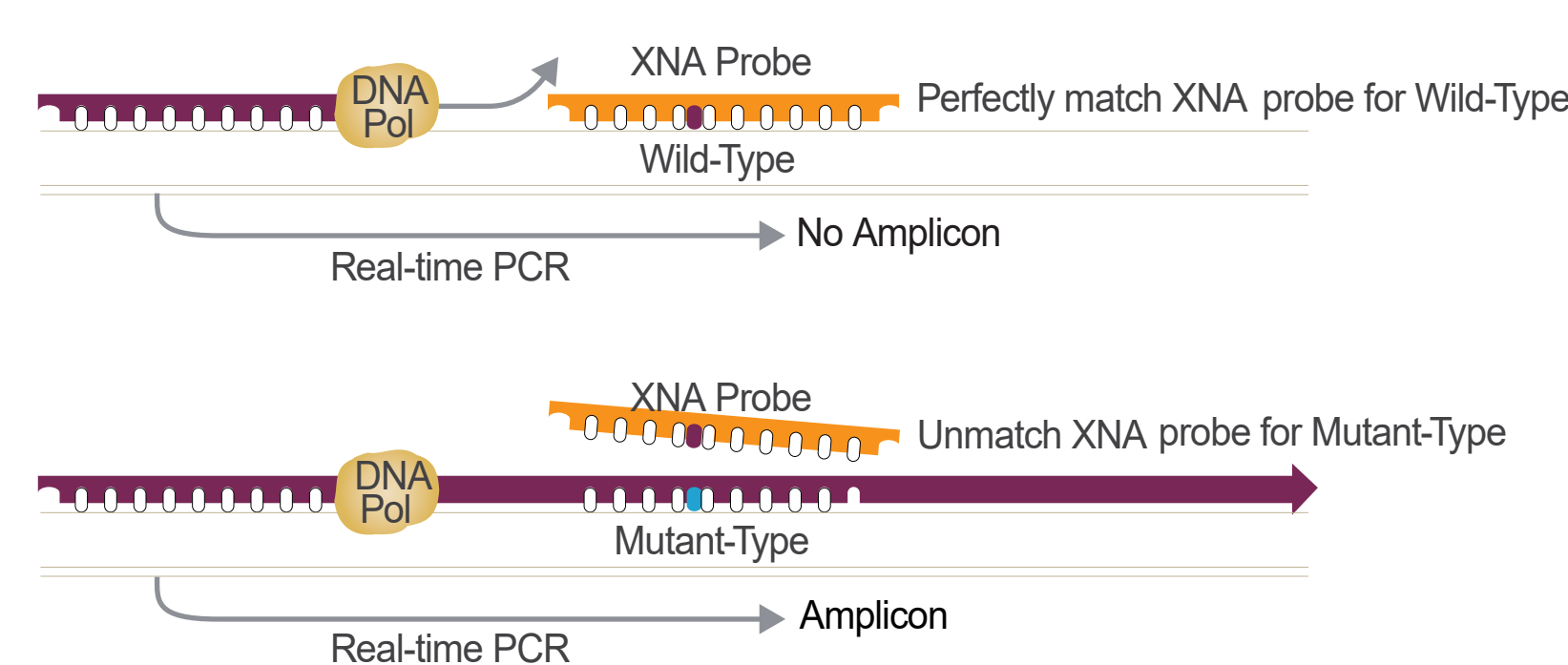
## INTRODUCTION

Colorectal cancer is a highly preventable disease as early detection increases rates of patient survival to near 100%. Herein we report the development and validation of a novel multiplex real-time PCR panel assay for simultaneous and qualitative detection of colorectal cancer-associated somatic mutations in the genes that are frequently mutated in colon cancer patients and are responsible for aberrant colonic epithelial cell proliferation. The multigene biomarker assay called ColoScape™ includes target gene mutation detection in APC (Exon 15), KRAS (Exon 2), BRAF (Exon 15) and CTNNB1 (Exon 3). The assay allows the sensitive detection of the presence or absence of mutations in the targeted regions of the genes interrogated in tissue biopsy (FFPE) and plasma samples.

## METHOD

The high sensitivity of this multigene biomarker assay is achieved due to xeno-nucleic acid (XNA) probe technology. XNA probes are novel backbone modified oligomers having natural nucleoside bases (A, T, C and G) that hybridize by Watson-Crick base pairing to natural DNA and RNA with much higher binding affinity than natural deoxyribonucleic acid oligomers of the same sequence. XNA probes are designed that bind to the selected wild-type sequences at the respective genetic loci in the target genes. These XNA probes have a much higher T<sub>m</sub> than the primer annealing temperature and suppress amplification of wildtype DNA templates and only allow amplification of the target mutant DNA templates in the sample. Both single nucleotide polymorphisms (SNP's) and insertion/deletions (indels) mutations can be detected. For each of the selected mutation sites primers and TaqMan hydrolysis probes were designed and tested together with the selected XNA oligomers and analytical assay performance confirmed. Human-Actin (ACTB) gene was selected as internal control for the assay. Singleplex detection reactions were optimized and integrated into multiplex qPCR reaction in which different probe fluorophore with well separated emission spectra were selected for each target for simultaneous detection of multiple target mutations in one reaction. Performance parameters of the assay were established on DNA of colorectal cancer patients extracted from FFPE, plasma as well as reference DNA materials (synthetic and cell line derived DNA). The assay demonstrates high sensitivity and specificity in detection of colon cancer and adenoma samples based on the set of biomarkers involved in colorectal cancer neogenesis and disease progression.

Figure 1: XNA technology



## RESULTS

### I. Analytical Sensitivity

The analytical sensitivity of the ColoScape™ kit was determined by studies involving APC, Beta-Catenin (CTNNB1), KRAS and BRAF-defined genomic DNA reference samples (Horizon Discovery, ATCC and SeraCare). Variant Allele Frequency (VAF) 1%, 0.5% and 0.1% mutant DNA template at 5ng and 10ng input were evaluated. The assay was validated on three qPCR instruments ABI QuantStudio 5, Roche LC480II and BioRad CFX384. For all tested purified reference gDNA inputs from 10ng/well to 5ng/well all target mutations were detected with 100% correct calls at 0.5% VAF (Figure 2). Interestingly, APC, CTNNB1 and BRAF assays sensitivity even down to 0.1% mutation in 5-10ng for each reaction.

For cfDNA from plasma samples, mutation detection limit of the ColoScape™ assay is 0.5% VAF (~7-8 copies of mutant DNA) with 5ng DNA input. Again, sensitivity testing for CTNNB1, KRAS12 and BRAF in plasma samples showed 0.1% mutation detection at 10ng DNA input level (Table 1).

Figure 2. Amplification profile of ColoScape™ multiplex qPCR assay on various concentrations of reference gDNA. Assay LOD Can detect up to 0.1% VAF DNA in 5ng gDNA

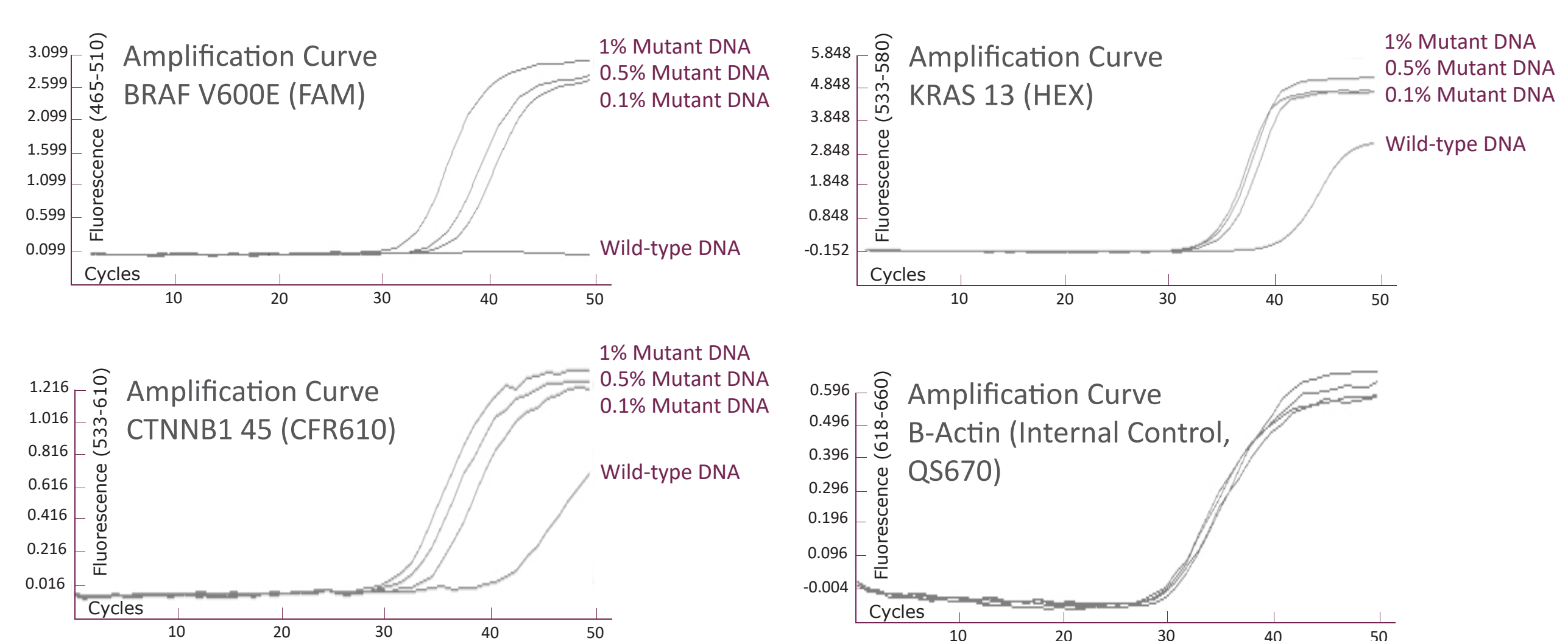


Table 1. Summary of LOD testing for cfDNA using ColoScape™

Gene Target	Reference DNA Input, ng/well	% Correct Call	
		10	5
APC 1309	1.0% Mutation	100%	100%
	0.5% Mutation	100%	95%
	0.1% Mutation	65%	40%
APC 1367	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	100%	100%
APC 1450	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	90%	90%
CTNNB1 c41	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	100%	100%
CTNNB1 c41	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	90%	85%
KRAS c12	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	100%	95%
KRAS c13	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	90%	95%
BRAF V600	1.0% Mutation	100%	100%
	0.5% Mutation	100%	100%
	0.1% Mutation	100%	95%

### II. Test of Assay Reproducibility

The high precision of the assays in the kit was verified by testing inter- and intra-assay, lot-to-lot (3 different lots), operator and instrument variability. Instruments tested produced consistent results for the 1% mutant and WT controls. The precision studies were summarized in Table 2.

Table 2. High analytical precision of ColoScape™

Assay Reproducibility	%CV
Intra-assay	≤ 3%
Inter-assay	≤ 5%
Operator Variability	≤ 4%
Lot-to-Lot Variation	≤ 3%

\* Based on Ct values

### III. Analytical Specificity

With known reference WT gDNA, the assay specificity is over 97%. There were no false positive calls for up to 320ng of gDNA per well and up to 20ng FFPE DNA from patients which are confirmed by NGS. Overall, analytical accuracy of the assay was shown to be over 95% on reference gDNA and DNA extracted from FFPE and plasma samples.

### IV. Clinical Performance on FFPE and Plasma Samples

Clinical sensitivity and specificity were tested on samples extracted from FFPE and plasma from patients with different stages of CRC from normal to advanced adenomas (AA), to colorectal cancer stages 1 through 4. In the initial testing of clinical samples, there were 10 pairs of matching tumor FFPE/adjacent normal tissues in the set (Test I in Table 3). Additional 97 FFPE samples and 10 cfDNA collected from CRC patients were tested by ColoScape™ and were confirmed by Sanger sequencing or NGS (Test II in Table 3). Plus, 52 plasma samples from FIT + patients were tested for pre-cancer scanning (Test III in Table 3). A sample was considered positive if at least one of the target mutations tested positive. The test result was considered correct if the CRC samples were positive and confirmed by Sanger sequencing or NGS and normal samples were negative. Pre-cancer detection sensitivity was 65%. Excluding pre-cancer samples, the assay specificity and sensitivity for FFPE samples were 100% and 92% respectively and 96% specificity and 95% sensitivity for cfDNA (Table 3).

Table 3. Clinical sensitivity and specificity for FFPE, plasma patient samples

Test	Types of Clinical Samples	Clinical Parameter	
		Specificity	Sensitivity
I	CRC (cfDNA)	N/A	100%
	CRC FFPE	N/A	90%
	Advanced Adenomas	95%	60%
II	FFPE	100%	93%
	cfDNA	100%	90%
III	cfDNA (Pre-Cancer)	92%	70%
Average	FFPE	100%	92%
	cfDNA	96%	95%

## CONCLUSION

The ColoScape™ kit was demonstrated to have robust analytical performance and clinical accuracy for FFPE and plasma samples. The rapid, precise and sensitive molecular assay for mutation detection in colon cancer has key benefits:

- **Unique:** proprietary XNA QClamp® TaqMan-based PCR technology with significantly increased mutation detection sensitivity. Only commercial qPCR kit available for all 4 gene mutations relevant for CRC.
- **Comprehensive:** Covers most clinically relevant mutations in 4 genes.
- **Ease of use:** Multiplex qPCR assay for simple qPCR setup and data analysis.
- **Efficient:** Only 15-30ng DNA as assay input.
- **Specific & Sensitive:** No cross-reactivity with wild-type up to 320ng purified gDNA 20ng FFPE DNA per reaction. Detection of 0.5% VAF and up to 0.1% VAF mutant DNA in WT at 5ng -10ng/well
- **Clinical Specificity:** 90-100% depending on sample type.
- **Rapid:** Total run time is less than 3 hours.
- **Versatile:** Assay validated on widely used real-time qPCR machines.

Note: ColoScape™ product is CE-IVD approved and is available as a research use only product in North America.

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