MSRE-qPCR for analysis of gene specific methylation can be accurately used for detection and validation of colorectal cancer-specific patterns

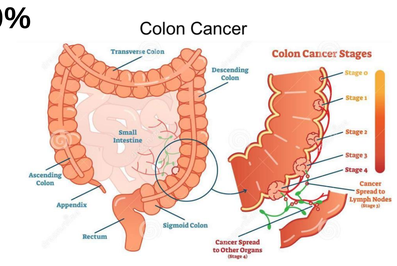
Kruusmaa K[1], Chersicola M[2], Bitenc M[1-2], Sanchez-Yague A[3], Schönthaler S[4], Pulverer W[4], Weinhaeusel A[4]

Universal Diagnostics S.L.[1], GenePlanet d.o.o.[2], Endoscopias Costa del Sol[3], AIT Austrian Institute of Technology GmbH[4]

Background

Colorectal cancer (CRC):

- **3rd** most commonly diagnosed cancer worldwide
- **90%** survival rate if caught early, **20%** survival if diagnosed at later stages



Current screening tests:

- Invasive
- Expensive
- Inaccurate/Low compliance rates

DNA methylation:

- Early, stable and tissue-specific event in cancer progression
- Detectable in circulating cell-free DNA (cfDNA) derived from blood
- Challenging due to low concentration of tumour-derived cfDNA (0.1- 1% against non-tumour cfDNA background)

Methylation-Sensitive Restriction Enzyme (MSRE)–qPCR:

- Detection of <10 copies of targets in highly multiplexed format
- Suitable for use in low tumour derived circulating DNA context

Methods

Patients:

- 4 ml of plasma from 133 participants (Table 1) attending CRC screening programs and oncology clinics in Spain and US from 2017- 2018.

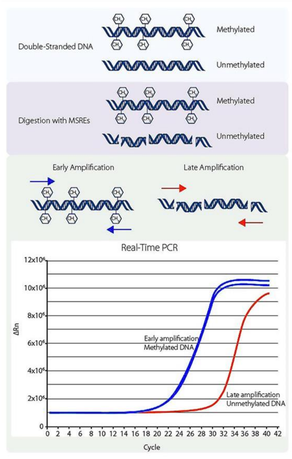
Markers:

- 250 CRC-specific CpGs were selected from publicly available tissue data
- 186 CpGs + 6 quality control markers successfully multiplexed into 2x96-plex assay

Protocol:

- 1/3 of extracted cfDNA pre-amplified and analysed by µ-fluidic qPCR
- 2/3 of extracted cfDNA digested with restriction enzymes-AciI, Hin6I or HpyCH4IV (1-15 cut sites per target) and analysed by µ-fluidic qPCR
- Data analysis based on ct-values subtracted from 45 (maximum cycle nr)

MSRE-qPCR



http://www.nxt-dx.com/epigenetics/msre-qpcr/

Results

- Data analysis over undigested DNA showed that 172 markers out of 186 could be successfully detected from plasma samples in multiplexed format
- CV < 15% for 6 control targets represents good technical reproducibility between assay runs
- Standard curve-s analysed for all experimental targets allowed accurate measurement down to 2-11 copies of methylated DNA
- Univariate analysis over methylation status of digested DNA identified number of markers with high individual accuracy p-value < 0.05 (Figure 1)
- Monte-Carlo cross validation (MCCV) was run for 50 iterations using balanced sub-sampling of the training set. The top 9 markers were used to build random forest classification model
- Validation of the 9-markar model on a separate set gave us AUC = 0.90 with overall cancer sensitivity at 80% and sensitivity for localized CRC at 77% and advanced at 86% with specificity set at 90% (Figure 2 A-B)

Table 1 Sample set. *An initial set of samples was split into a training set, for model building (blue) and to validation set for model validation (red).*

	Training (n = 70)					Validation (n = 63)			
	CRC	Healthy + Non-advanced adenoma	Healthy	Non-advanced adenoma		CRC	Healthy + Non-advanced adenoma	Healthy	Non-advanced adenoma
Female (%)	15 (21.4)	20 (28.6)	15 (21.4)	5 (7.1)	Female (%)	11 (17.5)	25 (39.7)	20 (31.7)	5 (7.9)
Male (%)	15 (21.4)	20 (28.6)	15 (21.4)	5 (7.1)	Male (%)	9 (14.3)	18 (28.6)	11 (17.5)	7 (11.1)
Age (range)	64 (41-80)	60 (46-84)	60 (46-77)	60 (48-84)	Age (range)	61 (45-84)	57 (47-80)	55 (47-80)	63 (55-70)
BMI (sd)	25.0 (± 4.4)	26.7 (± 4.3)	26.7 (± 4.7)	27.2 (± 3.0)	BMI (sd)	25.4 (± 3.8)	26.3 (± 4.0)	26.3 (± 4.0)	26.7 (± 4.0)
	CRC all	Proximal	Distal			CRC all	Proximal	Distal	
Localized	17	6	11		Localized	13	5	8	
Advanced	13	9	4		Advanced	7	4	3	

Figure 1 Detection of methylated markers in plasma. *45 – dCt values plotted for 4 markers for control (healthy+ non-advanced adenoma) samples (blue) and CRC samples (red). Higher 45 – dCt values correspond to higher methylation status*

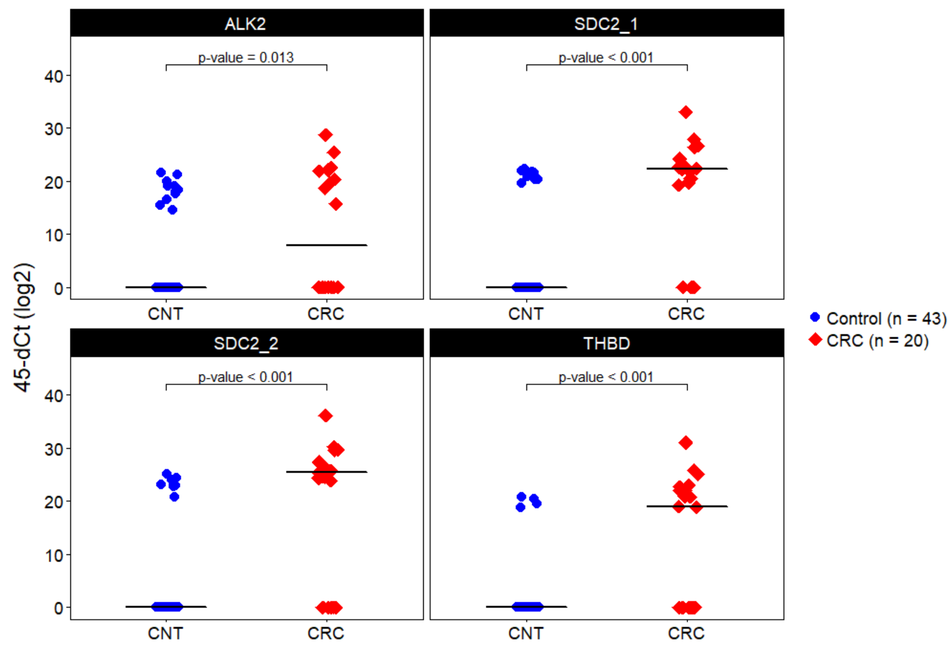
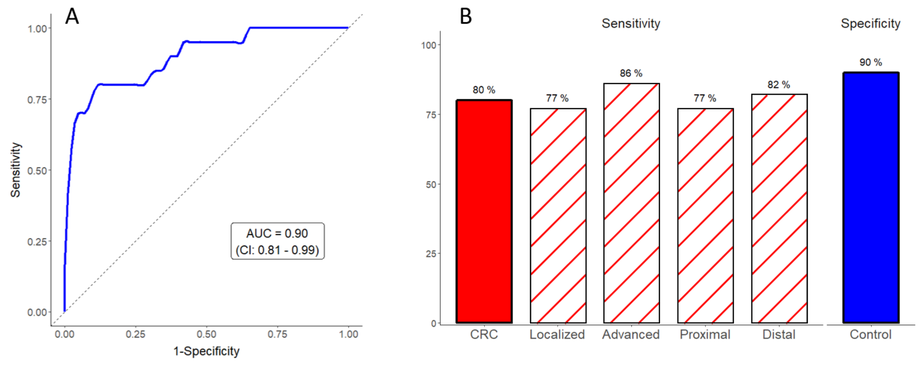


Figure 2 Performance of 9-marker panel on validation set *A. ROC curve and AUC for all samples. B. Accuracy values, where red bar represents the overall sensitivity for CRC, red-diagonal bars represent sensitivity by stage and cancer location and blue bar represents specificity for control (healthy + non-advanced adenoma)*



Conclusions

- MSRE-qPCR technology could be successfully used for accurate multiplexed analysis of tumour-related methylation markers from plasma samples
- Methylation markers can successfully be used for plasma-based detection of colorectal cancer patients
- 80% sensitivity at 90% specificity could be achieved for colorectal cancer with 77% sensitivity for early-localized cancer detection