Analytical Validation of a Circulating Tumor Methylated-DNA Assay for Detection of Colorectal Cancer Recurrence

John Alsobrook[1], Snigdha Gaur[1], Emily Kinnaman[1], Laura Schnetzler[1], David Murray[2], Sherin Alex[2], Robert Boorstein[1]

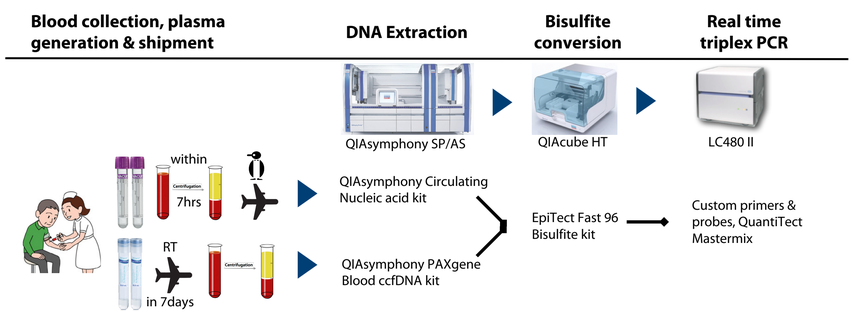
[1]Clinical Genomics Pathology Inc., Bridgewater, NJ, USA and [2]Clinical Genomics Technologies Pty Ltd, North Ryde, NSW, Australia

INTRODUCTION

Colvera™ is a qualitative epigenetic assay that detects circulating tumor DNA by measuring the methylation within two genes: *BCAT1* (Branched chain amino acid transaminase 1) and *IKZF1* (Ikaros Family zinc finger 1) that have been determined to be associated with colorectal cancer recurrence. Previous publications have described the assay's development [1], analytical validation [2] and clinical [3,4,5] validations in an EDTA based system. We report here the analytical validation of Colvera assay in EDTA based system and in a PAXgene based system as an LDT in a US CLIA licensed laboratory. PAXgene system allows specimen transport flexibility for up to 7 days at ambient temperature.

COLVERA TEST DESCRIPTION

Blood is collected either in 1) two EDTA tubes, processed to plasma within 7 hrs of collection & shipped frozen or in 2) two PAXgene Blood ccfDNA tubes (QIAGEN; Cat. No 768115; 1.5mL additive & 10mL blood draw) that are shipped at RT within 7 days from which plasma is generated in the lab. DNA is extracted on a QIAsymphony SP using the QIAsymphony DSP Virus/Pathogen kit (QIAGEN; Cat. No. 937055) from 4mL EDTA plasma and QIAsymphony PAXgene Blood ccfDNA kit (QIAGEN; Cat. No. 768536) from 4.8mL PAXgene plasma. Extracted DNA is bisulfite converted using EpiTect Fast 96 Bisulfite Conversion kit (QIAGEN, Cat. No. 59720) on QIAcube HT. Bis-DNA is measured in triplicate by realtime triplex PCR on LightCycler 480 II using custom methylation specific primers & probes (IDT) and QuantiTect Multiplex PCR No ROX Mastermix (QIAGEN; Cat. No. 204743). Colvera test is called positive when *ACTB* (*β*-actin) is positive and any replicate of *BCAT1* or *IKZF1* has a positive Ct.



MATERIALS AND METHODS

Blood from self-declared normal individuals, collected in EDTA or PAXgene blood tubes was processed to plasma within 7 hrs of draw and stored < -20℃ unless being used for PAXgene stability & accuracy.

Precision & analytical sensitivity: pooled plasma from self-declared normal donors spiked with methylated human gDNA (mDNA) at different conc. [1.6 – 250 pg/mL].

Interfering substances: pooled plasma (EDTA) or blood (PAXgene) with 50pg/mL mDNA, mixed with commonly found blood components at their upper reference ranges.

Accuracy (EDTA): split sample analysis with Clinical Genomics Lab in New Jersey (NJ) & Australia (AUS) using 20 donors (un-spiked and spiked at 5 different mDNA conc.)

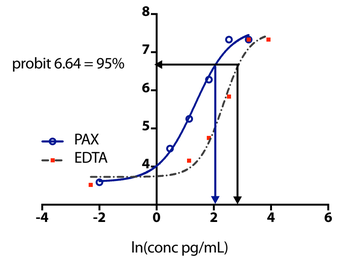
Accuracy (PAXgene): donor (colonoscopy confirmed 20 normal & 25 CRC) blood collected in 2 EDTA (plasma within 7hrs) and 2 PAXgene tubes (plasma after 7 days at RT).

Stability (PAXgene): pooled blood from self-declared normal individuals (un-spiked and 50pg/mL mDNA) stored at RT for 0, 5 & 8 days before processing.

All samples were processed as the Colvera Test described above.

RESULTS

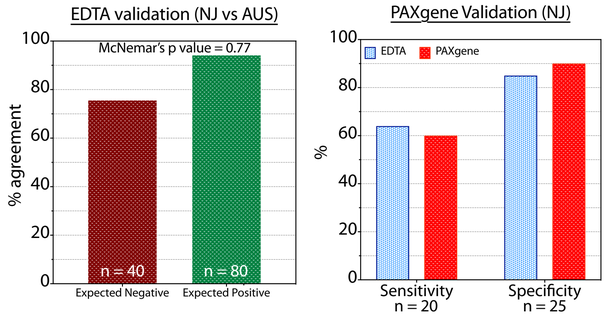
LoD: The LoD for Colvera in EDTA system is 17.1pg/mL (ln = 2.84) and in PAXgene system is 7.5pg/mL (ln=2.01).



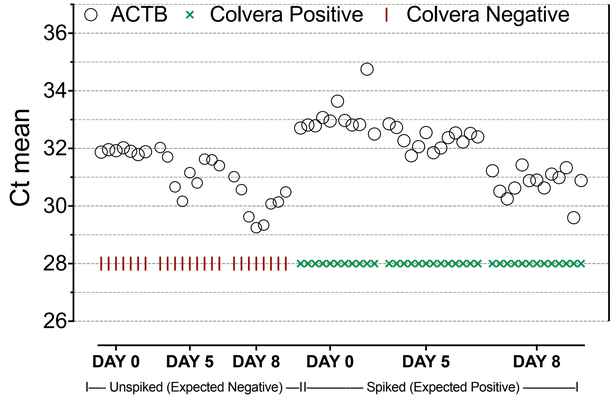
Precision: The test has excellent reproducibility for samples above its LoD.

	% agreement Colvera Result across 3 runs	
Spiked conc. (pg/mL)	EDTA Validation	PAXgene Validation
0	96.4	93.3
25	Not done	100
50	100	100
100	100	100
250	100	Not done

Accuracy : The test's accuracy is comparable between sites and between the two tube types.



Stability in PAXgene ccfDNA tubes: Amount of *ACTB* increased over time, likely due to WBC lysis, but that had no impact on Colvera results.



Interfering Substances:

Following did not interfere with the assay:

- Both validation studies: cholesterol (5 mg/mL), EDTA (20 mg/mL), genomic DNA (100 ng/mL), glucose (10 mg/mL), hemoglobin (1 mg/mL), RBC (0.4% v/v), triglycerides (12 mg/mL) & uric acid (0.24 mg/mL).
- PAXgene validation : albumin (40mg/mL), bilirubin (0.2 mg/mL).
- EDTA validation: EDTA (20mg/mL).

Following interfered with the assay:

- EDTA validation: albumin (40 mg/mL) & bilirubin (0.2 mg/mL); 90% concordance with control samples.
- PAXgene validation: When additive to blood ratio is higher than recommended, (1.5 : 10), it negatively affects Colvera results. Less than 9mL blood volume is NOT acceptable for testing.

Ratio	ACTB			BCAT1			IKZF1			# Positive replicates	Colvera Result
1.5 : 10	31.7	31.9	32.0	37.9	39.7	39.1	36.0	37.6	36.6	6 / 6	Positive
1.5 : 9	31.2	31.3	31.5	37.2	37.6	38.0	36.8	36.8	36.8	6 / 6	Positive
1.5 : 8	31.6	31.5	31.5	-	-	38.6	-	38.3	38.6	3 / 6	Positive
1.5 : 7	31.7	32.0	31.9	-	38.7	-	-	-	-	1 / 6	Positive
1.5 : 6	32.1	32.2	32.0	-	-	-	-	-	-	0 / 6	Negative
1.5 : 5	32.3	32.9	32.5	-	-	-	38.1	-	-	1 / 6	Positive
1.5 : 4	32.7	33.0	33.2	-	-	-	-	38.6	-	1 / 6	Positive

Colvera comparison with CEA: Colvera clinical sensitivity is twice that of CEA (61.5% vs 30.7%) and specificity is 85% & 100% respectively when tested in colonoscopy confirmed 20 normal and 26 CRC specimens.

Normals		CEA +	CEA -	CRC		CEA +	CEA -
Colvera	+	0	3	Colvera	+	7	9
	-	0	17		-	1	9

CONCLUSION

Colvera assay was successfully validated in a CLIA-licensed clinical lab (#31D2122075) as a LDT in both EDTA and PAXgene systems, thus allowing maximum flexibility for providers to get access to the test. The test detected two times the number of confirmed CRC cases as CEA. PAXgene system demonstrated similar sensitivity and specificity as the EDTA system with the added benefit of lower LoD and ease of specimen shipment.

REFERENCES

[1] A panel of genes methylated with high frequency in colorectal cancer. BMC 2014, 14(54)

[2] Validation of a circulating Tumor-Derived DNA blood test for detection of methylated *BCAT1* and *IKZF1* DNA. JALM 2017, 2(20: 165-175

[3] A cross-sectional study comparing a blood test for methylated *BCAT1* and *IKZF1* tumor-derived DNA with CEA for detection of recurrent colorectal cancer. Cancer Medicine 2016, 5(10): 2763-2772

[4] Evaluation of an assay for methylated *BCAT1* and *IKZF1* in plasma for detection of colorectal neoplasia. BMC Cancer 2015, 15: 654

[5] Effect of blood collection tubes on circulating tumor DNA (ctDNA) yield and specificity. AMP 2017 Poster # ST29