

A MODEL MINIATURIZED, AUTOMATED DIAGNOSTIC PLATFORM FOR COLORECTAL CANCER DIAGNOSTICS

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Abstract

Early detection of colorectal cancers (CRC) and neoplasias are critical for long-term patient survival. Here we present a study using stool DNA for CRC screening comparing the efficacy of our modified single base extension (SBE) diagnostic platform performed in a standard manual procedure with that of a miniaturized SBE methodology adapted for the Parallab 350 automated nanolitre liquid handler.

Keywords: molecular diagnostics, miniaturization, automation, population screening

1. Introduction

Numerous methodologies exist for early detection of colorectal cancer. While colonoscopy offers the highest detection sensitivity, many individuals refrain from getting screened due to the invasiveness of the procedure. Non-invasive tests such as PreGen-Plus™ and fecal occult blood test offer alternatives, each with distinct sensitivity and specificity performance characteristics, for anyone interested in CRC screening by methods other than colonoscopy. In PreGen-Plus™, we have developed a multi-target, stool DNA-based molecular diagnostic assay¹ that addresses sensitive, non-invasive CRC testing. Achieving the highest possible sensitivity and specificity of detection while reducing assay cost become important considerations when offering commercially viable technologies. The study described in this report adapts our standard manual SBE method to a miniaturized, automated, proof-of-concept platform utilizing the nanolitre, multi-tip, liquid handling capacity of the Parallab 350. We report 100% concordance in sensitivity and specificity for mutation detection by miniaturized, automated SBE compared to our standard, manual methodology.

2. Experimental

Stool DNA samples used in this study included 8 samples previously genotyped as mutation-positive and 20 genotypically negative (normal) samples by a manually-performed, modified SBE for the panel of cancer markers included in this test (Fig.1), as described in the Whitney et al manuscript. The 28 samples were tested in parallel by a) the manual modified SBE protocol as a process control, and b) the automated, miniaturized SBE protocol carried out on the Parallab 350 liquid handler (Parallabs, Worcester MA, USA). Figure 2 outlines the differences in PCR[®] and SBE reaction setup between the two methods. PCR and SBE reaction multiplexing was also incorporated in both control and Parallab protocols for this study.

Sequence-specific purified human stool DNA was amplified by PCR[®] for each of the loci queried in 10 µl reactions in the Parallab protocol (vs. 50 µl for the control protocol). Amplified targets incorporated one 5'-biotinylated PCR[®] primer for the target analytical strand and a non-biotinylated PCR[®] primer for the opposite strand. Targets were bound to 1 µm Dynal Dynabeads[®] MyOne™ streptavidin-coated paramagnetic particles (“SA-beads”; Dynal Biotech LLC., Brown Deer WI, USA) at 1.25 µg/µl per

final miniaturized SBE reaction concentration (vs. 2.8 μ m Dynal M280 Dynabeads at 0.5 ug/ul final reaction concentration). Double-stranded bead-bound targets were chemically denatured by 0.1N NaOH treatment, neutralized and washed to remove the non-biotinylated strand. Single-stranded bead-bound targets were then placed in the Parallax 350 for miniaturized 1 μ l SBE reaction setup (vs. our original 10 μ l SBE reactions). See Figure 3 for Parallax process outline. The Parallax method accounts for a 10-fold reduction in SBE reaction volume, as well as automation of setup, mixing, cycling and reaction cleanup steps. Following rapid, air-driven thermal cycling in the system capillary channels, reactions were treated with shrimp alkaline phosphatase (SAP; Promega, Madison WI, USA) at 0.04U per reaction (vs. 0.2U in the control process). Finally, reactions were transferred to a multiwell plate containing a mix of formamide and ROX dye for fragment analysis on the ABI 3100 CE instrument (Applied Biosystems, Foster City CA, USA).

3. Results and discussion

Figure 4 compares results for all 28 stool samples tested for both manual and automated methodologies, demonstrating 100% concordance between the two protocols. The miniaturized procedure performed comparably to our established assay, introducing no false positives or negatives that could be attributed to the process.

Having demonstrated identical performance to our standard manual SBE protocol, the concepts of automation and miniaturization promise significant cost savings in reagents as well as a projected increase in throughput and reproducibility as multi-block systems are incorporated. Analysis of the automated method in this study suggests a significant overall assay cost reduction when factoring in the additional cost associated with stool sample collection, sample preparation, sequence-specific DNA capture/enrichment and data analysis. We expect to validate and further optimize this system in the near future using a significantly larger sample set.

4. Conclusions

Our modified SBE assay for CRC mutation detection adapted for the Parallax 350 nanolitre liquid handler was shown to detect all cancers (100%) in this proof-of-concept study relative to our standard, non-automated methodology. The process changes result in significantly reduced analytical assay costs reflected by the 5-fold scale-down of our PCR[®] reactions and up to 10-fold reduction of SBE reagents per sample reaction. By reducing cost and improving throughput and reproducibility, it is our hope to make this diagnostic technology available to an even wider segment of the general population interested in non-invasive CRC screening.

References

[1] D. Whitney, J. Skoletsky, K. Boynton, L. Kann, R. Brand, S. Syngal, M. Lawson and A. Shuber, *Journal of Molecular Diagnostics*, 2004 Nov; 6(4): 386-95.

MUTATION INDEX	MUTATION TYPE	ONCOGENE	SITE	CHANGE
A1	point mutation	Kras	K12p1	G→A, C or T
A2	point mutation	Kras	K12p2	G→A, C or T
A3	point mutation	Kras	K13p3	G→A, C or T
B1	deletion	APC		2-5 bp deletion
B3	point mutation	APC	1306	G→A, C or T
B4	point mutation	APC	1312	G→T
C1	point mutation	APC	1367	C→T
C2	point mutation	APC	1378	C→T
C3	point mutation	APC	1379	G→T
D1	point mutation	APC	1450	C→T
D3	point mutation	APC	1465	G→A
E1	point mutation	p53	175p2	G→A or T
F1	point mutation	p53	245p1	G→A or T
F2	point mutation	p53	245p2	G→A or T
F3	point mutation	p53	248p1	C→T
F4	point mutation	p53	248p2	C→T
G1	point mutation	p53	273p1	C→T or A
G2	point mutation	p53	273p2	G→A
G3	point mutation	p53	282	C→T
J1	deletion	BAT26		4-15 bp deletion
P1	point mutation	APC	876	C→T
Q1	point mutation	APC	1554	C→A

Figure 1. Panel of CRC-related mutations included in our current modified single-base extension DNA assay. More than one type of base change may be detected at any one particular locus.

	Control SBE	Parallab SBE	Component Reduction
PCR® reaction volume	50 µl	10 µl	5-fold
SBE reaction volume	10 µl	1 µl	10-fold

Figure 2. Comparison of reagent requirements between original and automated SBE protocols.

PARALLAB SBE METHOD DESCRIPTION

1	SA-bead resuspension and aspiration into capillaries
2	SBE reaction mix aspiration into capillaries (1000 nl final volume)
3	30-cycle SBE (90 °C/2 sec., 50 °C/2 sec., 72 °C/2 sec)
4	SAP (shrimp alkaline phosphatase) aspiration, mixing with SBE reactions (1200 nl final volume)
5	SAP incubation (37 °C/1 min)
6	Dispensation of SAP-treated reactions to 96-well plate containing 10 ul formamide/ROX mix for CE analysis

*SA-magnetic beads attached to 5'-biotinylated, single-stranded, amplified templates;

PCR® amplification, bead-binding and template denaturation performed offline.

Figure 3. Outline of miniaturized SBE protocol adapted for automation on the Parallax 350 nanolitre liquid handler. Denatured, single-stranded amplified templates are attached to the SA-beads prior to manipulation on the Parallax 350. PCR® amplification, bead-binding and template denaturation were all performed offline.

STOOL DNA	PARALLAB	CONTROL
1	A3,Q1	A3,Q1
2	NEG	NEG
3	NEG	NEG
4	NEG	NEG
5	D1,P1	D1,P1
6	NEG	NEG
7	NEG	NEG
8	NEG	NEG
9	F4	F4
10	NEG	NEG
11	NEG	NEG
12	NEG	NEG
13	B3,F3	B3,F3
14	NEG	NEG
15	NEG	NEG
16	NEG	NEG
17	NEG	NEG
18	NEG	NEG
19	A3	A3
20	NEG	NEG
21	NEG	NEG
22	NEG	NEG
23	NEG	NEG
24	NEG	NEG
25	NEG	NEG
26	F4,P1	F4,P1
27	J1	J1
28	E1	E1

Figure 4. Complete genotyping results for set of 28 stool DNA samples (8 previously genotyped mutation-positive samples, 20 previously genotyped mutation-negative samples). “Parallab” and “Control” data sets refer to re-genotyping of new, thawed frozen stool aliquots of same source samples (1-28) using established, manual protocol (“Control”) and automated protocol (“Parallab”). Please refer to Figure 1 for mutation index.