The use of Internal Controls in Next Generation Sequencing to improve BRCA1 and BRCA2 gene copy loss detection

Thomas Morrison[3], Venus Chirip[1], Benjamin Yeung[2], Bradley Austermiller[3], Bryan Lo[1,2]

1. Department of Pathology and Laboratory Medicine, The Ottawa Hospital, Eastern Ontario Laboratory Association
2. Program for Cancer Therapeutics, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada
3. Accugenomics, Wilmington, North Carolina

Abstract

Introduction

The loss of BRCA1 and BRCA2 tumor suppressor gene copies is an important cancer driver, however, detecting genomic copy loss by NGS in cancer FFPE specimens is not robust. Accurate copy number variant (CNV) detection by NGS needs to account for sources of testing bias. NGS, whether by hybrid capture, PCR primer enrichment, or direct long read sequencing, produces sequencing reads disproportional to their starting levels. Further, because of the high complexity of NGS testing, small drifts in reagents and instruments reduce reliability of CNV methods that depend on external calibration samples. In this report we examine the ability of SNAQ™-SEQ Internal Standards (IS) to improve CNV detection when integrated into the ThermoFisher Oncomine Comprehensive Assay v3 (OCAv3).

Methods

Accukit™ Mammo DNA IS were designed to each exon of the ATM, BRCA1, BRCA2 and PALB2 genes. Each exonic amplicon had at least two unique IS SNP for their bioinformatic separation from sample reads. Purified DNA (20 ng) from nine FFPE tumor specimens with 40% to 90% tumor cellularity and six FFPE normal tissue specimens were spiked with 200 copies IS prior to running OCAv3. Variant Call Files (VCF) created using the IonReporter pipeline utilized a modified hotspot.bed file to ensure the IS variants were reported. An R markdown script extracted the IS variant counts from the VCF, calculated the exon abundance from NT (Native Template):IS read count ratio, and the abundance was then used to detect gene copy loss.

Results

When tested in the absence of IS, the OCAv3 copy analysis had insufficient statistics to give a PASSing CNV analysis results in half the genes (18 of 36 genes); four of which were indicated as having copy loss by SNAQ™-SEQ analysis. Further, the OCAv3 CNV detection method did not detect a sample with a LOH and a partial loss BRCA1. Lastly, two OCAv3 copy loss estimations were not supported by SNAQ™-SEQ abundance, suggesting OCAv3 method may have indicated a false positive.

Conclusions

SNAQ™-SEQ copy loss detection was based on estimating the exon abundance of ATM, BRCA1, BRCA2, PALB2 genes, converting abundance into copies per cell and then detecting genes with significantly lower copy number. The CNV detection comparison between OCAv3 with and without SNAQ™ indicated that analysis with SNAQ™ was more robust, giving a result for every gene in every sample. Further, there were copy losses detected by OCAv3 that were not supported by SNAQ™ analysis. The improved CNV detection with SNAQ™-SEQ CNV detection supports its integration into Next Generation Sequency for testing of FFPE specimens where gene copy loss is clinically actionable.

Internal Standards (IS) Design

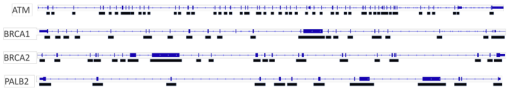

Figure 1. SNAQ™-SEQ Mammo DNA Internal Standards. The figure depicts the human genome mapping of exon locations (blue bars) and corresponding internal standards (black bars) for ATM, BRCA1, BRCA2 and PALB2 gene regions.

IS added to standard NGS workflow

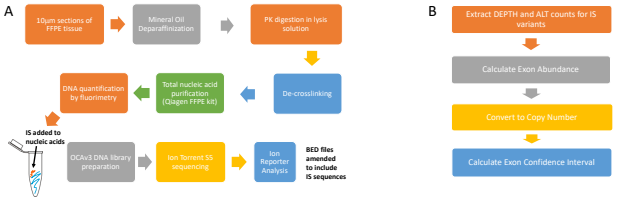

Figure 2. Schematic showing the standard NGS workflow for detecting genetic variants from FFPE tissue sections to bioinformatic analysis for copy number variation. Panel A - IS were added (spiked-in) following nucleic acid purification and quantification by fluorimetry and prior to Oncomine Comprehensive Assay v3 (OCAv3) DNA library prep. For each sample, 200 copies of IS were added to 20ng of DNA. Panel B - From the variant call files (VCF) generated by Ion Reporter Analysis, the DEPTH and ALT counts for IS variants were extracted and then used for abundance and copy number calculations.

FFPE normal tissue samples

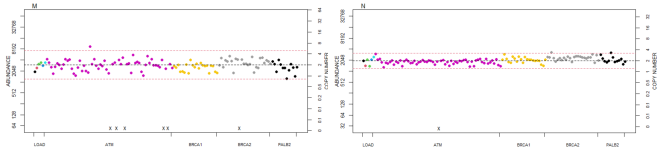

Figure 3. Two representative FFPE normal tissue sample cases (M and N) are shown. SNAQ™ based genomic abundance (left y-axis) or copies per cell (right y-axis) for each exon (circles) colored by gene name (x-axis). The x-axis region indicated by "LOAD" were GENE/EXON: CDK12/09, CHEK1/02, NBN/02,15 PIK3R1/02 RAD41/05 and will not be discussed in this poster. Each exon abundance calculation failing SNAQ™-SEQ QC were indicated by 'X' at bottom of each plot. The red lines indicate a modified Z-score 3-sigma cutoff used to remove outliers prior to establishing "normal" abundance. See Table1 for IonReporter's CNV detection results.

FFPE tumor samples with copy loss

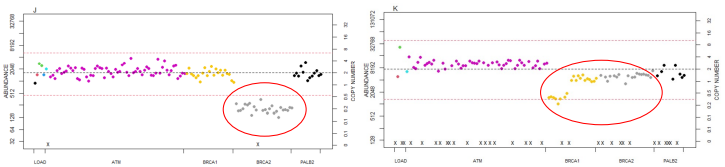

Figure 4. Two representative FFPE tumor sample cases (J and K) are shown. See Figure 3 for plot description. Red circles represent a Double BRCA2 knockout (sample J) or a LOH for BRCA1 & BRCA2 with a partial double knockout for BRCA1 (Sample K). See Table 1 for IonReporter's CNV detection results.

IS Improves Copy Loss Detection

SAMPLE	SNAQ™-SEQ	OCAv3
A	BRCA2	(ATM) BRCA1* BRCA2
B	BRCA1 BRCA2	(ATM BRCA1 BRCA2 PALB2)
H[2]	BRCA1[P] BRCA2 PALB2	(BRCA1) BRCA2 PALB2
I	BRCA2	(ATM BRCA1 BRCA2 PALB2)
J	BRCA2	ATM* (BRACA1) BRCA2
K[2]	BRCA1[P] BRCA2	ND
L	BRCA1 PALB2	(BRCA1) PALB2
M	none	(BRCA1 BRCA2 PALB2)
N	none	(BRCA1)
O[1]	BRCA1	(BRCA1 BRCA2)
P	none	ND

KEY
SAMPLE letters correspond to plots in Appendix A
none No copy observed copy loss
ND No Data
* SNAQ™-SEQ results do not support copy loss
GENE indicates loss of copy detected
(GENE) CNV analysis of gene did not pass QC.
[P] partial gene copy number loss
[1] Sample R had insufficient IS added, Sample O was a repeat
[2] Sample H was a repeat of sample K

Table 1. Summary of Copy Number Variants Detected by SNAQ-SEQ or OCAv3 pipeline.

IS Provides Abundance and Copy Number Quantification

SAMPLE	VAF	ABUND	ATM	BRCA1	BRCA2	PALB2
A	4.2%	5280	1.9-2.3 (40)	1.9-2.2 (14)	0.4-0.5 (24)	1.7-2.3 (10)
B	4.6%	4280	2.9-3.5 (49)	1.3-1.6 (21)	1.3-1.6 (28)	1.8-2.2 (12)
C	30.3%	467	1.8-1.9 (54)	2.0-2.3 (22)	2.0-2.4 (25)	1.8-2.1 (13)
D	6.7%	2790	1.9-2.1 (59)	1.9-2.0 (21)	2.1-2.3 (25)	1.7-2.0 (12)
E	7.1%	2630	1.9-2.0 (59)	1.9-2.1 (22)	2.1-2.4 (25)	1.8-2.1 (12)
F	1.3%	15900	1.9-2.3 (31)	1.9-2.4 (14)	2.0-2.5 (19)	1.5-2.1 (8)
G	10.2%	1800	2.0-2.1 (60)	1.8-2.0 (21)	2.0-2.2 (24)	1.7-2.0 (11)
H	2.4%	7610	2.5-2.8 (60)	0.7-1.0 (21)	1.1-1.3 (17)	1.5-1.7 (8)
I	16.2%	995	1.8-2.0 (58)	3.1-3.5 (21)	1.1-1.3 (25)	2.6-3.0 (10)
J	12.0%	1540	2.0-2.3 (58)	1.7-2.1 (22)	0.2-0.3 (24)	1.6-1.9 (11)
K	2.7%	7300	2.5-2.8 (44)	0.7-1.0 (20)	1.3-1.4 (17)	1.3-2.0 (8)
L	5.9%	3080	2.1-2.3 (46)	0.7-0.9 (21)	1.9-2.2 (24)	1.4-1.8 (13)
M	6.3%	3000	1.9-2.2 (55)	1.6-1.9 (22)	2.2-2.6 (25)	1.4-2.0 (13)
N	8.9%	1980	1.8-1.9 (60)	1.9-2.2 (21)	2.3-2.5 (25)	1.9-2.1 (11)
O	13.7%	1300	2.3-2.7 (59)	1.0-1.5 (16)	1.8-2.4 (24)	1.4-2.3 (12)
P	19.8%	810	1.8-2.2 (57)	1.9-2.6 (22)	1.7-2.0 (24)	2.0-2.7 (12)
Q	10.0%	1790	1.8-2.0 (60)	2.1-2.3 (18)	2.0-2.2 (25)	1.8-2.2 (12)
R	1.0%	20400		1.1-2.4 (4)	1.6-3.0 (4)	NA- NA (1)

Table 2. VAF indicates IS variant fraction, orange values suggest too little IS added to sample. ABUND indicates median NT gene copies. Gene data indicate 95% confidence interval for gene copies per cell with number in () indicating number of exons used in calculation. Red indicates possible copy loss.

Conclusions and Next Steps

- SNAQ™-SEQ Internal Standards (IS) were successfully added to the workflow for the DNA portion of OCAv3 (Oncomine Comprehensive Assay v3).
- After amending BED files, the Ion Reporter bioinformatics pipeline was able to map and analyze reads corresponding to SNAQ™-SEQ IS.
- SNAQ™-SEQ IS provided estimates of exon abundance for ATM, BRCA1, BRCA2, PALB2 and thus more robust copy loss detection and detection of exon level copy loss.

Next Steps:

- Complete an analysis of additional cases comparing NGS results with and without IS.
- Routine incorporation of IS into the workflow for BRCA1, BRCA2, ATM, PALB2 NGS variant and copy loss detection.

Acknowledgements Many thanks to Mathieu Lariviere and James Fraser, Ion Torrent Field Application Scientists, for technical support and assistance in amending BED files to include IS sequences and all the members of the Molecular Oncology Diagnostics Laboratory at The Ottawa Hospital, Eastern Ontario Laboratory Association, for their help in carrying out this project.