The optimization of copy number analysis of FFPE tumor DNA by array-CGH for implementation into a clinical diagnostic laboratory

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Introduction

Array comparative genomic hybridization (aCGH) has evolved as a valuable tool for the identification of genomic copy number alterations (gain/loss) in tumors. These studies have mostly used fresh frozen specimens for genomic DNA (gDNA) extraction. For retrospective studies and diagnostic purposes, often formalin-fixed paraffin-embedded (FFPE) tissue is the only available specimen or biopsy for study. Such specimens also permit identification of the tumor burden, which is an important issue considering the overall sensitivity of aCGH is 25-30%. However, gDNA extracted from FFPE specimens is of poorer quality and presents a challenge for aCGH using current protocols. The goal of this study was to optimize a procedure for the isolation and labeling of gDNA from FFPE specimens for aCGH and establish quality control parameters for implementation into a clinical diagnostic laboratory.

MatBA™

eARRAY-designed Agilent oligonucleotide 4 x 44K
80 regions ranging in size from 0.3-21.3 Mbp
35 kbp regional resolution (duplicate)
1 Mbp backbone (duplicate)
5 x 301 replicates

Region	Size (Mbp)	Region	Size (Mbp)	Region	Size (Mbp)
1p36.32-p36.23	7.9	6p21.31-p21.2	2	11q25	2
1p21	13	6p21.1	2	12p13.1	2.2
1p13.2-p13.1	6	6q12	0.3	12q13.1-q13.2	11.9
1q21	10.9	6q16	12.8	12q15	3.8
1q31	19.7	6q21	2.5	13q14	12.7
1q41-q44	8	6q22	16.5	13q31	16
2p25.3	1.7	6q23.3-q24	11.9	13q33-q34	13.6
2p16.1-p15	4.6	6q25	1	14q12	9
2p11.2-q11.2	2	7p22	7.2	14q32	15
2q13-2q14.1	0.6	7p21.3-p21.2	1.7	15q21.1	1.5
2q24	14.9	7q31	19.7	15q23-q24	10
3p22	9.9	8p23	12.7	16p13.3	6.3
3p14.1-p13	3.8	8p21.3	4.5	16p13.13	2
3q12.2-q12.3	1.2	8p12-p11.23	2	16p11.1-p11.2	10.6
3q21.2	0.3	8q21.2	0.3	16q24	5.2
3q22	8.9	8q24.21	4.2	17p13	11.2
3q26.1-q26.2	11.3	9p24.2-p24.1	2	17q22-q23.1	2
3q26.31	2	9p21	12.9	17q24.2-25.1	5.8
3q27	5.2	9q22	12	18p11	16.1
4p15	9.8	9q33.2-q34.1	10	18q21	18
4q11-q12	4	10p14	5.6	18q23	0.3
4q24	2.8	10p12.31-p12.2	2.5	19p13.3-p13.2	11
4q34.3-q35	11.6	10q23.2	1.9	19q13.33-q13.43	10
5p15	10	11p13	1.5	20q13	21.3
5q13.2-5q13.3	3	11q13	13.6	21q21	15.2
5q31.3	1	11q22.1-q22.2	1.4	22q12	15.9
6p25	7	11q22.3-q23	14		

Array sensitivity was found to be 40-50% using cell line dilution studies and FISH. MatBA™ has been implemented into the clinical diagnostic laboratory at CGI and is currently performed on chronic lymphocytic leukemia (CLL). The loss of 8p, 11q, 13q and 17p and gain of 2p, 3q, 8q and 12 are reported out by the laboratory. All detected aberrations are confirmed by quantitative PCR (QPCR) as an independent method.

Materials and Methods

aCGH for Fresh Frozen Tissue Specimens

Genomic DNA was isolated from a 25mm piece of fresh frozen tissue (test) using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The quality and quantity was measured by spectrophotometry and gel electrophoresis. An equimixture of male and female normal DNA (Promega) served as the reference DNA (MF). Test and reference (MF) DNAs (1ug) were digested with Rsa1 and Alu1 and differentially enzymatically-labeled with Cy5- and Cy3-dUTP respectively, essentially as described by the manufacturer (Agilent Technologies). Purified labeled DNAs were mixed and hybridized for 24hrs to MatBA™ essentially as described by the manufacturer (Agilent Technologies). After washing, the slides were scanned and Genomics Workbench Lite (Agilent Technologies) was used for aberration detection using the ADM2 algorithm (threshold 4, at least 8 contiguous probes with an average log ratio of ±0.4 after combining intra-array replicates).

aCGH for FFPE Specimens

In the case of the FFPE specimens, gDNA was isolated from either five 10 or 20 micron sections or 3 tumor-enriched core punches (1.5mm diameter x 2-3mm length) using the Qiagen DNeasy Blood and Tissue Kit test). The quality and quantity was measured by spectrophotometry. DNAs not meeting quality criteria were re-purified using Amicon Ultra-0.5, Ultracel-30 Membrane (Millipore, Billerica MA) and the quality checked prior to further procedures. The integrity of the DNA was assessed by agarose gel electrophoresis and gDNAs >0.8kbp in size were heat fragmented at 95^{0}C for the time required for the bulk of the DNA to range from 0.4kbp-0.8kbp in size. Reference (MF) DNA was also heat fragmented accordingly, and both test and reference DNAs (0.5ug/1ug) were then labeled either enzymatically using the CGH Labeling Kit for Oligo Arrays (Enzo Life Sciences) or non-enzymatically using the Genomic DNA ULS Labeling Kit (Agilent Technologies). Arrays were hybridized for 44hrs to MatBA™ essentially as described by the manufacturer (Agilent Technologies) and analyzed as described for fresh frozen tissue.

DNA Quality and Quantity Parameters

Fresh frozen: A_{260}/A_{280} = 1.6-2.0, A_{260}/A_{230} >2, yield >2ug, size >9kbp
The yield ranged from 14-54ug, all showed A_{260}/A_{280} and A_{260}/A_{230} within acceptable ranges, and the bulk >9kbp in size.
FFPE sections: A_{260}/A_{280} = 1.6-2.0, A_{260}/A_{230} >1.9, yield >2ug
The yield of ranged from 3-33ug. One specimen showed A_{260}/A_{230} <1.9 but after re-purification A_{260}/A_{230} was >2. The gDNA ranged from 0.5kbp-7kbp in size.
FFPE cores: A_{260}/A_{280} = 1.6-2.0, A_{260}/A_{230} >1.9, yield >2ug
The yield ranged from 1-20ug (3 cores per specimen). 102 specimens showed A_{260}/A_{230} <1.9 but after re-purification A_{260}/A_{230} was >1.9 except for 14 specimens (for these no further procedures were performed). The gDNA ranged from 0.3kbp-2kbp in size, with most of the DNAs not requiring heat fragmentation prior to labeling.

Non-Enzymatic VS. Enzymatic Labeling of FFPE Specimen DNAs

Six pairs of matched FFPE and fresh frozen tissue specimens (one follicular lymphoma [FL], one diffuse large B-cell lymphoma [DLBCL], four Hodgkin's lymphomas) were used to assess differences between enzymatic and non-enzymatic labeling of FFPE specimens.

Labeling Method	Frozen	FFPE-ULS	FFPE-ENZO
Signal Intensity (Cy5)	403-1064	84-227	417-1644
Avg Signal Intensity (Cy5)	647.5	135.5	1132.23
Signal Intensity (Cy3)	204-508	120-244	520-1043
Avg Signal Intensity (Cy3)	342.8	158	797.7
Signal to Noise (Cy5)	12-73	14-27	8-55
Avg Signal to Noise (Cy5)	33.84	20.7	31.49
Signal to Noise (Cy3)	7-27	14-30	11-61
Avg Signal to Noise (Cy3)	16.8	23.2	39.4
DLRS®	0.08-0.19	0.12-0.18	0.07-0.1
AvgDLRS®	0.13	0.15	0.08

The signal intensity of the non-enzymatically labeled FFPE gDNAs (FFPE-ULS) was overall lower compared with the gDNAs labeled using enzymatic labeling methods, but exhibited comparable signal to background noise. DNA from FFPE specimens labeled enzymatically (FFPE-ENZO) had lower DLRS values compared to DNAs labeled non-enzymatically (FFPE-ULS). Furthermore, FFPE-ENZO exhibited lower DLRS values than those obtained for DNA from frozen specimens (Frozen). Overall then enzymatic labeling was identified as the method of choice for FFPE specimens.

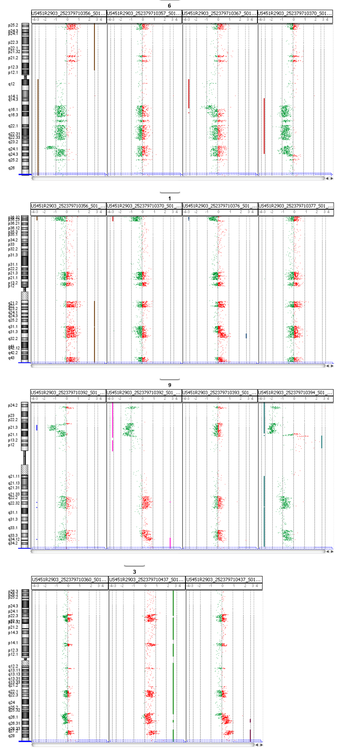
Aberration Detection in Fresh Frozen Tissue and Enzymatically Labeled FFPE Specimen DNAs

Aberrations ≥2Mbp in size, within regions represented on MatBA™ were assessed in all six pairs of matched fresh frozen and enzymatically labeled FFPE specimens.
For two of the four Hodgkin's lymphomas no aberrations were detected in fresh frozen tissue or the matched FFPE specimen at the same settings. This is not surprising given the 5-10% tumor burden for these specimens.
For one of the remaining two Hodgkin's lymphoma cases some aberrations were detected in the fresh frozen tissue specimen but not the matched FFPE specimens again, not surprising given the low tumor burden of the FFPE specimen (10%). For the last case, aberrations were detected in the FFPE specimen (70% tumor cell content) but not the fresh frozen tissue. Unfortunately, the tumor burden of the frozen specimens was not known.
For the FL specimen where the tumor burden was known to be 40-90% and the DLBCL specimen with 100% tumor burden, aberrations were detected in the FFPE specimens but not in the matched tissue when arrays were analyzed at the same settings. Again, the tumor burden was not known for the frozen specimens and a direct comparison of results could not be made

Clearly, assessment of tumor burden is an important component of aCGH in solid tissues for clinical implementation, given the sensitivity of aCGH.

Impact of Tumor Enrichment on Aberration Detection

Tumor burden is clearly a consideration for aCGH of solid tumors. One adaptation to improve aberration detection in FFPE specimens is to identify tumor rich regions in specimens (e.g. CD20+ areas in B-cell lymphomas) and extracting DNA from those regions (e.g. core punches). Core-punches of CD20+ regions of FL (86), DLBCL (146) and mantle cell lymphoma [MCL] (41) specimens were assessed by MatBA™ aCGH. Of these, 15 yielded unacceptable DLRS (>0.3) and were not analyzed further. The overall quality of hybridization was found to depend predominantly on the size of the original extracted DNA.



Loss of 6q in FL

Loss of 1p and gain of 1q in FL

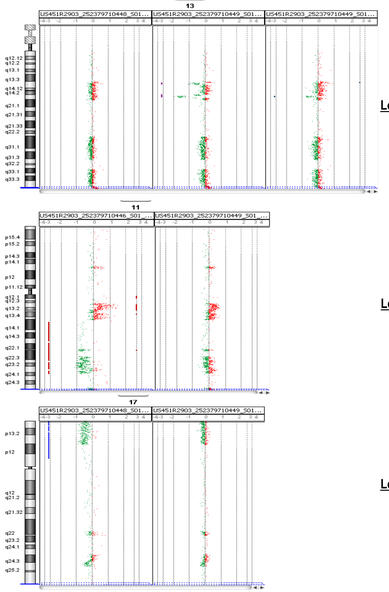
Loss of 9p in DLBCL

Gain of 3q in MCL

With tumor enrichment, the overall log ratio of aberrations was higher and many aberrations previously reported to occur in these tumors were frequently identified in the current study group.

Clinical Implementation

Clinical implementation of MatBA™ aCGH for small lymphocytic lymphoma (SLL), considered the same disease as CLL but presenting as a mass is near completion. Quality control criteria have been established for extracted DNA (see below) and for hybridization quality and aberration detection. Aberrations are confirmed by QPCR. FFPE sections of SLL with greater than 70% tumor burden are requested. Analytical sensitivity and specificity, reproducibility, and accuracy were assessed..



Loss of 13q14

Loss of 11q22

Loss of 17p13

Conclusions

- DNA extraction, fragmentation, labeling, and hybridization were optimized for FFPE specimens where enzymatic labeling performed optimally compared to non-enzymatic labeling.
- Quality control criteria were established for extracted DNA, size of fragmented DNA, labeling yield, and hybridization quality for aberration detection in FFPE specimens.
- Standard operating procedures for clinical implementation were developed for aCGH of FFPE specimens including confirmation of aberrations by QPCR.