Application Note

Genomics

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An Automated Bravo aCGH Workflow Using SPRI Bead Purification

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Abstract

Array comparative genomic hybridization (aCGH) is a technique enabling high-resolution, genome-wide screening of segmental genomic copy number variations (CNVs) and has been implemented in many clinical laboratories around the world. Agilent has developed an automated bead-based aCGH protocol on the Agilent Bravo NGS workstation (built on the Bravo automated liquid handling robot) which was adapted from an earlier Bravo protocol. This new method uses solid-phase reversible immobilization (SPRI) beads to replace the column-based method during the purification step. The automated bead-based protocol presented here demonstrates the possibility of an automation-friendly protocol to deliver reliable results for laboratories in need of an efficient aCGH workflow.

Introduction

aCGH is a molecular cytogenetic technique for the detection of chromosomal copy number changes on a genome-wide scale and at high-resolution relative to traditional cytogenetic techniques[1]. In aCGH, DNA from a reference (or control) sample and DNA from a test (or patient) sample are differentially labelled with two different fluorophores (cyanine 3 and cyanine 5, respectively). The labeled DNA samples are purified using purification columns (or plates) and subsequently used as probes that are co-hybridized to nucleic acid targets[2]. In most laboratories, the protocol is performed manually, resulting in a more laborious workflow and issues with scalability when faced with larger testing needs. Agilent currently has a column-based automated aCGH protocol compatible with the Bravo platform; however, this protocol is time-consuming and demands manual intervention at various steps. These factors limit its utility for high-throughput settings.

SPRI beads, introduced in 1998, are essential components in high-throughput nucleic acid cleanup and size selection protocols[3]. SPRI beads can be an appropriate alternative to replace the column-based plates used in the aCGH purification step. We took advantage of the fact that beads are routinely used for size-selection of amplicons in NGS library prep and could be adapted for similar DNA fragments.

In this application note, we developed an automated aCGH protocol based on the existing protocol for the Bravo platform using bead-based purification as an alternative to column-based purification. With minor changes to the existing column-based Bravo aCGH protocol and the addition of beads to the workflow, we believe the new bead-based protocol better addresses the needs of today's labs looking for a more seamless aCGH workflow.

Experimental Design

DNA samples were purchased from Coriell Institute (Camden, NJ, USA) and quality control was performed using the Agilent TapeStation 4200 system (p/n G2991AA), with the Agilent Genomic DNA ScreenTape assay (p/n 5067-5365). Sample information is described in Table 1. All of the gDNA isolated from samples were high-quality, showing DNA integrity numbers (DINs) greater than 9. This automated bead-based protocol was based on the earlier column-based Bravo aCGH automated protocol (G4410-90040 version 2.3) and uses 750 ng of total gDNA input per sample.

Samples were sex-mismatched to the same input mass of Agilent Human Reference Male and Female DNA (p/n 5191-3796 and 5191-3797, respectively).

Table 1. Sample information.

Sample ID	Gender	Sample Type	Chr	Cytoband	Class	Type	Start	End	Number of Probes	Length (bp)	Notes
NA13434	Male	Cell line	chrX	q22.2	CGH	DEL	103786713	103787815	3	1103	Pathogenic - small detection
NA04520	Female	Cell line	chr16	p13.3	CGH	LOSS	1984455	2060016	21	75562	Pathogenic - small loss
NA17885	Female	Cell line	chr17	p13.2	CGH	LOSS	3609260	3655099	19	45840	Pathogenic - small loss
NA11496	Female	Cell line	chr2	q32.3 - q33.1	SNP	LOH	195696408	198384404	73	2687997	Small cnLOH >2.5Mb
			chr5	q23.3 - q31.1	SNP	LOH	130057698	132669355	54	2611658	Small cnLOH >2.5Mb
			chr7	p22.3 - p11.2	SNP	LOH	80500	57333988	2104	57253489	UPD chr7
			chr7	q11.21	SNP	LOH	62554452	65939105	55	3384654	UPD chr7
			chr7	q11.22 - q36.3	SNP	LOH	67740561	158779582	2686	91039022	UPD chr7
			chr10	q22.1 - q22.3	SNP	LOH	71834742	76122423	83	4287682	Small cnLOH >2.5Mb

We used a custom labeling kit configuration of the SureTag Labeling kit (p/n 5190-4240) that allows 3 batches of 8 samples each, for a total of 24 samples (48 total labeling reactions) to label the samples and DNA references with cyanine 5 and cyanine 3, respectively. For purifying labeled DNA, we used AMPure XP SPRI beads (Beckman Coulter, Cat # A63880/1/2) in a standard protocol that has been broadly used in all SureSelect automation protocols. This protocol uses variable amounts of starting material and bead ratios, followed by 2 washes with 70% ethanol, drying of the purified sample bound to the bead at 37 °C, and elution in variable volumes of nuclease-free water. To verify the optimal sample-to-bead ratio, different proportions were tested. The optimal ratio of bead volume to sample volume was determined using the Agilent D1000 ScreenTape assay (p/n 5067-5582).

Ratios were tested from 0.5:1 to 1.8:1 (bead volume:sample volume). Sample was eluted with 20 µL of water, the same final volume from the standard column-based protocol (G4410-90010).

Sample and reference pairs were hybridized for 24 hours to the Agilent SurePrint G3 CGH+SNP 4x180K array (p/n G4890A - AMADID 029830) or for 40 hours to the Agilent GenetiSure Postnatal Research CGH+SNP 400K array (p/n G5974A - AMADID 078737). Arrays were then washed and scanned with the default settings for CGH G3 arrays using the Scanner Control software (version 9.1.11.13). Agilent Feature Extraction software (version 12.1.0.3) was used to extract data from raw microarray image files. The Agilent CytoGenomics software (version 5.1.2) was used to analyze aberrations and quality metrics. Samples were processed in duplicates and the experiments were performed in two independent runs.

Updates to the Current Bravo aCGH Protocol

The original automated Bravo aCGH protocol uses AutoScreen A 96-well plates (Cytiva Life Sciences, Cat # 25900598) that contain Sephadex G-50-based material in each well for the purification step. However, this approach is relatively expensive, requires additional equipment (e.g., an adaptor for plate centrifugation), involves many user interventions during the purification process, and frequently leads to inconsistent recoveries of purified DNA. Moreover, this protocol has a single, continuous step for digestion and labeling (including a two-hour incubation step) which does not allow the user to use the equipment during this time. Table 2 outlines the key changes between the existing Bravo aCGH protocol with columns and the new SPRI bead-based protocol.

Table 2. Changes from the original column-based aCGH protocol to the alternative proposed protocol using beads.

Steps	Current Column-Based Protocol (Version 2.3)	Proposed Bead-Based Protocol
Workflow	Universal Linkage System and enzymatic protocols	Enzymatic only
Workflow	CGH and CGH+SNP	CGH+SNP only
Workflow	Digestion and labeling in a single step of the protocol	Digestion and labeling in different steps
Labeling	Requires a 384-well plate for master mix	Requires an Agilent DeepWell 96-well plate (Agilent p/n 203426-100) for master mix
Purification	Sephadex column (plate-based)	AMPure XP beads
Elution	Low-Tris EDTA Buffer (pH 8.0)	Ultrapure molecular-biology-grade water
Hybridization Plasticware	Requires tall chimney plates (ABgene p/n AB-1184) for 2x arrays and a deep-well plate (ABgene p/n AB-0859) for 1x arrays	Requires a 96-well sample plate (Agilent p/n 5042-8502), ABI PCR plate, and an Agilent DeepWell 96-well plate (Agilent p/n 203426-100)

Results and Discussion

To see if there were major differences in the fragment profile between the standard column-based method and the SPRI bead-based method, we compared different bead-to-sample ratios to the profile observed for the standard column purification protocol using the D1000 assay from the TapeStation system. We tested bead-to-sample ratios of 0.5, 1, 1.5, and 1.8:1, and observed that a ratio of 1.8:1 had the profile (Figure 1) that was closest to the column-based purification. This indicated the 1.8:1 ratio was the most suitable option for purification step for the bead-based automated CGH protocol.

Our findings were consistent with the expected fragment profiles according to the purification protocol from the bead manufacturer. While we observed decreased retention of <100 bp fragments versus the column-based method, such fragments are not expected to contribute meaningfully to hybridization of target sequences. Moreover, the bead-based protocol shows superior recovery of >200 bp fragments, fragments that are within the targeted size range of restriction digestion.[4]

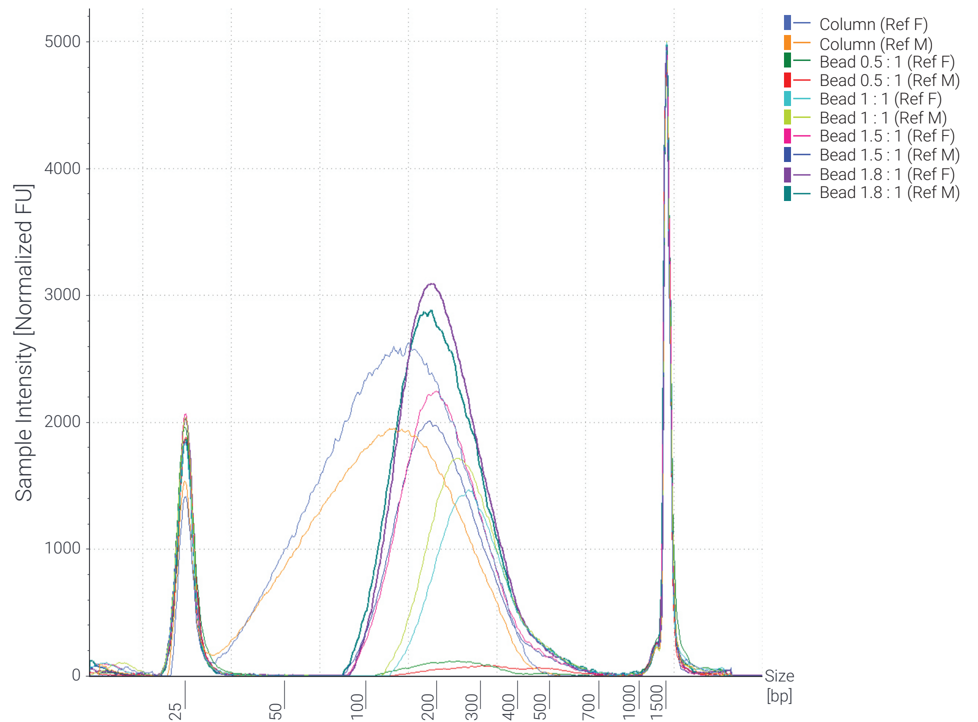

Figure 1. Comparison between bead purification and column purification. Electropherogram traces of digested and labeled DNA using the D1000 ScreenTape assay. Ref F, reference female, Ref M, reference male.

Automated Processing of the DNA Samples

Four Coriell samples were processed in duplicate with two different array formats: the SurePrint G3 CGH+SNP 4x180K array (p/n G4890A) and the Agilent GenetiSure Postnatal Research CGH+SNP 400K array (p/n G5974A). Two automation batches of 32 reactions (16 samples and 16 references) were done, in a total of 32 assays (32 samples against 32 references).

Due to the pipetting volume limits for the bead-based protocol, there were changes in the volume of the final reactions after each step when compared to the original column-based method. The final reaction volumes after each step are shown in Table 3. In order to have enough dead volume for the automated pipetting of all 8 rows of a 96-well plate, an extra dead volume equivalent to 8 samples was used for each aliquoting step. The final volumes of each reaction master mix, as well as the final volume to be added to the master mix plate, is available in the appendix of the new proposed protocol. The volumes of bead, sample, and final elution of purified samples are shown in Table 4. The final elution volumes reflect the expected volumes for the hybridization step in the original column-based protocol.

Table 3. Final volumes, in µL, of reaction mixes after each step.

Steps	4x180K array	2x400K array
Sample Input	22 µL (20 µL used)	22 µL (20 µL used)
After Enzymatic Digestion	26 µL	26 µL
After Labeling	50 µL	50 µL

Table 4. Volumes used for the bead purification step (µL). All formats used the 1.8:1 bead to-sample ratio.

	4x180K array	2x400K array
Reaction After Labeling	50 µL	50 µL
Bead Added	90 µL	90 µL
Final Elution Volume	22 µL	22 µL

In the original column-based aCGH automation protocol, samples are placed in columns 1 to 6 and the references are placed in columns 7 to 12 (Figure 2). Although this layout is compatible with the aliquoting and mixing steps of the enzymatic digestion and labeling processes, it causes incompatibilities of the position of the pipetting head for the bead purification step. If running less than 48 samples per run, an additional step was added to transfer the samples to a new 96-well sample plate (Agilent p/n 5042-8502). In this step, both samples and references are placed in the leftmost columns of the plate. This setup allows more efficient pipetting of samples for mixing purified references and samples in the next step.

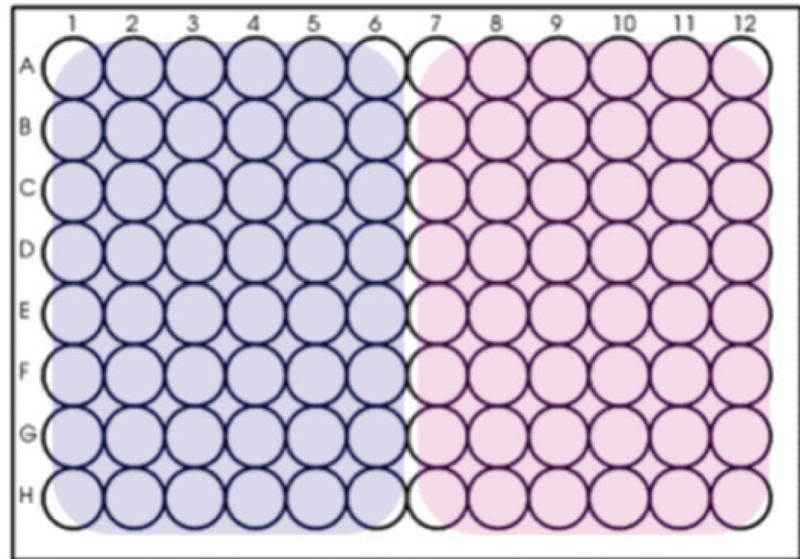

Figure 2. Layout of samples and references in an 96-well sample plate for automation. Columns 1 to 6 (purple) represent test gDNA samples, while columns 7 to 12 (pink) are reference gDNA samples.

Quality Control Metrics of Hybridized Samples

We achieved good or excellent QC metrics for hybridization in 94.2% of QC metrics. The main metric considered when evaluating a 'good' performance for the CGH assay is the derivative log ratio standard deviation (DLRSD). This metric reflects the measurement of probe-to-probe noise along the chromosomes in log ratio space and is critical for aberration calling. The DLRSD values were measured at 0.18 and 0.22 for the 4x180 and 2x400 formats, respectively (Figure 3), a value of 0.3 is acceptable, and a value below 0.2 is considered optimal.

Another important array QC metric, the signal-to-noise ratio, was also analyzed for both the green and red channels due to the potential impacts of the change in purification method (Figure 4). Values above 30 are considered "*Good*" and values above 100 are considered "*Excellent*" by the standard QC metrics settings of the Feature Extraction Software. All samples had signal-to-noise values above the minimum threshold of 30, indicating no significant impact on signal intensity due to the change in purification method.

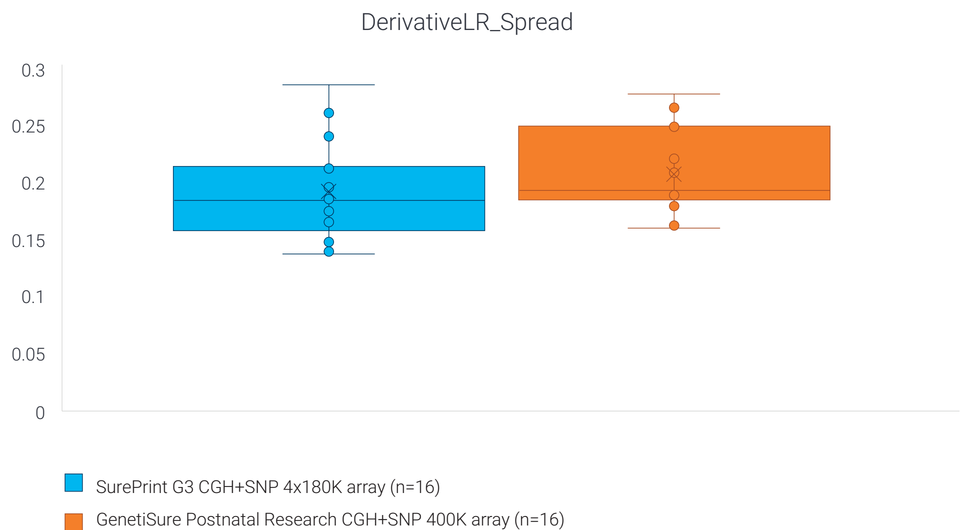

Figure 3. DLRSD values for arrays.

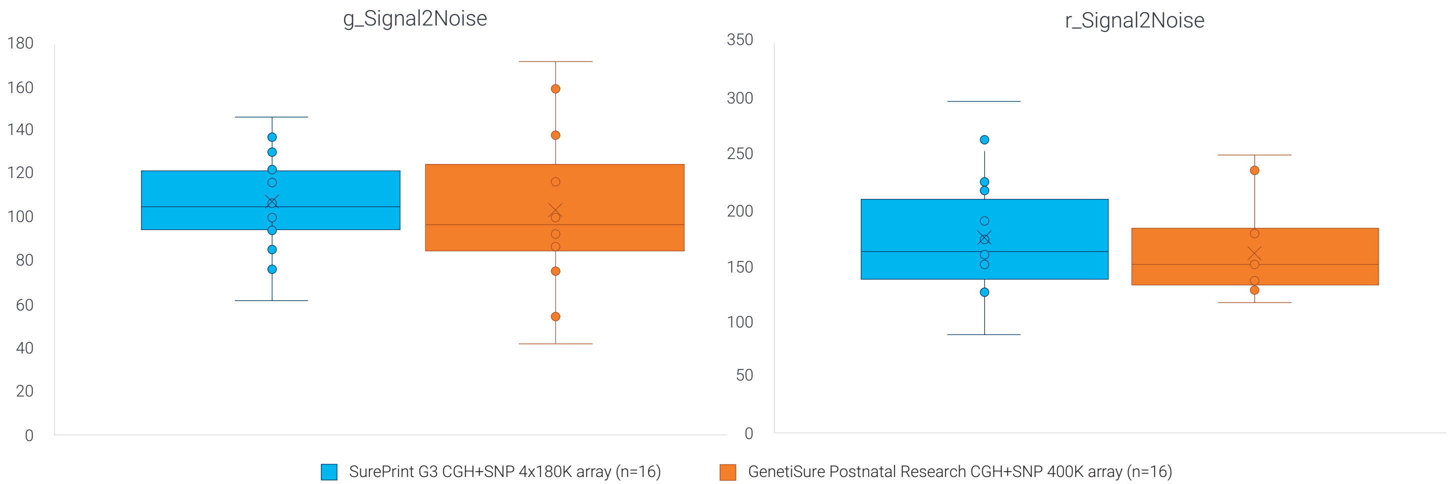

Figure 4. Signal-to-noise ratios for Cy3 (green) channel and Cy5 (red) channel.

Aberration Detection and Reproducibility Between the Two Different Workflows

Following detection of the expected aberrations in each of the four Coriell samples (Table 5 and Figure 5), we next verified the reproducibility of quality metrics and aberration calls between the column- and bead-based protocols. All 4 samples generated a 100% call concordance to known aberrations for each cell line with no false negative calls, resulting in a 100% sensitivity/ positive predictive value[5]. We did not test samples known to be negative for aberrations, hence negative predictive value and specificity are not reported.

Table 5. Known aberrations for samples tested.

Sample ID	Coriell Aberration	Current Study Aberration
NA13434	arr(1-22)x2,(XY)x1	Present
NA04520	arr 2q13(110212011-110519397) x3	Present
NA17885	17p13.2(3540391_3562018)x1	Present
NA11496	arr(7)x2 hmz	Present

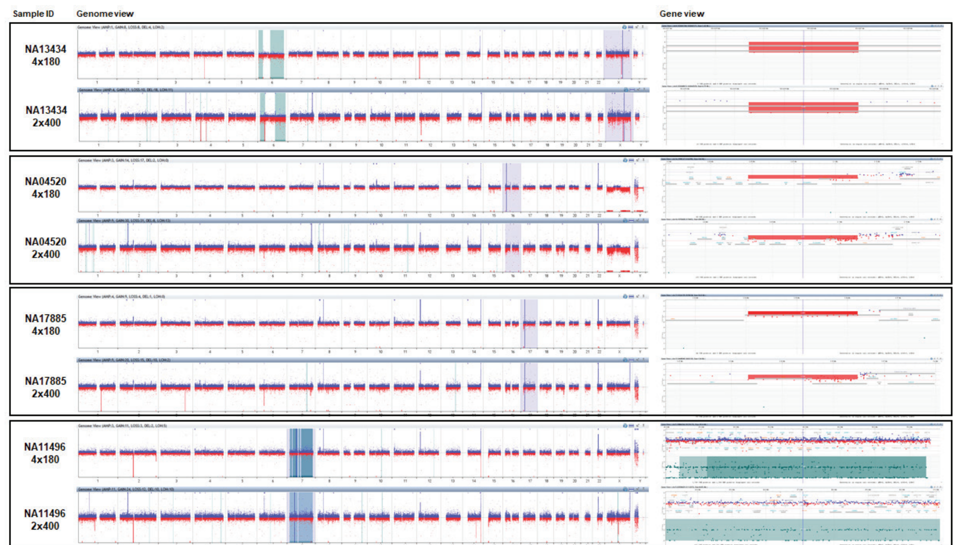

Figure 5. Genome and gene views contain the pathogenic gain or loss calls for the four Coriell samples tested on 4x180K and 2x400K arrays.

Repeatability of Aberration Calls Among Replicates Using SPRI Beads in an Automated Protocol

We also evaluated the repeatability of these results using the automated bead-based workflow. Thirty-two additional reactions were performed to verify the repeatability of our results and QC metrics within 2 sample preparation runs, using either CGH+SNP 180K (16 reactions, in duplicate) or CGH+SNP 400K (16 reactions, in duplicate). Considering only pathogenic aberrations (Table 5), we were able to detect 100% of known aberrations in the Coriell cell lines tested for a repeatability score of 16. Finally, all samples demonstrated '*Good*' to '*Excellent*' QC metrics.

Conclusion

We have demonstrated an automated, bead-based purification method that is a viable alternative to the current column purification protocol. This protocol enables a more automation-friendly workflow on the Bravo NGS platform. Furthermore, we provided an updated version of this bead-based protocol that enables use of the instrument for both NGS applications and aCGH automation. The plasticware in the protocol is the same as what is commonly used for NGS protocols. Additionally, this new protocol allows better utilization of instrument time, allowing it to be used for other tasks (e.g. NGS library preparation) during thermocycler incubation steps, thereby increasing productivity.

Taken together, the automated bead-based protocol extends the utility of the Bravo NGS platform to other critical applications such as CGH and drives gains in operational efficiency for clinical laboratories engaged in high-throughput genetic testing.

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