

# An Oligonucleotide Based Array-CGH System for Detection of Genome Wide Copy Number Changes Including Subtelomeric Regions for Genetic Evaluation of Mental Retardation

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Developmental delay (DD) and mental retardation (MR) are important child health issues with a one percent prevalence. Karyotyping with or without subtelomeric FISH (fluorescent in situ hybridization), unless the phenotype of the patient suggests a specific aberration for a specific FISH assay, is the most common procedure in cytogenetic evaluation of MR/DD. In addition, there are several platforms utilizing microarray based comparative genomic hybridization technology (array-CGH) for genetic testing. Array-CGH can detect deletions or duplications in very small segments of chromosomes and the use of this technology is expected to increase the diagnostic yield. The major limitation of the current BAC based array technologies is the low resolution (~1 Mb) of the chip and suboptimal coverage particularly in the subtelomeric regions. Our aim was to design a novel array-CGH chip with high-density of probes in the subtelomeric regions as well as to maintain sufficient density in other regions of the genome to provide comprehensive coverage for DD/MR. For this purpose, we used Human Genome CGH Microarray 44B chip (Agilent) as the template

for the novel design. Using e-array 4.0 (Agilent), one third of the probes were randomly removed from the array and replaced by 14,000 subtelomeric probes. The average density of the probe coverage is 125 kb and 250–400 probes interrogate subtelomeric regions. To evaluate the array, we tested 15 samples (including subtelomeric aberrations and other microdeletion syndromes), which were previously analyzed by karyotyping and/or FISH. The concordance rate between array results and previous results is 100%. In addition we detected two novel aberrations that were not detected by karyotyping. These results demonstrate the utility of this format of array-CGH in detecting genome wide submicroscopic copy number changes as well as providing comprehensive coverage of all subtelomeric regions.

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## INTRODUCTION

Clinical genetic evaluation and laboratory genetic testing play an important part in identification of the etiology of mental retardation and developmental delay (MR/DD). The main problem with current genetic tests for MR/DD is their low diagnostic yield. The test for aberrations in subtelomeres by FISH probes is recognized as standard practice in molecular cytogenetics evaluation of MR/DD following negative results of chromosome analysis unless the phenotype suggests known syndrome [Bejjani et al., 2005].

Array based comparative genome hybridization (array-CGH) is a novel powerful technique to detect

the chromosomal aberrations that can not be detected by traditional genetic techniques. In the last three years, several studies have established validity of this technique for use in MR/DD genetic testing [Shaw-Smith et al., 2004; Bradinova et al., 2005; de Vries et al., 2005; Schoumans et al., 2005; Miyake et al., 2006]. The variation in diagnostic yield

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reported in various studies (8.9–24%) is presumably due to differences in the ascertainment of the phenotype [Moog, 2005] and array-CGH technique. Although it is not currently officially endorsed by AAP for evaluation of MR/DD [Moeschler and Shevell, 2006], array-CGH is becoming a mainstream diagnostic tool for the evaluation of MR/DD. As of July 2006, eight academic and commercial labs (based on www.genetests.org) offer array-CGH as a diagnostic genetic test.

There are many considerations in designing an array based CGH assay. In an ideal situation, the array should cover the entire genome, should have a high resolution and should be cost-effective. Most of the published studies (and diagnostic applications) on MR/DD were performed on arrays, which are manufactured by spotting of bacterial artificial chromosomes (BAC) on glass slides. There is only one recent study on MR/DD by oligonucleotide arrays [Friedman et al., 2006]. In theory, during genetic testing, it is possible to use full genome tiling BAC arrays developed many groups [Ishkanian et al., 2004; de Vries et al., 2005]. One group in University of Nijmegen in Netherlands carried out a study using a BAC array with ~33,000 probes for genetic testing of MR/DD [de Vries et al., 2005]. In practice, tiling BAC arrays are currently expensive for clinical testing; 3,000 BAC arrays are a practical limit for most laboratories. For this reason, two alternative approaches are used to study MR/DD using array-CGH. In the first approach, BAC clone arrays encompassing the whole genome (usually 1 Mb resolution) are used, but have a very limited resolution and coverage in subtelomeric regions [Shaw-Smith et al., 2004; Schoumans et al., 2005; Miyake et al., 2006]. The second approach developed BAC arrays having very good coverage and resolution in subtelomeric regions, but no other areas in the genome [Veltman et al., 2002; Harada et al., 2004; Kok et al., 2005].

Ideally, an array-CGH based approach for genetic analysis of MR/DD should detect all known genetic aberrations, and should also provide sufficient probe density for discovery of novel genetic aberrations. It should also reliably test for the presence of subtelomeric aberrations, without sacrificing coverage of the other regions of the genome. For these reasons, we developed an oligonucleotide array spanning the entire human genome having an average resolution of 5 kb in subtelomeres and 125 kb in the remaining genome by modifying the commercially available Agilent 44 K array to enrich the array for subtelomeric probes. In order to validate our design we analyzed samples from 15 patients with various known genetic aberrations, which were determined in diagnostic lab settings and found total concordance between the array-CGH results and previously detected cytogenetic aberrations.

## MATERIALS AND METHODS

### Microarray

Agilent's Human Genome CGH Microarray Kit 44B is the template for our design. The array-CGH platform in this study is a customized, high definition microarray was designed using 44,000 probes comprised of 60 mer oligonucleotides designed by Agilent and printed using the Agilent Sureprint technology. We designed the array with E-array software 4.0 (Agilent, Palo Alto, CA), every third probe was removed and replaced by probes recognizing subtelomeric region (1 Mb proximal to telomeres) for 41 subtelomeric regions. That average resolution of the array is 5 kb in subtelomeres (see Table I for number of probes in each 41 region) and 125 kb in the remaining human genome software.

### Specimens and DNA Isolation

The test samples were comprised of genomic DNA extracted from cell lines (Coriell Institute, Camden, NJ), discarded whole blood samples or cell pellets (fixed 3:1 methanol:acetic acid). The MagnaCompact system (Roche Diagnostics Indianapolis, IN) was used to isolate DNA from whole blood and cell lines according to the manufacturer's instructions. Male or female DNA (Promega, Madison, WI) was used as reference to DNA obtained from whole blood and cell lines. The DNA from fixed cells was extracted by the Puregene DNA isolation kit (Gentra Systems, Minneapolis MN). Twelve independent male DNAs, all appear normal after karyotyping, were extracted from fixed cell pellets by the Puregene DNA isolation kit and combined to serve as a male reference for

TABLE I. Number of the Probes in Specific Subtelomeric Regions\*

Locus	# of probes	Locus	# of probes
1p	197	11q	361
1q	327	12p	332
2p	230	12q	302
2q	228	13q	289
3p	356	14q	326
3q	330	15q	312
4p	316	16p	360
4q	301	16q	300
5p	328	17p	309
5q	242	17q	312
6p	355	18p	316
6q	273	18q	351
7p	289	19p	297
7q	323	19q	279
8p	366	20p	363
8q	305	20q	362
9p	323	21q	317
9q	339	22q	383
10p	340	Xq	227
10q	284	Yq	7
11p	281		

\*Subtelomeric regions are defined as the region 1 Mb proximal to telomere.

female DNA extracted from fixed cells. A female control was created with the same protocol. All DNAs were quantitated with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

### Microarray Experiments

The test and reference DNAs are digested with Alu I and Rsa I (Promega), and purified with the QIAprep Spin Miniprep kit (Qiagen, Germantown, MD). Test DNA (3 µg) and reference DNA (3 µg) were labeled with either Cy3-dUTP or Cy5-dUTP (Perkin Elmer, Boston, MA) using the Bioprime Array CGH Genomic Labeling kit (Invitrogen, Carlsbad, CA). Following the labeling reaction the individually labeled test and reference samples are combined and concentrated using Microcon YM-30 filters (Millipore, Billerica, MA). The hybridization mixture contained the labeled DNAs, 2× Hybridization buffer (Agilent), 10× blocking agent (Agilent), and Human Cot-1 DNA (Invitrogen). The microarrays were hybridized in an Agilent SureHyb chamber in a rotisserie oven for 40 hr at 65°C. Four washing steps were done: room temperature with Agilent's Oligo CGH Wash buffer 1 for 5 min, a 37°C wash with Agilent Oligo CGH Wash buffer 2 for 1 min, an acetonitrile rinse at room temperature for 1 min and a 30 sec wash in Agilent's Stabilization and Drying Solution at room temperature. All slides were scanned on an Axon 4000B scanner using the Genepix 4.0 software. Data was obtained by Agilent Feature extraction software 8.0, and then imported into Agilent CGH analytics 3.2.1 software for analysis.

### Data Analysis

During analysis with CGH analytics software, the statistical algorithm was ADM-1, sensitivity threshold was 6.0 and the moving average window was 1 Mb. In order to determine that there was a copy number change in a particular locus, three criteria must have been met. These were positive call by the software, presence of 10 consecutive probes pointing out the same direction, and 1.5-fold average fold difference in the test DNA compared to the reference normal DNA.

### RESULTS

In this study 15 specimens with known aberrations were analyzed. Very diverse specimens (blood, methanol fixed cell lymphocytes, and cell lines) and genetic aberrations (subtelomeric deletions, subtelomeric duplications, small deletions, small duplications, large deletions, and large duplications) were used in CGH analysis. Eleven samples included aberrations in subtelomeric regions. Five of the samples were known syndromic phenotypes (Miller–Dieker syndrome, Charcot-Marie-Tooth

disease, Velo-cardio-facial/DiGeorge syndrome, Wolf–Hirschhorn syndrome, Down syndrome). Detected aberration changes varied from 0.6 to 154 Mb. Examples of genetic aberrations are shown in Figure 1 and detailed results of array CGH experiments are shown in Table II.

Concordance of the microarray and prior karyotyping/FISH results were 100%. Two additional novel aberrations not found in cytogenetic analysis were detected. A 3.5 Mb deletion in 14q11 was found in sample 1 (Table II, Fig. 1B) and a 3 Mb deletion in 17q24.3-17q25.1 in sample 5 (Table II).

### DISCUSSION

The main challenge in genetic testing for MR/DD is to provide a method which will yield a complete analysis of quantitative genomic associated changes associated with the disorders. In some cases an obvious phenotype and/or family history suggest a specific syndrome. Currently a variety of tests including karyotyping, subtelomeric FISH and BAC clone CGH are used and often all three.

Probe length and density are the primary determinants of the resolution of an array. Since the size of the BAC clones spotted on BAC arrays is 150 kb on average, this delimits their maximum sensitivity. Most of the BAC arrays in mental retardation research use contain 3,000 probes [Shaw-Smith et al., 2004; Schoumans et al., 2005; Miyake et al., 2006] and have a sensitivity of 1 Mb. Other commercial BAC arrays use ~460 probes (Spectral Genomics Constitutional Array 2.0) (<http://www.spectralgenomics.com>) and ~1890 probes (Signature Genomics Version 4.0).

Array CGH studies on MR/DD have been carried out mostly with BAC arrays—except one recent study [Friedman et al., 2006]—so far, but BAC arrays are not the only array CGH platform. Commercial high-density oligonucleotide platforms from Nimblegen [Selzer et al., 2005], Illumina [Peiffer et al., 2006], Affymetrix [Zhou et al., 2004] and Agilent [Barrett et al., 2004] are available. In this study we used a high density oligonucleotide platform from Agilent despite the fact alternative oligonucleotide platforms provide denser coverage. Microarrays on Agilent platform can be customized and at 44 K resolution level standard dual channel microarray scanners can be used. Customized arrays, such as this, are useful for providing increased power to detect rearrangements in regions of the genome which are known to be disease-associated. To be compatible with a standard scanner is also an advantage.

Any array-CGH system to be used in the molecular diagnosis of the MR/DD should detect subtelomeric aberrations, since subtelomeric FISH studies indicate genetic aberrations including subtelomeric regions are common (2.5–6.2%) in MR/DD [Davies et al., 2003; Jalal et al., 2003; Bocian et al., 2004; Yu et al., 2005]. Therefore we designed a unique modification

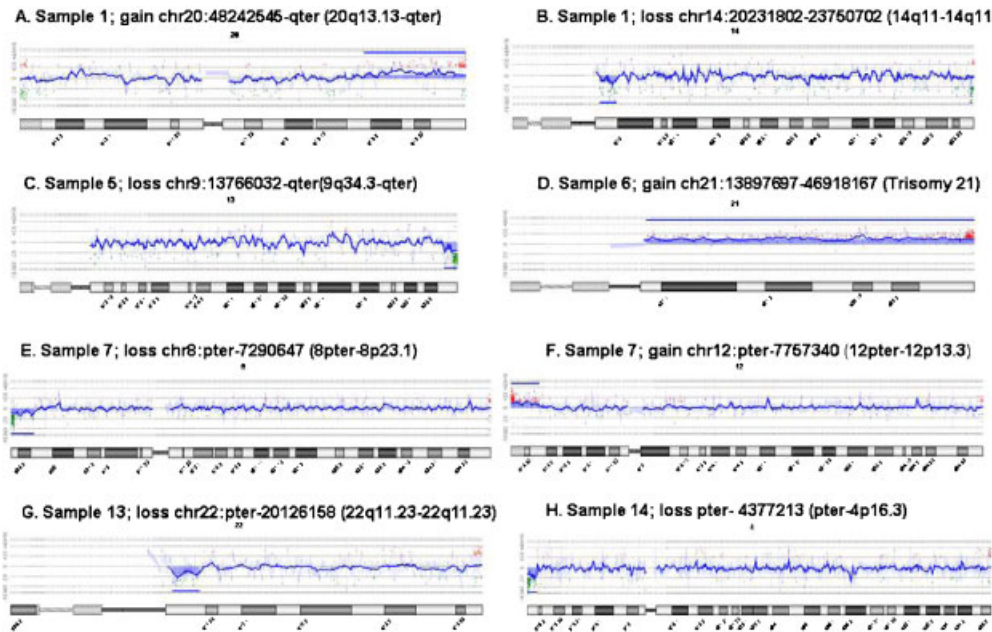


FIG. 1. Detection of copy number changes by array-CGH arrays. The weighted moving average (1 Mb) of ratios of probes was plotted as a function of chromosomal position using CGH Analytics 3.2.1 software. Copy number changes were determined by ADM-1 algorithm and indicated with light blue shaded areas. The blue line which on the top or the bottom of the graph demonstrated whether the aberration is a loss or gain and show the extent of the aberrations. Individual probes showing at least 1.5-fold change was plotted on the graph and shown in red (gain) or green colors (loss). Eight aberrations were shown in the figure. **A:** Duplication of 20q13.13-qter (Sample 1). **B:** Deletion of 14q11 (Sample 1). **C:** Deletion of 9q34.3-qter (Sample 5). **D:** Trisomy 21 (Sample 6). **E:** Deletion of 8pter-8p23.1 (Sample 7). **F:** Duplication of pter-12p13.3 (Sample 7). **G:** Deletion of 22q11.23 (Sample 13). **H:** Deletion of pter-4p16.3 (Sample 14).

of the Agilent 44 K chip to provide more complete coverage including subtelomeric regions.

Subtelomeric regions are defined as the terminal 500 kb of each eukaryotic chromosome arm [Riethman et al., 2005]. A commercial subtelomere FISH probe assay (Vysis TelVysion, Des Plaines, IL) screens for the rearrangement of 41 subtelomeres using BAC probes with a locus within 300 kb of the end of the chromosome. Taking all this information into account, we decided to probe for terminal 1 Mb of the chromosome of the known 41 subtelomeric regions. Our custom array provides 200–400 probes per subtelomeric region (Table I). The average subtelomeric and genome wide probe density of this custom array is 5 kb/probe and 125 kb/probe respectively.

We also tested and identified genetic aberrations for Miller–Dieker syndrome (14 probes), Charcot–Marie–Tooth disease (12 probes), Velo–cardio–facial/DiGeorge syndrome (52 probes), and Wolf–Hirschhorn syndrome (352 probes). The genomic loci for other important microdeletion or duplication syndromes associated with MR/DD were interrogated for the number of probes per locus based on the number of probes which are 1 Mb upstream and downstream of the target gene responsible from the phenotype. The syndromes and the number of probes per locus are as follows. Angelman/Prader–Willi:13; Beckwith–Widemann:15; Cri-du-Chat:32; Kallman:21; Rubin-

stein–Taybi:29; Smith–Magenis:20; Williams–Beuren:16. In addition to these syndromes, other important microdeletions such as 1p36, 3q29, 17q21.3 deletions were recognized by 212,113 and 385 probes, respectively. Based on our observation that 12 probes were sufficient for detection of all of genetic aberrations tested directly, the probe coverage for the aforementioned syndromic deletions or duplications should be sufficient.

During the course of this study, we also identified two novel copy number changes which were previously undetected by karyotyping/FISH. They are 3.5 Mb deletion on 14q11 and 3 Mb deletion on 17q24.3–17q25.1. Since normal structural variations (i.e., copy number polymorphisms) are quite common in human genome [Sebat et al., 2004], we checked the “Database of Genomic Variations” in The Centre for Applied Genomics, Toronto, Canada (<http://www.tcag.ca>) to see whether these deletions were reported previously. We found out that deletion in the 14q11 region (loss chr14:20,231,802–23,750,702) overlaps reported polymorphisms such as gain chr14:20,922,608–20,941,880 [Iafate et al., 2004], loss chr14:21,581,346–22,040,241 [Sebat et al., 2004], gain chr14:23,475,038–23,545,053 [Tuzun et al., 2005] and chr14:23,539,508–23,574,552 [Tuzun et al., 2005]. The deletion in 17q24.3–17q25.1 (loss ch17:65,681,113–68,545,988) overlaps with gain chr17:65,539,248–65,725,860 [Iafate et al., 2004] and gain chr17:68,361,

TABLE II. Genetic Aberrations in the Analyzed Samples

Sample	Sample type	Array-CGH results <sup>a</sup>	Probes	Interval	Size	Cytogenetic analysis
1	Fixed pellet	Loss chr13:110744785-qter Loss chr14:20231802-23750702 Gain chr20:4824245-qter Gain chr17:14003913-15432414 Gain chr10:47074824-87815522 Loss chr20:pter-625517 Loss chr9:13766032-qter Loss chr17:65681113-68545988 Gain chr21:13897697-46918167 Loss chr8:pter-7290647	329 84 555 12 545 237 268 16 703 410	13q34-qter 14q11-14q11 20q13.13-qter 17p12-17p12 10q11.22-10q23.2 20pter-20p13 9q34.3-9qter 17q24.3-17q25.1 21pter-21qter 8pter-8p23.1	3.5 Mb 3.5 Mb 14 Mb 1.5 Mb 30.5 Mb 0.6 Mb 1 Mb 3 Mb 33 Mb 7.3 Mb	46, XX, der(13)t(1;3;20)(q34q13.3 (VIJyRM2002-;20QTELL14+) 46, XY, dup(17)(p11.2) 46, XY, dup(10)(q11q23) 46, XX, ish del(20)(p13p13)(D20S1157-) 46, XX, ish del(9)(q34.3)(D9S325-) 47, XX,+21 46, XY, der(8)t(8;12)(p23.3p13.3).ish der(8)t(8;12)(p23.3p13.3)(D8S504;STS SAVH27+) 46, XX, del(2)(q37.1).ish del(2)(q37.3q37.3)(D28447-) 46, XY, ish der(2)t(2;19)(p25.3;q13.4) (VIJyRM2052-;D19S238E+) 46, XX, dup (8)(p11.2p23).ish dup(8)(WCP8+) 47, XXX 46, XY, ish del (17)(p13.3p13.3)(LISI-) 46, XX, del (22)(q11.21;q11.23).ish del(22)(q11.2q11.2)(TUPLE-) 46, XX, ish del(4)(p16.3p16.3)(WHSCR-) 46, XX, del(11)(p11.2p13)
2	Cell line	Gain chr12:pter-7757340 Loss chr2:233966794-qter Loss chr2:pter-764887 Gain chr19:57774256-qter Loss chr8:pter-7290647 Gain chr8: 12869106-42816942 Gain chrx:pter-qter Loss chr17:1613876-3141877 Loss chr22:pter-20126158 Loss chr4: pter-4377213 Loss chr11: 31755085-46363416	439 322 126 487 410 292 1542 14 52 362 130	12pter-12p13.3 2q37.1-2qter 2pter-2p25.2 19q13.41-19qter 8pter-8p23.1 8p22-8p12.21 Xpter-Xqter 17p13-17p13 22q11.23-22q11.23 4pter-4p16.3 11p11.2-1p13	7.7 Mb 9 Mb 0.7 Mb 6 Mb 0.7 Mb 30 Mb 154 Mb 1.5 Mb 20 Mb 4.3 Mb 14.5 Mb	
3	Cell line					
4	Fixed pellet					
5	Fixed pellet					
6	Blood					
7	Fixed pellet					
8	Blood					
9	Blood					
10	Blood					
11	Blood					
12	Blood					
13	Blood					
14	Blood					
15	Blood					

<sup>a</sup>Coordinates are based on Human May 2004 (hg17) assembly provided by University of California in Santa Cruz (UCSC).

430-68,390,648) [Tuzun et al., 2005]. Although deletions overlap some structural polymorphisms (most of them are small segmental duplications), the size of the deletion and presence in affected individuals strongly suggest that they might be associated with MR/DD. We do not have access to parental DNA specimens to ascertain for certain nature of these findings. Nevertheless the ability to detect such copy number changes also reflects the power of the custom designed array.

Our results demonstrate that our custom designed array CGH platform can detect deletions, duplications, unbalanced translocations (both in subtelomeric regions and elsewhere in the genome) and trisomies associated with DD/MR. We also expect that this system will be a cost-effective method due to development of multi-pack array arrays by Agilent Technologies, which allows testing of four specimens on one slide.

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