HUMAN MUTATION 27(4), 376–386, 2006

METHODS

Determination of Genomic Copy Number With Quantitative Microsphere Hybridization

Heather L. Newkirk,[1] Peter K. Rogan,[2] Mauricio Miralles,[1] and Joan H.M. Knoll[1]*

[1]*Laboratory of Genomic Disorders, Children's Mercy Hospitals and Clinics, University of Missouri-Kansas City, Kansas City, Missouri;* [2]*Laboratory of Human Molecular Genetics, Children's Mercy Hospitals and Clinics, University of Missouri-Kansas City, Kansas City, Missouri*

Communicated by Jing Cheng

We developed a novel quantitative microsphere suspension hybridization (QMH) assay for determination of genomic copy number by flow cytometry. Single copy (sc) products ranging in length from 62 to 2,304 nucleotides [Rogan et al., 2001; Knoll and Rogan, 2004] from *ABL1* (chromosome 9q34), *TEKT3* (17p12), *PMP22* (17p12), and *HOXB1* (17q21) were conjugated to spectrally distinct polystyrene microspheres. These conjugated probes were used in multiplex hybridization to detect homologous target sequences in biotinylated genomic DNA extracted from fixed cell pellets obtained for cytogenetic studies. Hybridized targets were bound to phycoerythrin-labeled streptavidin; then the spectral emissions of both target and conjugated microsphere were codetected by flow cytometry. Prior amplification of locus-specific target DNA was not required because sc probes provide adequate specificity and sensitivity for accurate copy number determination. Copy number differences were distinguishable by comparing the mean fluorescence intensities (MFI) of test probes with a biallelic reference probe in genomic DNA of patient samples and abnormal cell lines. Concerted 5′ *ABL1* deletions in patient samples with a chromosome 9;22 translocation and chronic myelogenous leukemia were confirmed by comparison of the mean fluorescence intensities of *ABL1* test probes with a *HOXB1* reference probe. The relative intensities of the *ABL1* probes were reduced to 0.59 ± 0.02 fold in three different deletion patients and increased 1.42 ± 0.01 fold in three trisomic 9 cell lines. *TEKT3* and *PMP22* probes detected proportionate copy number increases in five patients with Charcot-Marie-Tooth Type 1a disease and chromosome 17p12 duplications. Thus, the assay is capable of distinguishing one allele and three alleles from a biallelic reference sequence, regardless of chromosomal context. Hum Mutat 27(4), 376–386, 2006.
© 2006 Wiley-Liss, Inc.

KEY WORDS: copy number; *TEKT3*; *PMP22*; *ABL1*; mutation detection; microsphere; flow cytometry

INTRODUCTION

Flow cytometry has become an indispensable platform for the simultaneous multiparametric analysis of fluorescent markers within or attached to either cells or synthetic particles, such as microspheres. In a dual laser cytometer, one laser excites specific mixtures of internal fluorochromes in a set of microspheres, each having a distinct spectral signature. A second laser excites a secondary fluorochrome at a different wavelength (for example, streptavidin-conjugated phycoerythrin; SPE) bound to a biological moiety on the microsphere surface. Both emissions are simultaneously detected with a photomultiplier tube (PMT).

In this study, spectrally-encoded fluorescent microspheres coupled to synthetic DNA sequences were hybridized to homologous targets in labeled genomic DNA, which were then reacted with a fluorescently labeled antibody or SPE. Individual, codetected microsphere signatures and SPE intensities were counted in a single reaction, acquired in real-time, and digitally processed by a flow cytometer. These SPE signals can be expressed as the geometric mean fluorescence intensity (MFI), which has been shown to be an accurate measure of the approximately log-normal distribution of fluorescence intensities produced by flow cytometry [Coder et al., 1994; Kirkwood, 1979]. The instrumentation software also determines the standard deviation, coefficient of variation, and median and peak channel intensities (highest peak within the signal quantification curve) (Becton Dickinson, San Jose, CA; www.bdbiosciences.com).

Hybridization of amplified products to short oligonucleotide probes conjugated to fluorescent microspheres has facilitated high throughput genotyping by flow cytometry [Vignali, 2000]. However, amplification of genomic target DNA is a prerequisite to hybridization with conjugated probes in order to score discrete genotypes [Hadd et al., 2004; Rockenbauer et al., 2005]. Because amplification is inherently logarithmic and thus difficult to control, hybridization of these target sequences to conjugated

The Supplementary Material referred to in this article can be accessed at http://www.interscience.wiley.com/jpages/1059-7794/suppmat.

Received 11 August 2005; Revised 19 December 2005.

*Correspondence to: Joan H.M. Knoll, Ph.D., Children's Mercy Hospital, 2401 Gillham Rd., Kansas City, MO 64108.
E-mail: jknoll@cmh.edu

Grant sponsor: Katherine B. Richardson Foundation; Grant number: 4186; Grant sponsor: National Institutes of Health; Grant number: 1R21CA95167-02.

Presented at the 55th Annual Meeting of the American Society of Human Genetics, October, 2005.

DOI 10.1002/humu.20312
Published online 15 March 2006 in Wiley InterScience (www.interscience.wiley.com).

© 2006 WILEY-LISS, INC.

WILEY InterScience® DISCOVER SOMETHING GREAT

oligonucleotide probes does not reproducibly and accurately quantify genomic copy number [Earley et al., 2002; Sekar et al., 2005; Vignali, 2000]. Oligonucleotide probes may, in some cases, also lack adequate sensitivity and specificity for reliable determination of genomic target levels (see Results). By contrast, longer, single-copy DNA probes hybridized to unamplified, directly-labeled genomic targets can be used to unequivocally determine genomic copy number [Southern, 1975; White et al., 2004].

We examined the possibility that direct hybridization of patient-derived DNA to longer single-copy probes conjugated to microspheres could produce signals adequate for copy number determination by comparing the MFI values of test and reference probes in the same sample (Fig. 1). Previously, we validated computationally-derived single copy (sc) probes to detect a wide variety of chromosomal abnormalities by fluorescence in situ hybridization (FISH) for many different chromosomal regions [Knoll and Rogan, 2003; Rogan et al., 2001]. In this study, we developed quantitative microsphere suspension hybridization (QMH) assays with sc probes from three different chromosomal regions, two of which were associated with aneusomic conditions, and demonstrate the use of this method to determine copy number gains or losses in archival, methanol and acetic acid fixed cytogenetic specimens.

MATERIALS AND METHODS

Selection, Coupling, and Hybridization of Microsphere-Conjugated Probes

Probe selection. Sc probes specific for three genomic regions were developed [Rogan et al., 2001; Knoll and Rogan, 2003, 2004] and used to distinguish genomic copy number differences in patient samples and cell lines. They include chromosome 9q34 probes (16-1a, 16-1b, 16-1c, 16-1d, 16-2a, and 16-2b) from within intron 1b of *ABL1* (MIM# 189980) that are deleted in a subset of chronic myelogenous leukemia (CML; MIM# 608232) patients, chromosome 17p12 probes recognizing *TEKT3* and *PMP22* (MIM# 601097) from within the Charcot-Marie-Tooth syndrome Type 1A (CMT1A; MIM#118220) duplicated region [Inoue et al., 2001], and chromosome 17q21 probes from *HOXB1* (HOXB1a, HOXB1b, HOXB1c, HOXB1d, and HOXB1e; MIM# 142968) (presumed to be present in two copies per diploid genome, since intragenic deletions at this locus are likely to result in clinical abnormalities [Studer et al., 1996]). These probes were selected based on their single copy sequence composition, their GC content (40–55%), the lack of potential stable secondary structures predicted by MFold software (www.bioinfo.rpi.edu/applications/mfold/old/dna/), and their length (62–2,304 nucleotides). Probes with either suboptimal GC content (<40%) or stable secondary structures were found to hybridize inefficiently and were generally avoided (for example, probe 16-1d, which probably contains a stable hairpin loop close to its 3′ end; Supplementary Table S1 [available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat]). Within the predominantly single-copy *HOXB1* gene region, we detected a higher density of potential sc intervals, more consistent GC composition among different probes, and fewer predicted stable secondary structures that might interfere with hybridization. Although longer sc sequences for the *ABL1* and *CMT1A* regions were more limited in number, shorter probes could be designed and produced from within longer sc intervals that were originally used for scFISH [Knoll and

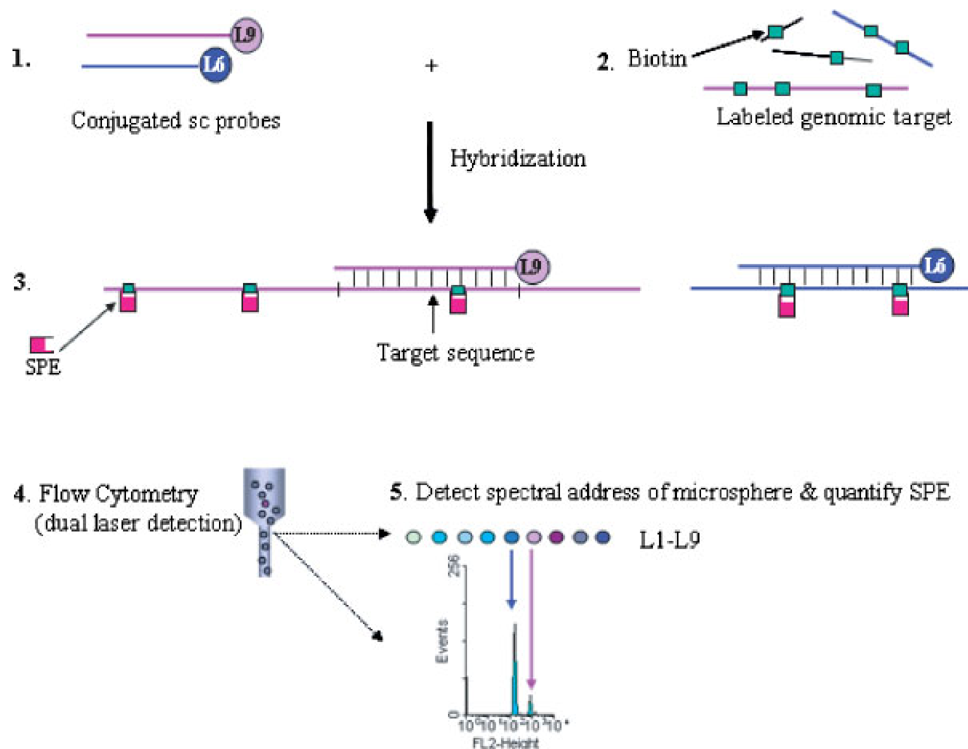

FIGURE 1. **Schematic of sc probe-coupled microsphere hybridization assay. 1) Each sc probe is synthesized by PCR using an amino-modified forward primer and conjugated to spectrally distinct carboxylated microspheres. 2) Sample genomic target DNA is extracted from fixed cytogenetic cell pellets and nick-translated to incorporate biotin dUTP. 3) Sc probe-coupled microspheres are hybridized to the labeled genomic DNA, stained with streptavidin-phycoerythrin (SPE), and then washed to remove unbound SPE. 4) Hybridized, probe-conjugated microspheres are analyzed by flow cytometry. 5) Dual laser detection of distinct microsphere spectral addresses and quantification of homologous genomic targets annealed to each microsphere-coupled probe.**

Rogan, 2003] (Table 1). Potential SNPs within each probe region were collated from dbSNP (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = snp); however, SNP genotypes of the samples used in the present study were not determined because the paucity of SNPs and their low population frequencies at the selected loci. Characteristics of all probes used in this study are summarized in Table 1.

Single copy (sc) probe synthesis. Sc probes were synthesized by PCR using Pfx (Invitrogen, Carlsbad, CA; www.invitrogen.com) with normal human genomic templates (Promega, Madison, WI; www.promega.com) as previously described [Rogan et al. 2001], except that only one oligonucleotide of each primer pair was chemically modified. A single 22- to 24-nucleotide primer of each sc probe-specific pair was synthesized with a 5′amino-modifier C-12 for coupling to microspheres (Integrated DNA Technologies, Coralville, IA; www.idtdna.com). PCR products were separated by electrophoresis in low EEO agarose (Seakem; FMC Bioproducts, Rockland, ME; www.cambrex.com), extracted by microspin column centrifugation (Qiagen, Valencia, CA; www.qiagen.com), and product concentration was determined by spectrophotometry.

Coupling of probes to microspheres. Fluorescent microspheres, from two different manufacturers were used, each with distinct spectral addresses. Cyto-Plex Microspheres, designated as levels L1–L9, were obtained from Duke Scientific (Palo Alto, CA; www.dukescientific.com) and microspheres designated as levels R1–R9, were obtained from Molecular Probes (Eugene, OR; www.probes.invitrogen.com). The surface of the 4-μm microspheres is coated with approximately 1.6×10^8 carboxyl sites (Molecular Probes). Aside from their unique spectral signatures, the chemical properties of the microspheres produced by each manufacturer were indistinguishable in terms of their efficiencies of conjugation with different amino-modified sc probes and of hybridization. Coupling of purified sc probes to microspheres was carried out via a modified carbodiimide reaction [Dunbar et al., 2003; Fulton et al., 1997]. Each probe was initially heat denatured and then snap-cooled on ice. Approximately 3.125×10^5 microspheres with identical spectral characteristics were pipetted into a 1.5-mL microcentrifuge tube, centrifuged for 2 minutes at 10,000 *g*, and drained of supernatant. 150 μL of 0.1 M MES buffer [2-(*N*-morpholino) ethanesulfonic acid] pH4.5 was added to each tube and the microspheres were vortexed briefly followed by centrifugation for 2 minutes at 10,000 *g*. Supernatant was removed and the microspheres were resuspended by vortexing in 80 μL of 0.1 M MES. 50 nmol of one sc probe was added to each tube and mixed by vortexing, which corresponds to a 600-fold excess of probe to potential binding sites. A 1.25 μL volume of fresh 10 mg/mL solution of 1-ethyl-3-3-dimethylaminopropyl carbodiimidehydrochloride (EDC) was added and the reaction was vortexed briefly and incubated in the dark for 30 minutes with occasional mixing. Mixing and incubation of EDC was repeated twice, using 1.25 μL of freshly prepared EDC solution each time. The reaction was stopped by addition of 500 μL 0.02% Tween20 followed by vortexing and centrifugation for 2 minutes at 10,000 *g*. Following removal of the supernatant, 250 μL of 0.1% SDS was added to each tube, vortexed, and then centrifuged at 10,000 *g* for 2 minutes. The supernatant was carefully removed, 25 μL of 0.1 M MES pH4.5 was added, and the tube was vortexed and stored in the dark at 4°C. Coupled microsphere concentrations were quantitated by adding 1 μL of each microsphere suspension to 100 μL of 1 × PBS and analyzing on the FACSCalibur flow cytometer (Becton Dickinson) using the conditions given below. We estimate that an average of 4.9×10^9 molecules (or 8.1 fmol) of probe were coupled to each microsphere (based on a 98% coupling efficiency), as determined below (see Results, first paragraph).

Genomic DNA Template Preparation

Genomic template was prepared from methanol and acetic acid fixed cell pellets of either patient samples remaining after clinical cytogenetic characterization or from cell lines obtained from the NIGMS Coriell Cell Repository (Camden, New Jersey; http://locus.umdnj.edu/ccr). The cells were washed twice with 1 × PBS and their concentrations were determined with a hemocytometer. Genomic DNA template was then extracted from ~600 cells per sample. This DNA was replicated in vitro using the GenomiPhi kit (Qiagen), a procedure that faithfully maintains the copy number of sequences present in the original genomic template [Little et al., 2005]. Genomic DNA was then nick-translated (1 μg) with biotin-16 dUTP for 60 minutes at 15°C to obtain labeled products of 300 bp to 1 kb in length [Knoll and Lichter, 1994]. Fifty nanograms of nick-translated patient sample was analyzed in each hybridization assay.

The samples used to validate the method were known from previous cytogenetic and/or FISH studies to exhibit differences in copy number within chromosome 9q34 and chromosome 17p12. The cytogenetic findings for these samples are listed in Table 2 and

TABLE 1. Characterstics of Hybridization Probes

Gene	Probe	Length (bp)	Hybridization Temperature (°C)	Coordinates (Hg17)	SNPs[a]	% Heterozygosity[b]
HOXB	HoxB1a[c]	2286	51	Chr17:43965662–43967948	6	0.0030
	HoxB1b[c]	1343	50	Chr17:43962053–43963396	5	0.0037
	HoxB1d[c]	485	50	Chr17:43966759–43967243	0	0.0000
	HoxB1c	102	50	Chr17:43964237–43964330	0	0.0000
	HoxB1e[c]	62	50	Chr17:43963520–43963581	0	0.0000
ABL1	16-1a[c]	2304	48	Chr9:130623551–130625854	7	0.0030
	16-1b	100	45	Chr9:130624671–130624771	0	0.0000
	16-1c[c]	62	50	Chr9:130625551–130625608	0	0.0000
	16-1d[c]	500	50	Chr9:130621702–130622202	1	0.0020
	16-2a[c]	1381	48	Chr9:130627353–130628735	4	0.0029
	16-2b	101	50	Chr9:130627353–130627454	0	0.0000
TEKT3	TEKT3	98	50	Chr17:151491108–15149206	0	0.0000
PMP22	PMP22	101	50	Chr17:15073475–15073576	0	0.0000

[a]SNP reported to be present within corresponding genomic sequence (collated from dbSNP).
[b]Genotyped SNP from dbSNP with the maximum heterozygosity determined in the probe interval.
[c]QMH results using the indicated probes can be found in Supplementary Table 1.

TABLE 2. Flow Cytometric Detection of Chromosome Abnormalities

Sample	Cytogenetic findings	Test probe	Mean intensities			Genotype
			Test probe	Reference probe[a]	Ratio[b]	
47[c]	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	48.72	50.94	0.96	wt
38	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	39.22	40.22	0.98	wt
195	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	1240.47	1179.37	1.05	wt
195[d]	*ibid*	16-1b	1121.98	1132.11	0.99	wt
CMT1A-1	nuc ish 17p12(PMP22x3)	16-1b	41.04	44.74	0.92	wt
177	t(9;22)(q34;q11.2).ish der(9)(ABL1)	16-1b	182.41	173.86	1.05	wt
177[d]	*ibid*	16-1b	162.15	161.31	1.01	wt
86	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	107.40	104.20	1.03	wt
86[d]	*ibid*	16-1b	124.63	114.80	1.09	wt
124	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	721.63	678.48	1.06	wt
77	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	122.30	119.63	1.02	wt
47	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-2b	44.31	51.74	0.86	wt
38	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-2b	40.22	44.99	0.89	wt
47	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-2b	27.41	26.77	1.02	wt
86	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	PMP22	84.99	82.70	1.03	wt
81[e]	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	PMP22	203.47	202.15	1.01	wt
86[e]	t(9;22)(q34;q11.2).ish der(9)(ABL1)	PMP22	84.99	82.70	1.03	wt
86	*ibid*	PMP22	84.99	82.70	1.03	wt
81	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	PMP22	203.47	202.15	1.01	wt
81[d]	*ibid*	PMP22	190.66	194.03	0.98	wt
86	t(9;22)(q34;q11.2).ish der(9)(ABL1)	TEKT3	97.22	99.60	0.98	wt
33	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	12.87	26.35	0.49	del
33	*ibid*	16-1b	58.76	91.19	0.64	del
81	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	28.02	41.48	0.68	del
33	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	12.87	26.35	0.49	del
46	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-1b	82.77	142.28	0.58	del
33[c]	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-2b	69.43	103.90	0.67	del
81	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-2b	46.78	91.19	0.51	del
33	t(9;22)(q34;q11.2).ish der(9)(5'ABL1)	16-2b	46.78	88.81	0.53	del
GM09286	47,XY,+9.ish 9p11q11(D9Z1x3)	16-1b	90.58	72.62	1.25	dup
GM10186	47,XY,+9.ish 9p11q11(D9Z1x3)	16-1b	75.23	59.16	1.27	dup
GM06074	46,XX,rec(9)dup(9)inv ins(9)(q22.1;q34.3q34.1)	16-1b	97.95	77.63	1.26	dup
GM10186 [d]	47,XY,+9.ish 9p11q11(D9Z1x3)	16-1b	104.00	74.37	1.40	dup
GM09286 [d]	47,XY,+9.ish 9p11q11(D9Z1x3)	16-1b	65.10	48.72	1.34	dup
GM09286	47,XY,+9.ish 9p11q11(D9Z1x3)	16-1b	55.47	37.93	1.46	dup
GM10186	47,XY,+9.ish 9p11q11(D9Z1x3)	16-1b	57.94	35.61	1.63	dup
CMT1A-1	nuc ish 17p12(PMP22x3)	PMP22	965.98	564.61	1.71	dup
CMT1A-2	nuc ish 17p12(PMP22x3)	PMP22	556.73	342.57	1.63	dup
CMT1A-3	nuc ish 17p12(PMP22x3)	PMP22	964.76	521.22	1.85	dup
CMT1A-4	nuc ish 17p12(PMP22x3)	PMP22	626.91	356.77	1.76	dup
CMT1A-1[e]	nuc ish 17p12(PMP22x3)	PMP22	196.31	121.56	1.61	dup
CMT1A-2[e]	nuc ish 17p12(PMP22x3)	PMP22	159.39	110.60	1.44	dup
CMT1A-3[e]	nuc ish 17p12(PMP22x3)	PMP22	90.11	64.04	1.41	dup
GM12214	D17S122x3[f]	PMP22	869.93	530.49	1.64	dup
CMT1A-1	nuc ish 17p12(PMP22x3)	TEKT3	104.09	74.17	1.40	dup
CMT1A-1[g]	nuc ish 17p12(PMP22x3)	TEKT3	3313.34	1990.96	1.66	dup
CMT1A-1[d]	*ibid*	TEKT3	125.56	79.62	1.58	dup
CMT1A-1[d]	*ibid*	TEKT3	70.65	48.21	1.47	dup
CMT1A-1[d]	*ibid*	TEKT3	107.43	76.43	1.41	dup
CMT1A-2	nuc ish 17p12(PMP22x3)	TEKT3	70.65	48.21	1.47	dup
CMT1A-2[d]	*ibid*	TEKT3	122.33	86.02	1.42	dup
CMT1A-2[d]	*ibid*	TEKT3	104.09	74.17	1.40	dup
CMT1A-2[d]	*ibid*	TEKT3	107.62	74.36	1.45	dup
CMT1A-3	nuc ish 17p12(PMP22x3)	TEKT3	122.33	86.02	1.42	dup
CMT1A-4	nuc ish 17p12(PMP22x3)	TEKT3	107.43	76.43	1.41	dup
GM12214	D17S122x3[f]	TEKT3	181.33	131.19	1.38	dup

wt: wild type; del: deletion; dup: duplication.
[a]HOXB1c probe included in each hybridization as reference for two copies unless otherwise noted.
[b]Ratio of geometric mean fluorescence for test: reference probe.
[c]Samples run in blind study.
[d]Samples run in reproducibility experiments for hybridizations performed on different days.
[e]16-1b used as reference probe.
[f]Duplication reported at locus by Lupski et al., 1991.
[g]HOXB1d used as reference probe.

in Supplementary Table S1. The sources of the samples included: 11 patients with CML, of which three possessed concerted chromosome 9q34 *ABL1* deletions (found in 10% of patients [Sinclair et al., 2000]) in addition to the characteristic chromosome 9;22 translocation (designated Samples 33, 46, and 81), and eight with the chromosome 9;22 translocation without a deletion (designated Samples 38, 47, 77, 86, 124, 138, 177, and 195); two from cell lines with complete trisomy 9 (GM09286 and

GM10186); a single cell line with a chromosome 9q34 duplication (GM06074); and five patients with Charcot-Marie-Tooth disease type 1A and a chromosome 17p12 duplication (designated CMT1A-1 through 4, and GM12214). An institutional review board exemption was approved for this research study.

Hybridization reactions. For hybridization, 50 ng of each sample was diluted in 40 μL 1.5 × TMAC hybridization buffer (3 mol/L tetramethylammonium chloride, 50 mmol/L Tris-HCl, pH8.0, and 1g/L sarkosyl) containing 10,000 sc probe-coupled microspheres. Optimal target DNA concentration was determined by dilution of labeled genomic template prior to hybridization (Supplementary Table S2a). Addition of 10 and 25 ng of genomic target to the conjugated sc probes was insufficient to reliably assess copy number; however, 50 ng of template DNA (corresponding to ~0.05 amol of a 100-nucleotide sc target sequence) resulted in reproducible and accurate copy number determination. If 98% of the carboxy sites are conjugated, we estimate that the molar ratio of the probe exceeds that of the homologous genomic target sequence in the reaction by $\sim 1.6 \times 10^9$-fold.

The hybridization reactions were heat denatured at 95°C for 3 minutes and then hybridized overnight between 45 and 51°C (the temperature selected was dependent upon probe nucleotide composition and length [Lewin, 1980]) (Table 1). The hybridized microspheres were then washed with 250 μL 1.5 × TMAC [Dunbar et al., 2003] followed by centrifugation at 10,000 *g* for 2 minutes. The supernatant was removed and 12 μL of a 1:50 dilution of a streptavidin-phycoerythrin reporter (SPE; Molecular Probes) in 1.5 × TMAC was added to detect microsphere-bound genomic targets containing biotin. The reactions were incubated at their hybridization temperature for 12 minutes. Following labeling, 250 μL of 1.5 × TMAC was added to each reaction, mixed, and centrifuged at 10,000 *g* for 2 minutes. The supernatant was removed and the hybridized microspheres were resuspended in 70 μL of 1.5 × TMAC.

Flow cytometry detection of hybridized microsphere-coupled sc probes. Hybridization reactions were diluted in 300 μL 1 × PBS prior to analysis on a FACSCalibur flow cytometer (Becton Dickinson). Approximately 5,000 microspheres of each set were analyzed per reaction. The signal of each target, hybridized to its complementary probe coupled to microspheres, was determined from the fluorescence intensity of SPE. Compatible microsphere spectral addresses selected to minimize overlap with the emission wavelengths of phycoerythrin (PE) were confirmed by comparing results obtained with otherwise identical unconjugated and hybridized microspheres. For each reaction, a reaction tube with all the components except target DNA was used as a negative control to determine background fluorescence in the FL2 (PE) detection channel, which consistently measured a fluorescence intensity $< 10^1$. Fluorescent microsphere standards (LinearFlow Flow Cytometry Intensity Calibration Kit; Molecular Probes) were used for relative fluorescence intensity (RFI) calibration of different fluorochrome detection channels of the flow cytometer. The instrument was also calibrated with fluorescent reference standards (Quantum R-PE MESF Medium Level Kit; Bangs Laboratories, Fishers, IN; www.bangslabs.com), based on surface-labeled microspheres calibrated in molecules of equivalent soluble fluorochrome (MESF) units.

Optimal PMT voltage tube settings minimized differences between MFI values of two different microsphere-conjugated probes hybridized to the same control genomic DNA sample. These settings were determined from instrument derived fluorescence measurements (CellQuest; Becton Dickinson) producing tight clusters of spectrally distinct microspheres in the SSC (side scatter) vs. microsphere signal plot. Typical PMT voltage settings for the FACSCalibur instrument were FSC (forward scatter) = E00 (no signal amplification), SSC (side scatter) = 344 V, FL1 = 727 V, FL2 = 640 V, FL3 = 300 V, and FL4 = 500 V. Thresholds for FL1, FL2, and FL3 were set at the default of 52 V. The FSC threshold was selected as the primary parameter and had a value of 52 V and the secondary parameter was set at SSC with a value of 125 V. The flow rate was set on low and the sheath fluid used was FACsFlow (Becton Dickinson). The system was flushed between runs with 1–3 mL of sheath fluid to remove any residual microspheres. CellQuest was used for data collection and analysis using the logarithmic transformation option for output values of non-normal distributions. The data analysis was also carried out with the WinMDI2.8 flow cytometry package (WinMDI; J. Trotter, Salk Institute, La Jolla, CA; www.cyto.purdue.edu/flowcyt/labinfo/images/TutorialWinMDI).

Aside from respective differences in annealing temperature, most probes did not require significant optimization because their single copy composition assured highly specific and reproducible detection of the corresponding genomic sequence. In fact, probes that were paralogous to other genomic loci produced inconsistent MFI ratios for different individuals with the same genotype (data not shown). Voltage parameters that resulted in either poor separation of spectrally-distinct multiplexed microspheres or broad FL2 peaks were avoided [Bagwell et al., 1989; Brown et al., 1994].

Each of the probes tested was able to detect the corresponding labeled PCR product in a hybridization reaction, establishing that probe conjugation to microspheres and hybridization to homologous targets was efficient. Purified *ABL1* (16-1, 16-2), *TEKT3*, *PMP22*, and *HOXB1* products were amplified from normal genomic DNA (Promega) using a single 5′ biotinylated primer. The biotinylated PCR products were heat denatured and hybridized to complementary microsphere-conjugated probes followed by detection with SPE. The conjugation efficiency of the carbodiimide coupling procedure was estimated by comparing the quantity of single microspheres (from the gated side scatter microsphere count) to the number of sc probes hybridized to homologous target (gated microsphere signal count and MFI level). A MFI from microspheres with properly attached probes hybridized to their homologous product is approximately 0.35% of RFI, which was determined during instrument calibration [Lowe et al., 2001].

Hybridization of each probe was optimized across a range of annealing temperatures (45–60°C) by gradient thermocycling (BioRad Laboratories, Hercules, CA; www.biorad.com). For each sc probe, the optimum temperature for hybridization was the one that produced MFI ratios closest to the expected ratio for paired controls with known genotypes; i.e., for a normal sample and a patient harboring a deletion (expected MFI ratios of 1 and 0.5, respectively), or from a normal genotype and a locus-specific duplication (expected ratios of 1 and 1.5, respectively). The reference HOXB1 probe included in optimization reactions was matched in length and relative GC content to the test probe (i.e., HOXB1a and 16-1a hybridizations were multiplexed, HOXB1b and 16-2a were multiplexed, and HOXB1c was multiplexed with either 16-1b, 16-2b, TEKT3, or PMP22).

We confirmed that the MFI ratios based on geometric MFIs of the test and reference probes exhibited among the lowest dispersion around the expected values. The relative fluorescence intensities of the test and reference probes were correlated with the expected copy number ratios for these genotypes: geometric mean and median (both with $r = 0.89$), arithmetic mean ($r = 0.86$), and peak channel intensities ($r = 0.42$).

Verification of Probe Specificity and Sensitivity

Genomic reconstruction experiments. To test the specificity of sc probes in a complex genomic environment, we hybridized sc probe-coupled microspheres to their corresponding purified PCR products in the presence of excess, sheared *Bos taurus* DNA (Amersham Biosciences, Piscataway, NJ; www.amershambiosciences.com). Reactions with 10,000 microspheres independently conjugated to 16-1a, 16-2a, TEKT3, and HOXB1a probes were hybridized to 5 ng of the corresponding identical PCR product (labeled with biotin) and 10 to 50 ng of nick-translated *Bos taurus* genomic DNA (unlabeled). To estimate the lowest detectable amount of hybridized product, a dilution series of PCR product (5–150 genomic equivalents per hybridization reaction) in 10 ng of sheared *Bos taurus* DNA (a molar ratio of PCR product: *Bos taurus* DNA ranging from ~1:300 to 1:10; Supplementary Table S2b) were hybridized to microsphere-conjugated probes.

Microsphere swap experiments. Additionally, microsphere swap experiments were performed in which sc probe 16-1a was conjugated to two spectrally distinct microsphere levels (R2 and R9), hybridized to corresponding PCR product in a multiplex reaction. The MFI values were compared for each microsphere set.

Statistics

Analyses were performed with the MATLAB Statistical Analysis Toolbox (MATHWORKS, Natick, MA; www.mathworks.com) and StatCrunch (Integrated Analytics, LLC; www.statcrunch.com). Probe hybridization was estimated as a ratio of the geometric MFIs [Coder et al., 1994; Kirkwood, 1979], measured from test and reference probes that were prepared and analyzed in the same reaction. Means of MFI ratios, standard deviations and 95% confidence intervals were computed for either individual for linked probe sets or for patients with the same genotype.

RESULTS

Specificity of the Hybridization Assay

We first optimized the specificity of the QMH assay by hybridizing target PCR products homologous to sc probes conjugated to microspheres. The specificity of the probe for homologous target sequence also provides a measure of the conjugation efficiency of the carbodiimide coupling procedure. Based on the SPE mean signal (in the FL2 channel) above the calculated background fluorescence ($<10^1$) and the number of conjugated microspheres present in the reaction, close to 98% ($\pm$0.4%) of the microspheres had hybridized to homologous target PCR product (relative to the fluorescence intensity of the calibration standard; see Materials and Methods; Supplementary Table S2b). The majority of the carboxyl sites coating the surface of the microspheres were thus linked to single-stranded DNA probes, though the microspheres were not completely saturated.

Genomic reconstruction experiments were performed to evaluate probe hybridization in a heterogeneous, complex genomic environment. Each microsphere-coupled sc probe was hybridized to its corresponding biotin-tagged PCR product diluted in an excess of unlabeled, nick-translated *Bos taurus* genomic DNA. In silico sequence comparisons (BLAT; www.genome.ucsc.edu) indicated that probes 16-1a, 16-2a, and TEKT3 were not similar to sequences in the *Bos Taurus* genome. Hybridization results confirmed that sc probes 16-1a, 16-2a, and TEKT3 and PMP22 were not homologous to sequences in the *Bos taurus* genome (data not shown), since only background fluorescence ($<10^1$) was evident from reactions lacking the homologous PCR target. By contrast, HOXB1a showed a MFI of 12.81 in one hybridization assay, which was anticipated, as this probe shares 90% sequence similarity to *HOXB1* sequences in the *Bos taurus* genome.

The sensitivity of detection of these microsphere-conjugated probes was evaluated by hybridization to varying amounts of the corresponding PCR products seeded into *Bos taurus* genomic DNA. The sensitivity of detection was linearly related to the amount of target present, based on increases in SPE MFI at higher product concentrations (Supplementary Table S2b). The limit of detection was approximately five genomic equivalents of product for both 16-1a and 16-2a; however, signal above background was evident for 2.5 genomic equivalents by hybridizing with probe 16-1a. Microsphere swap experiments showed that MFIs for different microspheres conjugated to the same probe were related to the concentration of product, and were essentially independent of the spectral address of the microsphere. For example, for Sample 47, multiplex hybridizations of probe 16-1a conjugated to microsphere R2 had an MFI of 32.8, whereas 16-1a conjugated to microsphere R9 of the MFI was 32.02, a difference of ~2%. For sample 33 hybridized to this probe, the MFI varied by ~13% (41.88 vs. 36.16) for microspheres with different spectral addresses.

Optimization of Probe Length

The effect of probe length on the hybridization signal was then assessed by conjugating probes of varying lengths from the same genomic interval to microspheres. Overlapping sc probes 16-2a (1,381 nucleotides) and 16-2b (101 nucleotides) were coupled to the surface of spectrally distinct microspheres and multiplexed in a single hybridization reaction to Sample 33 (a chromosome 9 deletion patient) and Sample 47 (which has a normal genotype at the 16-2 locus). These experiments showed that probes that were shorter than those typically used in scFISH studies more accurately estimated correct copy number, regardless of genotype. A multiplex experiment with DNA from Sample 33 showed 16-2a and 16-2b probes to have lower MFI values (0.67 and 0.51, respectively) compared to the HOXB1c reference probe (Supplementary Table S1; Table 2). Overall, the signal obtained with the shorter 16-2b probe appears to more precisely reflect the actual copy number in these deletion positive individuals (n = 3; Supplementary Table S1). The MFI ratios for these probes using a normal control sample in which both of these loci were biallelic (Sample 47) were 1.17 for 16-2a, and 1.02 for 16-2b, again showing that 16-2b more accurately measures known copy number than 16-2a (Supplementary Table S1; Table 2). Analogous results were obtained with subsets of probe 16-1 [16-1a (2,304 nucleotides) and 16-1b (100 nucleotides)] conjugated to microspheres when independently hybridized to control genomic DNA. Both probes successfully hybridized to both PCR product and control genomic DNA; however, the distribution of SPE fluorescence for probe 16-1a hybridization reactions was significantly broader than for 16-1b (the results of other multiplex QMH assays in which sc probe lengths were compared are shown in Table 1 and in Supplementary Table S1).

We found that microspheres conjugated to the shorter probes produced well-defined mean fluorescence distributions and consistently higher PE values. The MFI ratios of the ~100-nucleotide probes produced the lowest intra- and inter-sample variances (Fig. 2; Supplementary Table S1). Shorter

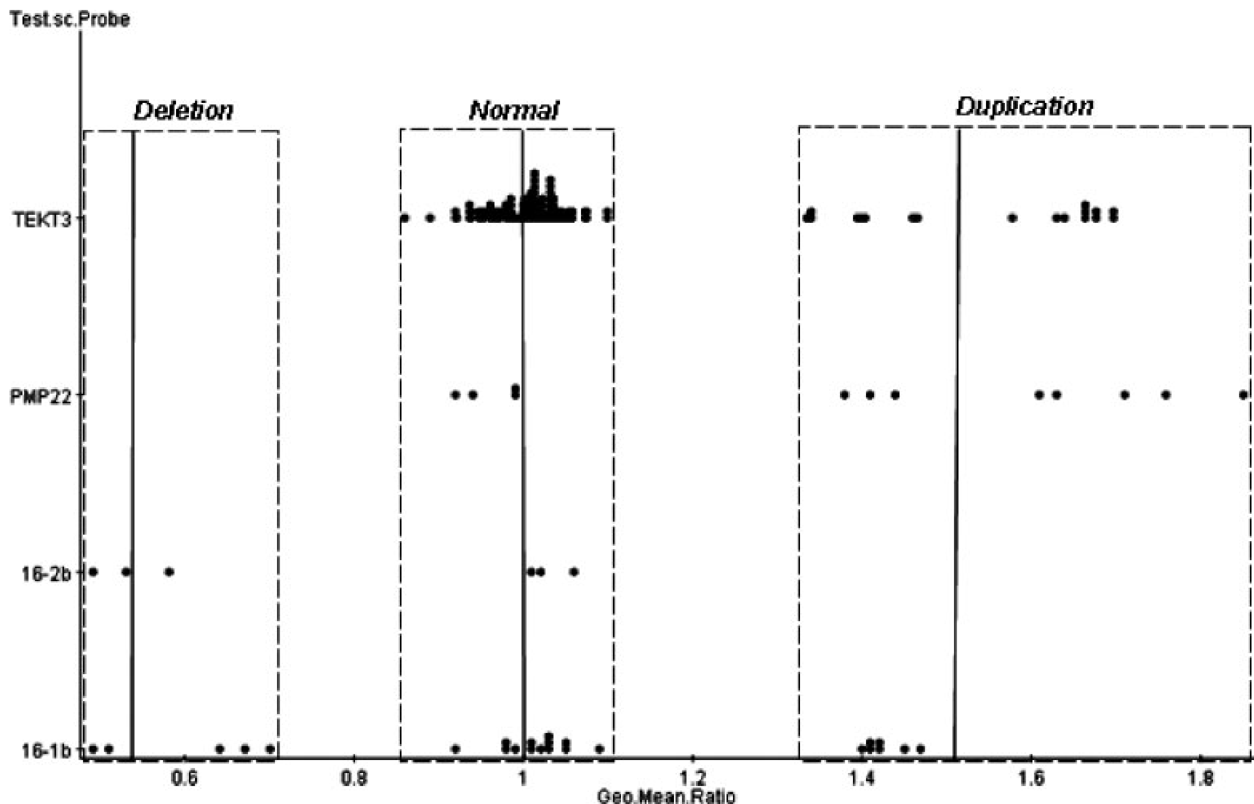

FIGURE 2. **Comparison of geometric mean ratios (x-axis) for pairs of sc test probes (16-1b, 16-2b, PMP22, and TEKT3) to reference probe (HOXB1c) compiled from 170 reactions. Samples are grouped by test probe (y-axis). A box (dashed lines) is drawn around each distinct genotypic group (left = chromosomal deletion, middle = normal, right = chromosomal duplication). Vertical lines indicate the theoretical MFI ratio for each genotype (0.5, 1.0, and 1.5 for deletion, normal, and duplicated samples, respectively). As indicated, the MFI ratios for all samples within each genotype do not overlap with other genotypes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]**

oligonucleotide probes (HOXB1e and 16-1c) proved to be inadequate for characterizing the genotypes of known samples. They produced test to reference probe MFI ratios that could not consistently distinguish copy number (Supplementary Table S1), a finding consistent with other recent reports [Borucki et al., 2005].

In some instances, analyses using longer probes also did not consistently produce the expected MFI ratios based on known genotypes at these loci (Supplementary Table S1). This suggested either that these sequences form structures that alter hybridization to genomic targets or that make them inaccessible to these targets. Furthermore, we found that longer probes conjugated to microspheres have a reduced shelf life that also affected the reproducibility of hybridization with the same batch of probe. Microspheres conjugated to 100- to 102-nucleotide sc probes stored at 4°C in the dark gave reproducible MFIs after more than 2 months, whereas longer conjugated probes (485–2,304 nucleotides) appeared to degrade within 2 weeks of preparation (resulting in lower MFIs).

Detection of Reduced Genomic Copy Number in Samples With Deletion of 9q34

The genotypes of chromosome 9q34 deletion and nondeletion control samples were distinguishable based on comparisons of MFI ratios obtained by QMH using optimized sc probes. In a blinded study, microspheres conjugated to 16-2b and HOXB1c were hybridized in separate reactions to Sample 33 and Sample 47 genomic DNA as well as to normal controls (Fig. 3). The MFI ratio of the 16-2b probe to the HOXB1c reference probe (Table 2) indicates a reduced copy number for 16-2b relative to HOXB1c in Sample 33 (ratio = 0.67), consistent with a deletion of a single copy of this locus. This result is consistent with our previous FISH results using sc probe 16-2a. The MFI ratios of 16-1b to HOXB1c in Samples 33 (ratio = 0.49 and 0.64) and 81 (ratio = 0.68) were also consistent with the FISH results using 16-1a in both patients. Hybridizations carried out in parallel with a biallelic genomic control (Sample 47) produced MFI intensities consistent with disomy at these loci (ratio = 0.96).

Multiplex hybridizations with two *ABL1* probes were then carried out to determine if genotypes at tightly linked genomic loci were consistent in the same individual. A known deletion patient, Sample 33, and a biallelic control, Sample 38, were hybridized with probes 16-1b, 16-2b, and HOXB1c in separate multiplexed reactions. Relative to the HOXB1c, Sample 33 exhibited MFI ratios of 0.61 for probe 16-1b and 0.51 for 16-2b. Hybridization with Sample 38 also gave consistent MFI ratios with these probes (16-1b: HOXB1c was 0.87, and 16-2b:HOXB1c was 0.89).

The MFI ratios for 16-1b to HOXB1c and for 16-2b to HOXB1c are indicated for samples containing either one, two, or three alleles at the 16-1 (*ABL1*) locus in Figure 2. The normal samples have average MFI ratios for either 16-1b- or 16-2b-derived probes that are nearly concordant with the expected ratios predicted from the genotypes themselves. Patients with CML whose samples have a deletion within *ABL1* have, as expected, distinctly lower mean MFI ratios using these probes, which were approximately equivalent to the expected 50% reduction in copy number (Table 3).

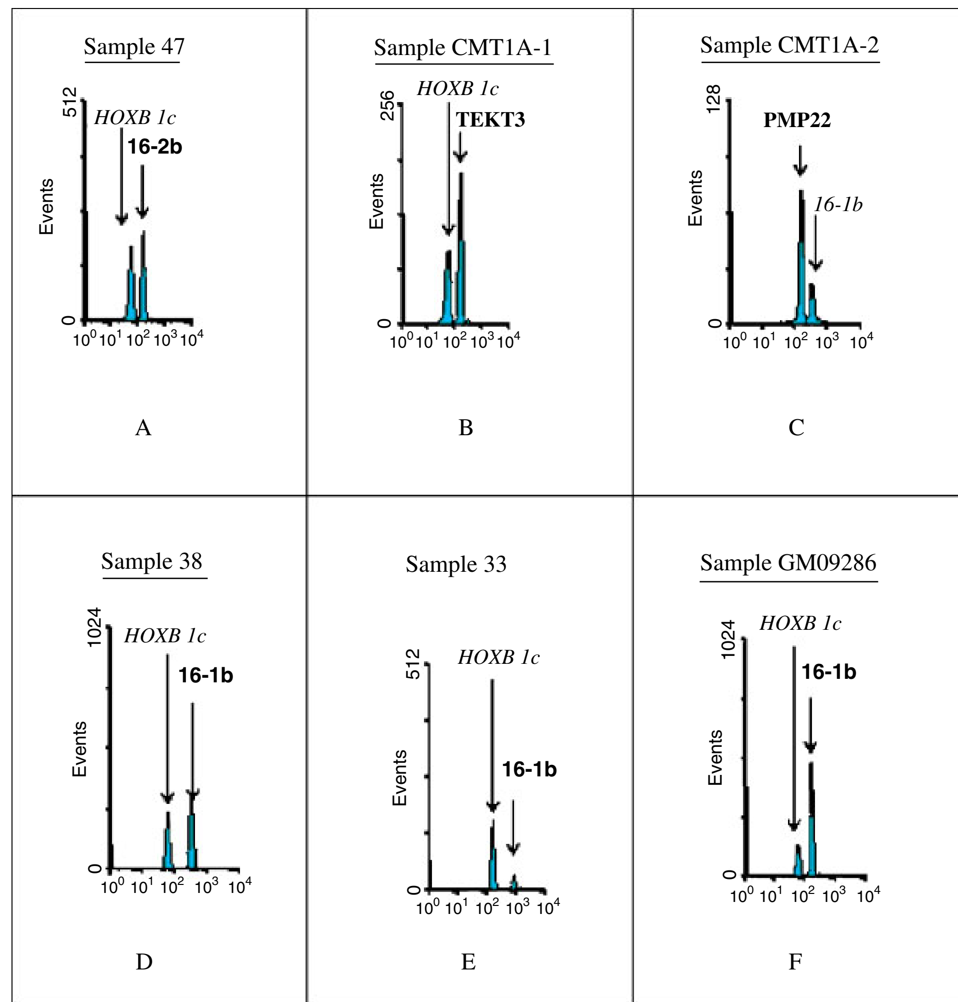

FIGURE 3. **Fluorescence detection of multiplexed sc probe-microspheres hybridized to samples. The number of events (i.e., the number of microspheres analyzed) is displayed on the y-axis and the fluorescence intensity per microsphere spectral address is shown on the x-axis. Reference probes are indicated in italics and test probes are in bold. The interpretation of results and corresponding cytogenetic nomenclature (consistent with ISCN [2005]) are shown in the panels: (A) intact for probe 16-2b in Sample 47 (MFI ratio of 16-2b:HOXB1c probes is 0.86; Table 2), indicated as t(9;22)(q34;q11.2).ish der(9)(B35:CHR9:130627353-130627454+); (B) duplicated for TEKT3 in Sample CMT1A-1 (MFI ratio TEKT3:HOXB1c is 1.40; Table 2), indicated as ish 17p12(TEKT3x3) or ish17p12(B35:CHR17:15149108-15149206x3); (C) duplicated for PMP22 in Sample CMT1A-2 (MFI ratio PMP22:16-1b is1.44; Table 2), indicated as ish 17p12(PMP22x3) or ish 17p12(B35:CHR17:15073475-15073576x3); (D) intact for 16-1b in Sample 38 (MFI ratio16-1b:HOXB1c is 0.98), indicated as t(9;22)(q34;q11.2).ish der(9)(B35:CHR9:130624671-130624771+); (E) deleted for 16-1b in Sample 33 (MFI ratio 16-1b:HOXB1c is 0.49), t(9;22)(q34;q11.2).ish der(9)(B35:CHR9:130624671-130624771-); (F) duplicated for 16-1b in Sample GM09286 (MFI ratio 16-1b:HOXB1c is 1.25), indicated as 47,XY,+9.ish 9(B35:CHR9:130624671-130624771x3). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]**

Detection of Increased Copy Number of Chromosome 9q34 in Trisomic 9 Cell Lines

To determine if samples with three alleles could be distinguished from the expected disomic genotype, probes 16-1b and HOXB1c were hybridized to DNA from cell lines that were triallelic for 9q34 and compared with Sample 47, which contains two copies of this locus (Table 2). Partial trisomy 9 cell lines (GM09286, GM10186, and GM06074), exhibited MFI ratios for 16-1b:HOXB1c ranging from 1.25 to 1.63, whereas both test and reference probes had similar MFI ratios for the normal control sample (ratio = 0.96). While these ratios are clearly different, the MFI ratios for these cell lines deviate somewhat from the expected value of 1.5. This may be related to specific characteristics of the 16-1b probe, since other probes from this genomic region did not show this effect to the same extent (see Discussion). Nevertheless, the mean MFI ratios for 16-1b:HOXB1c consistently demonstrated increased copy number over samples with disomic genotype (Tables 2 and 3).

Detection of Increased Copy Number of Chromosome 17p12 Region in CMT1A Patients

Genomic DNA from 5 CMT1A patients with FISH-confirmed chromosome 17p12 duplications and a control sample with a normal chromosome 17 genotype was hybridized with the HOXB1c reference sc probe and a probe from either the *TEKT3* or the *PMP22* gene. The patient samples displayed similar increased MFI ratios for hybridization to both TEKT3 (1.38 to 1.58) and PMP22 probes (1.41 to 1.85), reflecting the presence of three copies of this locus (Table 2; Figs. 2 and 3). The control sample (sample 86) exhibited comparable MFI ratios for both probes (0.98 and 1.03) (Table 2). Similarly, multiplex replicate

TABLE 3. Average MFI Ratios for Different Genotypes

Genotype	Locus specific abnormality	Probe(s)[a]	MFI ± SE (n)	95% CI
Comparison of MFI ratios with expected ratios from known genotypes				
Biallelic (nondeletion)	None	16-1b or 16-2b: HOXB1	1.01 ± 0.01 (16)	1.00–1.03
	None	TEKT3 or PMP22: HOXB1	1.01 ± 0.01 (4)	0.97–1.05
Deletion	del (5′ *ABL1*)	16-1b or 16-2b HOXB1	0.59 ± 0.02 (11)	0.54–0.64
Triallelic	+9 or int dup(9)	16-1b: HOXB1	1.37 ± 0.05 (7)	1.25–1.50
	int dup 17p12	TEKT3 or PMP22 HOXB1	1.53 ± 0.04 (17)	1.46–1.61
	int dup 17p12	PMP22: 16-1b	1.48 ± 0.06 (3)	1.21–1.75
Identification of genotypes in blinded study of 20 control and 2 abnormal samples[b]				
Biallelic	None	TEKT3 HOXB1	1.00 ± 0.01 (60)	1.00–1.01
Triallelic	CMT1A	TEKT3 HOXB1	1.51 ± 0.07 (6)	1.33–1.70

[a]Average of ratios of geometric MFIs for test: reference probes.
[b]Variance in MFI ratios among triplicate sets was 6.5×10^{-6} to 1×10^{-3}.

hybridizations of samples with known genotypes with microsphere-coupled TEKT3 and HOXB1 demonstrated these results were reproducible, yielding MFI ratios that varied by less than 4.8% for CMT1A-2 and by 11% for CMT1A-1 (Table 2). The TEKT3 and PMP22 MFI ratios were, respectively, slightly less than and greater than 1.5, the expected MFI ratio. Nevertheless, the average MFI ratios for both probes analyzed on all CMT1A positive samples were consistent with locus-specific 17p12 duplication (Table 3), and excluded the possibility of a false-negative result (Fig. 2).

Since most patients with CMT1A duplications typically have a normal genotype at the *ABL1* locus, the QMH assay was run using the chromosome 9 probe, 16-1b, as the reference probe (Table 2). We observed MFI ratios close to expected values for the CMT1A patients (Table 3), which themselves were distinct from those obtained from two normal controls (Samples 81 and 86, with MFI ratios of 1.01 and 1.03, respectively; Table 2). This indicated that the chromosome 17p12 duplications were detectable with TEKT3 and PMP22 probes, regardless of the chromosomal context of the reference sequence.

In a blinded study, triplicate reactions of 22 coded genomic samples (consisting of 20 normal and two CMT1A patient samples with previously confirmed duplications) were hybridized to TEKT3 and HOXB1c probes. Reproducibility for triplicate runs of each of these samples was excellent, with variances in the MFI ratios ranging from 6.5×10^{-6} to 1×10^{-3}. Patient samples with CMT1A duplications were readily distinguishable from normal controls, based on the respective TEKT3:HOXB1c hybridization MFI ratios for these groups (summarized in Table 3; individual MFI ratios are listed in Supplementary Table S3). These are consistent with our previous results showing no overlap between these genotypes in our non-blinded analysis (Fig. 2).

Distinguishing Copy Number Genotypes

The MFI ratio is a robust metric for determining genomic copy number, regardless of which loci are analyzed. The average test to reference probe MFI ratio was 1.03 ± 0.12 ($n = 126$) in all normal individuals in the present study (Table 2; Supplementary Table S4). The ratio was 0.57 ± 0.08 ($n = 8$) for all samples with hemizygous deletions, and was 1.53 ± 0.14 ($n = 33$), for triallelic samples. While several probes showed a modest degree of skewing of MFI ratios, abnormalities were clearly distinguishable from normal genotypes for all of the sc probes at the 95% confidence level, and for the normal vs. deleted genotypes, at the 98% confidence level.

DISCUSSION

We developed a quantitative microsphere hybridization (QMH) assay utilizing single copy (sc) probes to detect genomic copy number differences (patent pending). Prior amplification of locus-specific target DNA was not required since sc probes are designed to hybridize to a unique locus in the haploid genome sequence with high specificity. Loss or gain of sc sequences can be directly detected by hybridization to purified or archival patient genomic DNA. Copy number can be accurately determined using residual fixed cytogenetic cell preparations, in which genomic DNA is extracted from nuclei stored in methanol and acetic acid. This approach obviates the requirement for large sample quantities, additional blood draws, locus-specific genomic amplification, or time-consuming genomic DNA purification methods.

Hybridization experiments demonstrated adequate sensitivity to discern the presence of one vs. two copies and two vs. three copies of a genomic sequence. Use of multiple independent sc probes conjugated to microspheres with distinct spectral signatures can independently measure copy number changes in the same hybridization assay. Chromosome deletions were confirmed in the *ABL1* gene in three CML patients, trisomy of chromosome 9q34 was confirmed in three cultured cell lines, and duplication of chromosome 17p12 was confirmed in cells from five CMT1A patients. The same probe used to detect a hemizygous chromosome 9q34 deletion also detected three alleles in cell lines with trisomy at this locus. Test:reference probe ratios of geometric MFIs (and of median fluorescence intensities) were consistent for patients with the same genotype and were clustered around expected values, regardless of the chromosomal origin of the test probe.

We optimized several parameters during assay development. Specifically: 1) the sc probes conjugated to microspheres showed specificity for homologous sequences when examined in a heterologous complex genomic environment, i.e., as little as 5 ng of target sequence present in 1 μg of heterologous genomic sequence was successfully detected; 2) sc probes conjugated to microsphere sets with different spectral addresses showed negligible differences in MFIs, regardless of which probe was conjugated to which microsphere level; 3) the length of the sc probe attached to the microsphere surface affected the efficiency of hybridization, with shorter probes (~100 nucleotides) exhibiting greater MFIs and more accurate MFI ratios compared to longer probes (1–2 kb) or oligonucleotide probes (62 nucleotides). The shorter probes were more stable (i.e., had a longer shelf life) resulting in less lot-to-lot variation in conjugated microsphere stocks; 4) which, in turn, reduced the effort required to conjugate and quantify sc probes bound to microspheres.

The MFI ratios of probes 16-1b, TEKT3, and PMP22 deviated slightly, but consistently, from expected values for samples with aneusomic genotypes. There are several potential explanations for these differences. For 16-1b, the deviation could be related to specific characteristics that influence the MFI of this probe.

We speculate that hybridization to this probe may be affected by the stability of the probe-target duplex (16-1b has an overall GC content of 40%), or by the potential secondary structures formed by this sequence. The corresponding TEKT3:HOXB1c and PMP22:HOXB1c MFI ratios also deviated from the expected genotypic ratio for locus-specific duplications, possibly for the same reasons. Variation in replication timing between normal and abnormal chromosome structures could also potentially affect MFI ratios by producing transient differences in copy number at test and reference genomic loci (as we have shown elsewhere in the genome [White et al., 1996]).

QMH can be readily implemented in laboratories with access to a dual-laser flow cytometer. Assay optimization can be simplified with sc probe sets designed to minimize differences in composition and length and to avoid formation of stable secondary structures. Results were consistently reproduced for numerous samples and synthetic probes independently conjugated to microspheres. Surface carboxylated microsphere sets with distinct emission spectra and intensities which enable the concurrent analyses of hundreds or more target sequences are available from several manufacturers [Kellar and Iannone, 2002]. Carbodiimide coupling is a robust chemistry for probe attachment that has been standardized with routine laboratory protocols [Dunbar et al., 2003; Fulton et al., 1997]. QMH assays using sc probes conjugated to carboxylated polystyrene microspheres obtained from two different manufacturers (see Materials and Methods) produced comparable results. The microsphere sets have been engineered to discriminate many spectral signatures simultaneously. It should be feasible to automate multiplex coupling of different spectrally encoded microspheres to distinct sc probes. This should decrease both labor and reagent costs while increasing the amount of information obtained per assay. Furthermore, the minimal sample requirements and variety of sample types used in QMH assays should make it realistic to undertake high density, multilocus copy number determination for numerous genomic loci in the same individual.

The QMH platform may be a suitable diagnostic alternative for other methods that do not require context-dependent sequence identification. QMH cannot currently identify genomic translocations that are detected by FISH. However, it complements and extends the genre of existing methods for copy number determination, which include array comparative genomic hybridization (aCGH), Southern analysis, multiplex amplifiable probe hybridization (MAPH), multiplex ligation-dependent probe amplification (MLPA), and quantitative PCR (qPCR). QMH does not require either target DNA amplification or the addition of competitor DNA (i.e., C_ot-1 DNA) for repeat suppression, which compromises the reproducibility of quantitative hybridization measurements [Newkirk et al., 2005]. Excellent signal-to-noise ratio is achieved since simultaneous dual wavelength detection of both microsphere and hybridized target DNA significantly reduces any background from unbound target. Multiplexing hybridization probes conserves the available patient material and decreases the time needed to obtain test results. The abundant quantities of probe molecules conjugated to the surface of microspheres ensure that the efficiency of hybridization to homologous targets is unaltered by the presence of an excess of complex heterologous genomic sequences.

We found that differences in copy number are reliably detected at a genomic resolution of ≥ 100 bp at a level of ≥ 5 genomic equivalents per QMH reaction. This exceeds the resolution achieved by FISH, Southern hybridization, and aCGH (which is often limited by the size and density of cloned probes [Shaffer and Bejjani, 2004]), and is similar to MAPH and MLPA in probe density [Schouten et al., 2002]. QMH is not sensitive to the effects of SNPs, which potentially could occur at or near genomic target sequences used as ligation templates in MLPA or as primer binding sites in qPCR. QMH, with the possible exception of qPCR, requires less input DNA, which is a significant factor in designing high-throughput multilocus studies to determine copy number genotypes, and input DNA can be obtained from archival cells. Moreover, QMH does not show the significant variability in the levels of fluorescence signal seen in aCGH [Oostlander et al., 2004; Newkirk et al., 2005]. The technical skill required to set up the method and interpret QMH results is comparable to any of these other techniques, and in fact, may be less demanding.

Multiplex, parallel hybridization of probes to genomic targets is well established for aCGH, MLPA, and MAPH, and is currently being developed for QMH. With this capability, it should be feasible to precisely and rapidly delineate the extent of chromosomal abnormalities that alter normal copy number with genomic arrays of sc microsphere-conjugated hybridization probes. A dense set of sc probes spanning a chromosomal region could be used to initially screen for the boundaries of aneusomic domains and metaphase FISH studies could then provide a chromosomal context of the ends of the aneusomic regions in appropriate circumstances, such as defining the extent of deletion or duplication in a contiguous gene syndrome. Theoretically, 3,840 different products could be assayed on a single high-density microtiter plate using a set of 10 spectrally-distinct, encoded microspheres in each well. Assuming a minimum probe resolution of one per 20 kb [Rogan et al., 2001], a single plate could determine the copy number of a 20-Mb genomic domain within minutes. QMH based on sc probes also appears to be naturally extensible to other applications, such as copy number polymorphism determination and the detection and quantification of mRNA species and alternative splice forms in cDNA (Newkirk, Knoll, and Rogan, unpublished results).

ACKNOWLEDGMENTS

We thank Ruth Morgan, Dr. David Zwick, and Lisa Wheeler for their flow cytometry expertise; Dr. Steve Simon for valuable advice on statistical analyses; Dr. Yu-Zhong Zhang from Molecular Probes for providing unlabeled microsphere suspensions and quality-controlling these reagents; Joel Gaeddert for initial flow cytometric data analysis; and the anonymous reviewers for their insightful comments. This paper is dedicated to the memory of Dr. Samuel A. Latt.

REFERENCES

Bagwell CB, Baker D, Whetstone S, Munson M, Hitchcox S, Ault KA, Lovett EJ. 1989. A simple and rapid method for determining the linearity of a flow cytometer amplification system. Cytometry 10:689–694.

Borucki MK, Reynolds J, Call DR, Ward TJ, Page B, Kadushin J. 2005. Suspension microarray with dendrimer signal amplification allows direct and high-throughput subtyping of Listeria monocytogenes from genomic DNA. J Clin Microbiol 43: 3255–3259.

Brown RD, Zarbo RJ, Linden MD, Torres FX, Nakleh RE, Schultz D, Mackowiak PG. 1994. Two-color multiparametric method for flow cytometric DNA analysis. Standardization of spectral compensation. Am J Clin Pathol 101:630–637.

Coder DM, Redelman D, Vogt RF. 1994. Computing the central location of immunofluorescence distributions: logarithmic data transformations are not always appropriate. Cytometry 18: 75–78.

Dunbar S, Godbout R, Newkirk H, Hetzel J. 2003. Microsphere suspension array technology for SNP detection in cattle. IEEE Eng Med Biol Mag 22:158–162.

Earley M, Vogt RJ, Shapiro H, Mandy F, Kellar K, Bellisario R, Pass K, Marti G, Stewart C, Hannon W. 2002. Report from a workshop on multianalyte microsphere. Cytometry 50:239–242.

Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR Jr. 1997. Advanced multiplexed analysis with the FlowMetrix system. Clin Chem 43:1749–1756.

Hadd AG, Laosinchai-Wolf W, Novak CR, Badgett MR, Isgur LA, Goldrick M, Walkerpeach CR. 2004. Microsphere bead arrays and sequence validation of 5/7/9 T genotypes for multiplex screening of cystic fibrosis polymorphisms. J Mol Diagn 6: 348–355.

Inoue K, Dewar K, Katsanis N, Reiter LT, Lander ES, Devon KL, Wyman DW, Lupski JR, Birren B. 2001. The 1.4-Mb CMT1A duplication/HNPP deletion genomic region reveals unique genome architectural features and provides insights into the recent evolution of new genes. Genome Res 11:1018–1033.

ISCN. 2005. An international system for human cytogenetic nomenclature. Shaffer LG, Tommerup N, editors. Basel, Switzerland: S. Karger. p 55–84, 104–111.

Kellar K, Iannone M. 2002. Multiplexed microsphere-based flow cytometric assays. Exp Hematol 30:1227–1237.

Kirkwood TBL. 1979. Geometric means and measures of dispersion [Letter]. Biometrics 35:908–909.

Knoll JH, Lichter P. 1994. In situ hybridization to metaphase chromosomes and interphase nuclei. In: Dracopoli NC, Haines JL, Korf BR, Moir DT, Morton CC, Seidman CE, Seidman JG, Smith DR, editors. Current protocols in human genetics, vol. 1, unit 4.3 [Revised 2005]. New York: Green-Wiley.

Knoll JH, Rogan PK. 2003. Sequence-based, in situ detection of chromosomal abnormalities at high resolution. Am J Med Genet 121:245–257.

Knoll JHM, Rogan PK. 2004. Single copy genomic hybridization probes and method of generating same. US Patent No. 6,828,097.

Lewin B. 1980. DNA sequence organization: nonrepetitive and repetitive DNA. Gene expression 2 – eucaryotic chromosomes, 2nd edition. New York: Wiley-Interscience Publication. p 503–530.

Little SE, Vuononvirta R, Reis-Filho JS, Natrajan R, Iravani M, Fenwick K, Mackay A, Ashworth A, Pritchard-Jones K, Jones C. 2006. Array CGH using whole genome amplification of fresh-frozen and formalin-fixed, paraffin-embedded tumor DNA. Genomics 87:298–306.

Lowe M, Spiro A, Summers AO, Wireman J. 2001. Multiplexed identification and quantification of analyte DNAs in environmental samples using microspheres and flow cytometry. In: Spencer JFT, Spencer ALR, editors. Methods in biotechnology: environmental biology: methods and protocols. Totowa, NJ: Humana Press. p 51–74.

Lupski JR, Oca-Luna RMd, Slaugenhaupt S, Pentao L, Guzzetta V, Trask BJ, Saucedo-Cardenas O, Barker DF, Killian JM, Garcia CA, Chakravariti A, Patel PI. 1991. DNA duplication associated with Charcot-Marie-Tooth disease type 1A. Cell 66:219–232.

Newkirk H, Knoll JHM, Rogan PK. 2005. Distortion of quantitative genomic and expression hybridization by C_0t-1 DNA: mitigation of this effect. Nucleic Acids Res 14; 33:e191.

Oostlander AE, Meijer GA, Ylstra B. 2004. Microarray-based comparative genomic hybridization and its applications in human genetics. Clin Genet 66:488–495.

Rockenbauer E, Petersen K, Vogel U, Bolund L, Kolvraa S, Nielsen KV, Nexo BA. 2005. SNP genotyping using microsphere-linked PNA and flow cytometric detection. Cytometry A 64:80–86.

Rogan PK, Cazcarro PM, Knoll JH. 2001. Sequence-based design of single-copy genomic DNA probes for fluorescence in situ hybridization. Genome Res 11:1086–1094.

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57.

Sekar M, Bloch W, John PS. 2005. Comparative study of sequence-dependent hybridization kinetics in solution and on microspheres. Nucleic Acids Res 33:366–375.

Shaffer LG, Bejjani BA. 2004. A cytogeneticist's perspective on genomic microarrays. Hum Reprod Update 10:221–226.

Sinclair P, Nacheva E, Leversha M, Telford N, Chang J, Reid A, Bench A, Champion K, Huntly B, Green A. 2000. Large deletions at the t(9;22) breakpoint are common and may identify a poor-prognosis subgroup of patients with chronic myeloid leukemia. Blood 95:738–743.

Southern EM. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–518.

Studer M, Lumsden A, Ariza-McNaughton L, Bradley A, Krumlauf R. 1996. Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb-1*. Nature 384: 630–634.

Vignali D. 2000. Multiplexed particle-based flow cytometric assays. J Immunol Methods 243:243–255.

White LM, Rogan PK, Nichols RD, Wu BL, Korf B, Knoll JH. 1996. Allele-specific replication of 15q11-q13 loci: a diagnostic test for detection of uniparental disomy. Am J Hum Genet 59: 423–430.

White SJ, Breuning MH, den Dunnen JT. 2004. Detecting copy number changes in genomic DNA: MAPH and MLPA. Methods Cell Biol 75:751–768.