Evaluation of the performance of Amersham HyPer5 dye in comparative genomic hybridization microarray applications

***Key words**: BAC • array comparative genomic hybridization • fluorescent labeling • microarray*

Abstract

The labeling efficiency of HyPer™5 fluorescent dye was compared to that of Amersham Cy™5-dCTP and Amersham Cy3-dCTP in three different experiments. The labeled probes were then used in 3K bacterial artificial chromosome (BAC) array comparative genomic hybridization (aCGH) microarray analyses. HyPer5-dCTP incorporation into amplified genomic DNA was comparable to that of Cy5-dCTP incorporation. HyPer5-labeled probes exhibited greater stability over time compared to Cy5 probes; in addition, they performed similarly to Cy5-labeled probes on the 3K BAC aCGH microarrays.

Introduction

Karyotyping is an important diagnostic tool in the clinic for detecting chromosomal aberrations that underlie many genetic diseases. Initial karyotyping, based on chromosome banding techniques, allowed genome-wide detection of translocations and large chromosomal aberrations; however, the resolution was limited to 5 to 10 Mb. The subsequent development of fluorescence *in situ* hybridization (FISH) enabled the detection of submicroscopic chromosomal imbalances, but the technique can only be applied to analyze a limited number of specific DNA regions rather than the entire genome at once. Multicolor FISH derivatives aimed at the simultaneous detection of all chromosomes also suffer from low resolution.

High-resolution, genome-wide detection of submicroscopic chromosomal imbalances became feasible by aCGH. In this technique, well-characterized short DNA pieces of the genome are spotted on a glass slide matrix, and are then hybridized with differentially fluorescent-labeled sample and reference genomic DNA. Evaluation of the normalized fluorescence ratio per DNA spot allows the precise identification of DNA copy number alterations in the entire genome tested. The resolution limit is defined by the number of different DNA spots per genomic region and by the genomic size covered by a specific DNA spot. The DNA in these spots can be cloned genomic DNA derivatives or alternatively synthetic oligonucleotides. For successful aCGH, adequate signal-to-noise ratios and low standard deviations (SD) of fluorescence intensity ratios are a prerequisite. Both these factors are influenced by the stability of the fluorescent dyes used. In this report, we used aCGH to evaluate the performance of a new GE Healthcare fluorescent dye, HyPer5, that is photostable and resistant to ozone.

Materials

Products used

Amersham HyPer5-dCTP (25 nmol)	28-9231-83
Amersham Cy5-dCTP (25 nmol)	PA55021
Amersham Cy3-dCTP (25 nmol)	PA53021
Amersham CyScribe™ Array CGH Genomic DNA Labeling System (30 reactions)	28-9199-56

Other materials

BioPrime™ Array CGH Genomic Labeling System	18095-011

Methods

The labeling efficiencies of HyPer5-dCTP, Cy5-dCTP and Cy3-dCTP were tested in three independent experiments. In the first two experiments, a modification of the BioPrime Array CGH Genomic Labeling System (Invitrogen) was used, and labeled probes were subsequently purified with the BioPrime Purification module. In a third experiment, reagents from Amersham CyScribe Array CGH Genomic DNA Labeling System were used, and labeled DNA samples were purified with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit.

Identical modifications were applied to both kits. All experiments were started from 150 ng genomic DNA that was amplified overnight in a 25 µl reaction using 0.5 µl of Klenow DNA polymerase. The probes labeled in experiments 1 and 2 were further evaluated for CGH performance on 3K BAC microarray platforms.

The labeling efficiency, determined by the base-to-dye ratio (BDR) of HyPer5-dCTP, Cy5-dCTP, and Cy3-dCTP in newly synthesized DNA, was quantitated by spectroscopy at wavelengths of 220 nm up to 750 nm, with the maximum absorbance for Cy3 and HyPer5/Cy5 at 550 nm and 650 nm, respectively.

DNA yield, dye incorporation, and BDR were calculated (Table 1) according to following formulas:

DNA yield using HyPer5-dCTP (ng/µl) = $[A_{260} - A_{320} - (0.25 \times A_{650})] \times 50$
DNA yield using other Cy-dCTP (ng/µl) = $(A_{260} - A_{320}) \times 50$
Dye incorporation HyPer5 (pmol/µl) = $(A_{650} \div 125\,000) \times 1\,000\,000$
Dye incorporation Cy5 (pmol/µl) = $(A_{650} \div 250\,000) \times 1\,000\,000$
Dye incorporation Cy3 (pmol/µl) = $(A_{550} \div 150\,000) \times 1\,000\,000$

BDR = [DNA (ng/µl) × 1000] ÷ [dye incorporation (pmol/µl) × 324.5]

Table 1. Probe-labeling efficiency using HyPer5-dCTP, Cy5-dCTP, and Cy3-dCTP

	DNA sample	Dye	DNA yield (ng/µl)	DNA yield (total)	Dye incorp. (pmol/µl)	Dye incorp. (total)	BDR
Experiment 1	Patient 1	Cy5	57.25	2862.50	5.40	270.00	32.67
	Patient 2	Cy5	70.50	3525.00	5.84	292.00	37.21
	Patient 3	Cy5	72.75	3637.50	5.68	284.00	39.47
	Patient 1	Cy3	68.50	3425.00	7.53	376.67	28.03
	Patient 2	Cy3	72.75	3637.50	7.33	366.67	30.57
	Patient 3	Cy3	72.00	3600.00	7.10	355.00	31.25
	Patient 1	HyPer5	74.56	3728.13	6.04	302.00	38.04
	Patient 2	HyPer5	84.88	4243.75	6.00	300.00	43.60
	Patient 3	HyPer5	80.06	4003.13	5.56	278.00	44.38
	Patient 1	Cy3	68.00	3400.00	7.53	376.67	27.82
	Patient 2	Cy3	75.50	3775.00	7.47	373.33	31.16
	Patient 3	Cy3	75.25	3762.50	7.30	365.00	31.77
Experiment 2	Patient 4	Cy5	30.50	1525.00	3.72	186.00	25.27
	Patient 5	Cy5	65.25	3262.50	5.54	277.00	36.31
	Patient 6	Cy5	73.25	3662.50	5.88	294.00	38.39
	Patient 4	Cy3	34.75	1737.50	3.87	193.33	27.70
	Patient 5	Cy3	70.00	3500.00	6.73	336.67	32.04
	Patient 6	Cy3	74.75	3737.50	7.10	355.00	32.45
	Patient 7	HyPer5	69.56	3478.13	5.56	278.00	38.56
	Patient 5	HyPer5	80.13	4006.25	5.84	292.00	42.28
	Patient 6	HyPer5	84.56	4228.13	5.72	286.00	45.56
	Patient 7	Cy3	66.25	3312.50	6.67	333.33	30.63
	Patient 5	Cy3	72.50	3625.00	6.83	341.67	32.69
	Patient 6	Cy3	74.50	3725.00	7.13	356.67	32.18
Experiment 3	Patient 8	Cy5	39.00	1950.00	1.76	88.00	68.29
	Patient 2	Cy5	42.00	2100.00	1.76	88.00	73.54
	Patient 3	Cy5	39.00	1950.00	1.68	84.00	71.54
	Patient 8	Cy3	41.50	2075.00	2.47	123.33	51.85
	Patient 2	Cy3	42.50	2125.00	2.47	123.33	53.10
	Patient 3	Cy3	49.00	2450.00	2.67	133.33	56.63
	Patient 8	HyPer5	44.88	2243.75	1.36	68.00	101.68
	Patient 2	HyPer5	44.38	2218.75	1.36	68.00	100.55
	Patient 3	HyPer5	45.00	2250.00	1.28	64.00	108.34
	Patient 8	Cy3	51.50	2575.00	2.87	143.33	55.36
	Patient 2	Cy3	47.50	2375.00	2.60	130.00	56.30
	Patient 3	Cy3	46.00	2300.00	2.67	133.33	53.16

Table 2. Comparison of probe performance on 3K BAC microarrays

CGH array A (Cy5/Cy3)		CGH array A (HyPer5/Cy3)	
Cy5 sample	Patient 1	HyPer5 sample	Patient 1
Cy3 sample	Patient 2	Cy3 sample	Patient 2
Hybridization efficiency	98.98%	Hybridization efficiency	92.67%
Cy5 signal:noise	8.68	HyPer5 signal:noise	2.61
Cy3 signal:noise	7.86	Cy3 signal:noise	7.86
SD	0.25573	SD	0.25011
SD corrected for sex mismatch	0.11833	SD corrected for sex mismatch	0.14013
CGH array B (Cy5/Cy3)		**CGH array B (HyPer5/Cy3)**	
Cy5 sample	Patient 2	HyPer5 sample	Patient 2
Cy3 sample	Patient 3	Cy3 sample	Patient 3
Hybridization efficiency	99.49%	Hybridization efficiency	98.73%
Cy5 signal:noise	14.76	HyPer5 signal:noise	3.72
Cy3 signal:noise	8.71	Cy3 signal:noise	8.28
SD	0.08986	SD	0.10949
CGH array C (Cy5/Cy3)		**CGH array C (HyPer5/Cy3)**	
Cy5 sample	Patient 3	HyPer5 sample	Patient 3
Cy3 sample	Patient 1	Cy3 sample	Patient 1
Hybridization efficiency	98.87%	Hybridization efficiency	99.01%
Cy5 signal:noise	12.00	HyPer5 signal:noise	4.60
Cy3 signal:noise	7.46	Cy3 signal:noise	7.11
SD	0.24299	SD	0.25834
SD corrected for sex mismatch	0.10418	SD corrected for sex mismatch	0.12963

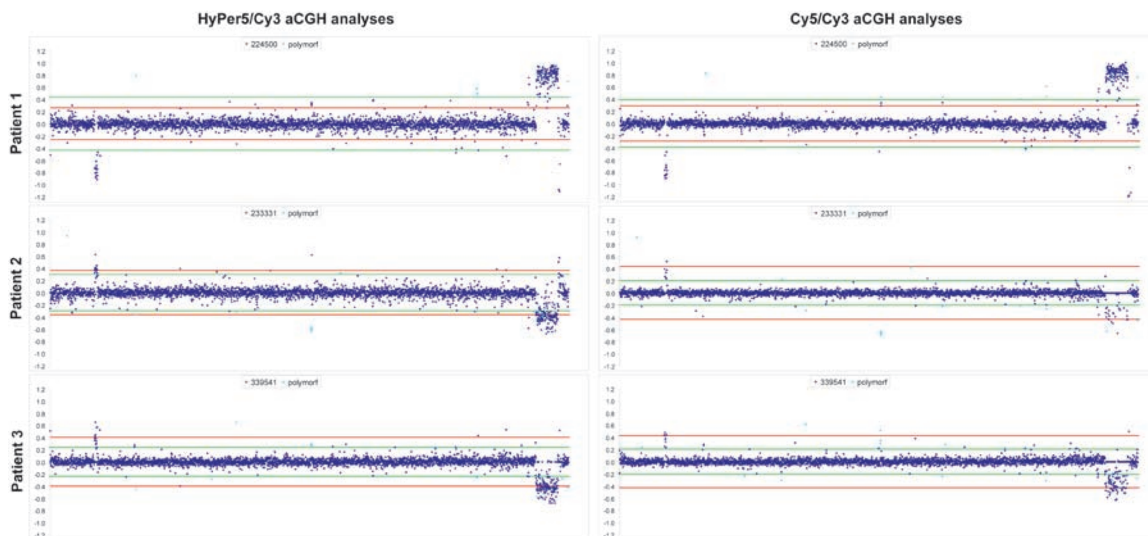

Fig 1. Comparison of combined 3K BAC microarray CGH results. Probes labeled with HyPer5-dCTP perform similarly to Cy5-labeled probes in 3K BAC CGH microarray analyses; however, they produced slightly increased standard deviations.

Results and Discussion

Labeling efficiency

Similar DNA-amplification and dye-incorporation efficiencies (and thus also BDRs) were obtained when HyPer5-dCTP or Cy5-dCTP was used in conjunction with the modified BioPrime Array CGH Genomic Labeling System (Table 1, Experiments 1 and 2). Higher BDRs were detected with a modified CyScribe Array CGH Genomic DNA labeling protocol (Table 1, Experiment 3) when compared to the modified Bioprime protocol: BDRs of Cy5 or Cy3 probes were doubled, while BDRs of probes generated with HyPer5-dCTP were tripled.

CGH microarray performance

Probes labeled with HyPer5-dCTP produced slightly higher SD and (initially) lower signal-to-noise ratios (Tables 2 and 3; Fig 1) in microarray scans. Cy5-labeled probe signals were 8 to 14 times higher than background, while HyPer5-labeled probe signals were 2.6 to 4.6 times higher than background. Signal-to-noise ratios for Cy3-labeled probes were identical (7 to 8 times higher than background). Although the initial signal intensity generated by the HyPer5 probes was slightly diminished, the obtained values were within our defined range for diagnostic CGH analysis.

Part of the CGH arrays were rescanned multiple times in a period of 14 days (Table 4; Fig 2). During this period, environmental ozone concentrations were monitored (Fig 3, time of rescans is indicated). From these rescans, it is obvious that HyPer5 signals are at least as stable as Cy3 signals under identical experimental conditions. HyPer5 and Cy3 probe performance was stable or even slightly improved on the array with respect to SD and signal-to-noise ratios (Table 4; Fig 2). On the contrary, Cy5 probe performance dropped quickly in a period of 5 to 8 days, resulting in a 250% to 350% increase in SD and a 700% to 800% decrease in signal-to-noise ratio (Table 4). HyPer5/Cy3 CGH arrays still allowed efficient detection of the chromosomal aberrations 14 days after the labeling experiment, while the same aberrations were not as easily detected on the Cy5/Cy3 CGH arrays, due to the higher variation caused by Cy5 decay.

Conclusions

HyPer5 outperforms Cy5 with respect to dye stability, as was observed in repeated scans. HyPer5-dCTP incorporation into amplified genomic DNA was comparable to that of Cy5-dCTP incorporation. Furthermore, probes labeled with HyPer5-dCTP could be efficiently purified from unlabeled material and performed similarly to Cy5-labeled probes on the 3K BAC microarray platform. Although the HyPer5 dye probes produced lower initial signal-to-noise ratios and slightly higher SD in HyPer5/Cy3 CGH array experiments when compared to Cy5-labeled probes, HyPer5/Cy3 CGH arrays were able to detect similar chromosomal aberrations as did Cy5/Cy3 CGH arrays. However, in a 14-day period, HyPer5/Cy3 CGH arrays allowed the repeated detection of these chromosomal aberrations whereas Cy5/Cy3 CGH arrays suffered from increasing variation and lowered signal-to-noise ratios introduced by Cy5 decay. We expect that chromosomal aberrations are easier to detect with HyPer5/Cy3 CGH arrays, compared to Cy5/Cy3 CGH arrays, in environmental conditions with high ozone concentrations.

We anticipate that the HyPer5 dye will become the standard in our microarray CGH analyses.

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Table 3. Combined 3K BAC microarray results per DNA sample (Experiment 1)

Patient	HyPer5/Cy3 CGH arrays		Cy5/Cy3 CGH arrays	
	SD	HE[1]	SD	HE[1]
1	0.109351	99.24%	0.097413	99.44%
2	0.074866	98.76%	0.049833	95.12%
3	0.060757	98.25%	0.05196	96.87%

[1] HE = hybridization efficiency

Table 4. Performance of probes labeled with HyPer5-dCTP, Cy5-dCTP, and Cy3-dCTP over time. A, Experiment 1; B, Experiment 2.

A	Date	7/9/2007	7/12/2007	7/13/2007	7/17/2007	7/23/2007
	Scans	1	4	5	6	7
HyPer5/Cy3 array 2007060419 high	HyPer5 sample	Patient 3	Patient 3	Patient 3	Patient 3	Patient 3
	Cy3 sample	Patient 1	Patient 1	Patient 1	Patient 1	Patient 1
	Hyb efficiency	99.01%	99.10%	98.93%	98.87%	98.93%
	HyPer5 signal:noise	4.60	4.85	4.99	5.55	6.01
	Cy3 signal:noise	7.11	8.86	9.67	9.03	8.88
	SD	0.25834	0.27209	0.26591	0.27419	0.27039
	SD corrected for sex mismatch	0.1296	0.13338	0.12145	0.12382	0.12547
Cy5/Cy3 equivalent 2007060418 low	Cy5 sample	Patient 3	Patient 3	Patient 3	Patient 3	Patient 3
	Cy3 sample	Patient 1	Patient 1	Patient 1	Patient 1	Patient 1
	Hyb efficiency	98.87%	98.87%	98.87%	98.87%	98.87%
	Cy5 signal:noise	12.00	5.52	4.95	1.88	1.75
	Cy3 signal:noise	7.46	9.34	10.37	10.03	9.17
	SD	0.24299	0.27052	0.26913	0.36510	0.34164
	SD corrected for sex mismatch	0.10418	0.13040	0.13921	0.25773	0.23620

B	Date	7/12/2007	7/13/2007	7/17/2007	7/23/2007
	Scans	1	4	5	6
HyPer5/Cy3 array 2007060428 low	HyPer5 sample	Patient 5	Patient 5	Patient 5	Patient 5
	Cy3 sample	Patient 6	Patient 6	Patient 6	Patient 6
	Hyb efficiency	98.53%	98.62%	98.90%	98.98%
	HyPer5 signal:noise	3.16	3.67	4.72	5.69
	Cy3 signal:noise	11.40	14.72	15.47	14.14
	SD	0.11046	0.08697	0.09426	0.09655
Cy5/Cy3 equivalent 2007060426 high	Cy5 sample	Patient 5	Patient 5	Patient 5	Patient 5
	Cy3 sample	Patient 6	Patient 6	Patient 6	Patient 6
	Hyb efficiency	99.38%	99.13%	83.84%	88.92%
	Cy5 signal:noise	11.06	5.31	1.31	1.53
	Cy3 signal:noise	9.08	13.87	14.84	14.00
	SD	0.10089	0.13343	0.36664	0.37150

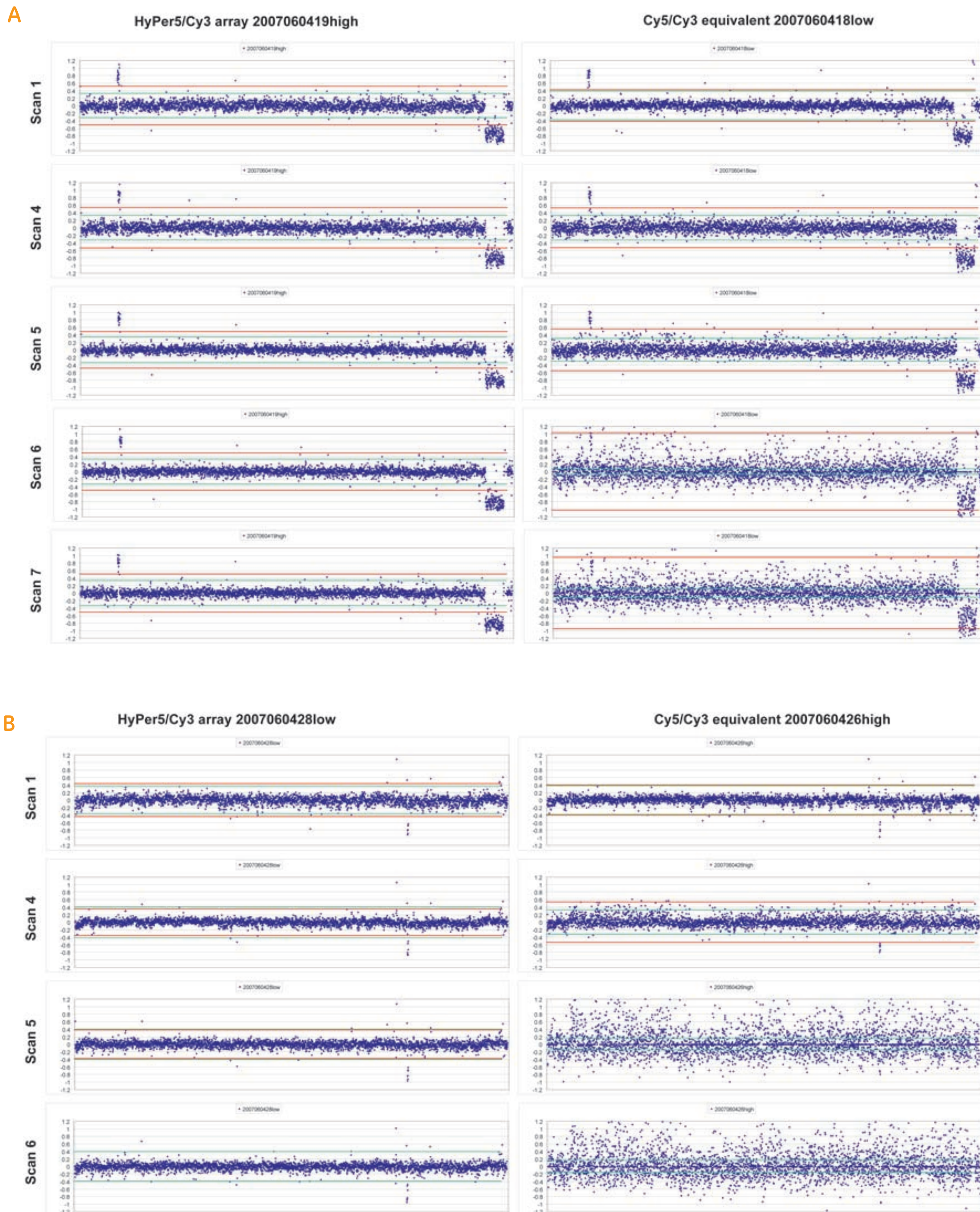

Fig 2. Performance comparison of probes labeled with HyPer5-dCTP, Cy5-dCTP, and Cy3-dCTP over time. Under similar environmental conditions, HyPer5 and Cy3 dyes are significantly more stable than the Cy5 dye. As such, rescans of HyPer5/Cy3 CGH arrays produce consistent results, while rescans of Cy5/Cy3 CGH arrays become uninterpretable over time (for timing of the different scans and ozone concentrations during the experiments see Fig. 3). Results are shown for Experiment 1 (Fig 2A) and Experiment 2 (Fig 2B).

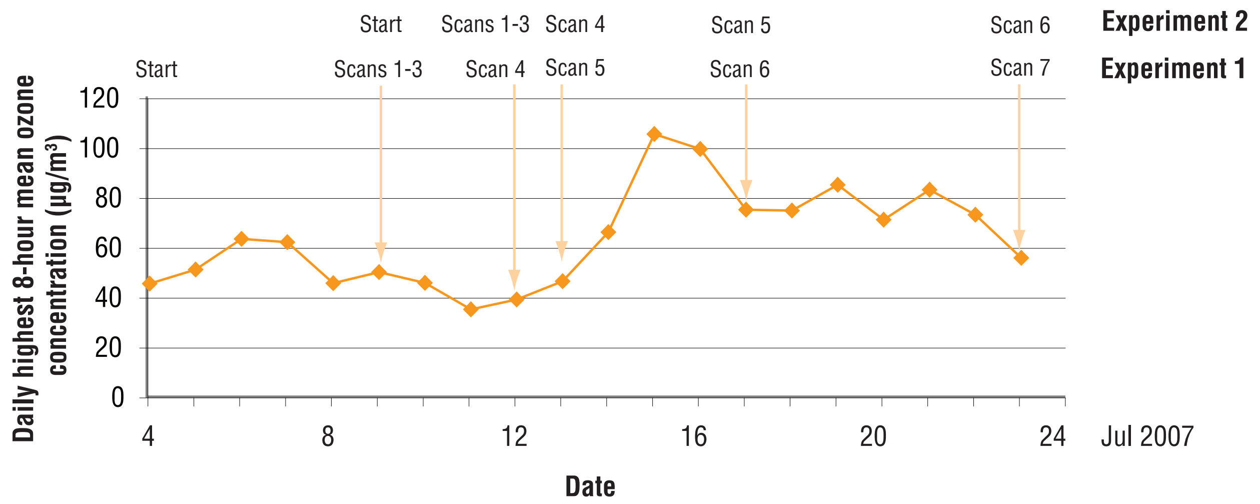

Fig 3. Dates of the different scans and daily monitoring of the ozone concentrations during the experiments (0000 h through 2400 h GMT). Ozone concentrations shown are the average of readings at three locations (Brussels, Woluwe, and Aarschot).

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