Cellular Oncology 29 (2007) 351–359
IOS Press

DNA quality assessment for array CGH by isothermal whole genome amplification

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Abstract. *Background:* Array Comparative Genomic Hybridization (array CGH) is increasingly applied on DNA obtained from formalin-fixed paraffin-embedded (FFPE) tissue, but in a proportion of cases this type of DNA is unsuitable. Due to the high experimental costs of array CGH and unreliable methods for DNA quality testing, better prediction methods are needed. The aim of this study was to accurately determine the quality of FFPE DNA input in order to predict quality of array CGH outcome. *Material and Methods:* DNA quality was assessed by isothermal amplification and compared to array CGH quality on 59 FFPE gastric cancer samples, one FFPE colorectal cancer sample, two FFPE normal uvula samples, one fresh frozen and six FFPE HNSCC samples. Gastric cancer DNA was also quality tested by *β-globin* PCR. *Results:* Accurate prediction of DNA quality using the isothermal amplification was observed in the colorectal carcinoma, HNSCC and uvula samples. In gastric cancer samples, the isothermal amplification was a more accurate method for selecting good quality DNA for array CGH compared to using PCR product lengths. The isothermal amplification product was used for array CGH and compared to the results achieved using non-amplified DNA in four of the samples. DNAs before and after amplification yielded the same segmentation patterns of chromosomal copy number changes for both the fresh DNA sample and the FFPE samples. *Conclusion:* The efficiency of isothermal DNA amplification is a reliable predictor for array CGH quality. The amplification product itself can be used for array CGH, even starting with FFPE derived DNA samples.

Keywords: Array CGH, FFPE, DNA quality, isothermal amplification

1. Introduction

Array Comparative Genomic Hybridization (array CGH) is a powerful method for identifying DNA copy number gains and losses in tumors on a genome-wide scale [9,19]. DNA copy number profiles of tumors can be used for prognosis prediction and therapy selection [6,7,15,16,18]. A major limitation for using array CGH remains the high experimental costs. High quality DNA, as obtained from cell lines or fresh frozen tissue, minimizes hybridization failures and therefore overall costs. Nowadays array CGH is increasingly applied to DNA extracted from formalin-fixed and paraffin-embedded (FFPE) tissue samples [1,3–5,10, 14]. Using DNA from FFPE for array CGH has great potential since it opens up the rich source of large clinical tissue archives, with detailed clinicopathological information including long-term follow up data. FFPE derived DNA often is of suboptimal quality, which may be due to degradation and/or fixation induced DNA cross links. Moreover, we have observed that DNA quality can vary depending on tissue origin; colorectal tumor samples generally give good results, while gastric cancer samples are intrinsically difficult. Overall, from a certain proportion of cases it is not possible to obtain good quality array CGH results. Several methods are available to test the quality of DNA obtained from archival tissue in order to predict the quality of the array CGH result, such as gel electrophoresis based size fractioning, wavelength coefficients by spectrophotometry or multiplex-PCR testing [13]. However, these methods still leave room for improvement, since in our hands they do not invariably correlate with array CGH quality. Reliable pre-

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diction of array CGH experimental success has evident financial advantages and can reduce work load. The aim of the present study was to test a new method, i.e. isothermal whole genome amplification efficiency, to evaluate genomic DNA quality from FFPE or other samples which may yield suboptimal DNA for array CGH analysis, and predict the quality of the array CGH result. In addition, we set out to test the potential of isothermal whole genome amplification as a method for applying array CGH to amplified genomic DNA of small samples.

2. Materials and methods

2.1. Material and DNA extraction

DNA was isolated from 59 FFPE gastric cancer (GC) tissue samples and one colorectal cancer (CRC) sample as described previously [17]. Briefly, areas containing at least 70% tumor cells were marked on a 4 μm hematoxylin and eosin stained section and 1–8 adjacent series of 10 μm sections, depending on the amount and cellularity of the tissue, were cut. After deparaffination, selected tumor areas were manually dissected and the tumor tissue was incubated overnight at 37°C with 1 M sodium thiocyanate to reduce the number of formalin induced cross links between the DNA strands. DNA was extracted by a column based method (QIamp DNA microkit, Qiagen, Hilden, Germany). In addition, DNA from seven head and neck squamous cell carcinoma (HNSCC) tissue samples (one fresh frozen biopsy and six FFPE samples) and one uvula sample (FFPE) were isolated by micro dissection under a stereomicroscope as described before [12]. One uvula sample (FFPE) was manually dissected. DNA was purified by proteinase K treatment and standard phenol chloroform extraction and collected by ethanol precipitation.

2.2. PCR-based DNA quality assessment

Quality of DNA isolated from GC tissue samples was tested by amplifying the human housekeeping gene *β-globin* by PCR, comparable to what has been described by van Beers et al. [13], with two primer sets that produce 209 bp (β3 forward primer acacaactgtgttcactagc and β5 reverse primer gaaacccaagagtcttctct) and 300 bp (β3 forward primer acacaactgtgttcactagc and β6 reverse primer catcaggagtggacagatcc) products. Of each FFPE sample, 50 ng DNA was added to a mixture containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase (Applied Biosystems, Nieuwerkerk aan den IJssel, NL), 0.5 μM forward primer (β3) and 0.5 μM reverse primer (either β5 or β6) with a final volume of 25 μl. The PCR reaction was performed for 40 cycles (1 min at 94°C, 2 min at 58°C and 90 s at 72°C) with an initial denaturation of 4 min at 94°C and a final extension 4 min at 72°C. Human placenta DNA and deionised water were used as positive and negative control, respectively. PCR products were visualized on a 2% agarose gel stained with ethidium bromide. DNA quality was scored based on the largest PCR product, as good (300 bp), intermediate (200 bp) and poor (no product). DNAs scored as good and intermediate are regarded to be suitable for array CGH [13].

2.3. DNA quality assessment by isothermal amplification

100 ng of genomic DNA was combined with 20 μl of primers (Enzo Life Sciences, Farmingdale, USA) and Nuclease-free water (Enzo Life Sciences, Farmingdale, USA) to a final volume of 39 μl. After 10 min of incubation at 99°C, followed by 5 min on ice, 10 μl of nucleotide mix (Enzo Life Sciences, Farmingdale, USA) and 1 μl enzyme (Enzo Life Sciences, Farmingdale, USA) were added, followed by incubation for 1 h at 37°C. Next, 5 μl Stop Buffer (Enzo Life Sciences, Farmingdale, USA) was added to stop the reaction. The amplified DNA was purified using the QIAquick PCR Purification Kit (Qiagen, Westburg, Leusden, NL) according to the manufacturer's procedures (Enzo BioScore™ Screening and Amplification kit, Enzo Life Sciences, Farmingdale, USA). The DNA yields after amplification were measured with a Nanodrop ND-1000 spectrophotometer (Isogen, IJsselstein, NL) and DNA quality was scored according to the total yield of the DNA as excellent (>10 μg), good (3–10 μg), intermediate (1–3 μg), poor (<1 μg) and no DNA input (<0.5 μg). DNAs scored as excellent, good and intermediate can be labeled for array CGH analysis according to the manufacturer.

2.4. Array CGH procedures

DNA from GC samples was hybridized onto 5K BAC arrays and CRC, HNSCC and uvula derived DNA on 30K oligonucleotide arrays. BAC CGH and oaCGH were performed as previously described [11, 14]. Briefly, 600 ng tumor and reference DNAs, either

amplified or non-amplified, were labeled by random priming (Bioprime DNA Labeling System, Invitrogen, Breda, NL) in a 50 µl reaction with Cy3 dCTP and Cy5 dCTP (Perkin-Elmer Life Sciences) respectively. Test and reference labeled genomic DNA were combined and co-precipitated with 100 µg Cot-1 DNA (Invitrogen, Breda, NL) for BAC CGH arrays and with 10 µg Cot-1 for oaCGH arrays by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. The precipitate was collected by centrifugation at 14.000 rpm for 30 min at 4°C, and dissolved in 130 µl hybridization mixture containing 14.3% dextran sulphate (USB), 35% formamide (Invitrogen, Breda, NL), 2 × SSC (Sigma) and 4% SDS (Sigma). The hybridization solution was heated for 10 min at 73°C to denature the DNA, and then incubated at 37°C for 60–120 min to allow the Cot-1 DNA to block repetitive sequences. Hybridization was performed in a hybridization station (Hybstation12 – Perkin-Elmer Life Sciences, Zaventem, BE) and incubated for 38 h at 45°C. After hybridization, slides were washed six times in 50% formamide, 2 × SCC, pH 7.0 at 45°C, and twice in PN buffer (0.1 M sodium phosphate, 0.1% nonidet P40, pH 8.0) at room temperature, twice with 0.2 × SCC and twice with 0.1 × SCC. Slides were dried by centrifugation at 1000 rpm for 3 min at room temperature.

2.5. Array CGH image acquisition and data analysis

Digital images of the arrays were acquired by using a dedicated microarray scanner G2505B (Agilent technologies, Palo Alto, USA). Spot analysis and quality control were performed fully automatically, using BlueFuse 3.4 software (BlueGnome, Cambridge, UK). When the BlueFuse quality flag was below 1 or the confidence value was below 0.1, spots were excluded from further analysis. The $\log_2$ tumor to normal fluorescence ratio was calculated for each spot and normalized against the mode of the ratios of all autosomes. Breakpoints for gains and losses were defined over chromosomes 1–22 using a segmentation algorithm [8].

2.6. Array CGH quality assessment

Array CGH quality was assessed by means of the median absolute deviation (MAD) of the $\log_2$ ratios of a chromosome arm without a breakpoint. In all tumors, $\log_2$ ratios of (part of) chromosome 2p were used for MAD value calculations, except for one tumor in which the MAD value was calculated over chromosome 2q because of a breakpoint on chromosome 2p. MAD values <0.12, $\geqslant 0.12$, <0.18 and $\geqslant 0.18$ were considered as representing good, intermediate and poor quality array CGH result, respectively.

2.7. Statistical analysis

One way ANOVA with Bonferroni p-value correction was used to evaluate pair-wise differences of MAD values between the different categories of the total DNA yield after isothermal amplification (good, intermediate and poor). Linear regression was used to calculate and test the slope between MAD values and total DNA yield (SPSS 12.0 for Windows, SPSS Inc., Chicago, IL, USA). Two cases were excluded from statistical analysis since the MAD values were extremely high (0.510 and 0.960).

3. Results

3.1. DNA quality testing for array CGH

DNA quality of all 59 GC tissue samples was tested using two methods, *β-globin* PCR and the isothermal amplification, and results of both methods were compared to the MAD value of the array CGH results. When using the PCR-based quality test, 29 samples (49.2%) showed a 209 bp and a 300 bp *β-globin* PCR product and 30 samples (50.8%) showed only the 209 bp product (Fig. 1). There where no samples without any PCR product. Using the isothermal amplification method, DNA of six samples (10.2%) was classified as good quality, 29 samples (49.2%) as intermediate quality and 24 samples (40.7%) as poor quality. As to the array CGH results, 22 tumors (37.3%) showed good profiles with a median MAD value of 0.107 (0.092–0.157), 29 tumors (49.1%) showed an intermediate quality profile, median MAD value 0.120 (0.088–0.171) and eight tumors (13.6%) showed poor profiles, median MAD value 0.164 (0.096–0.960) (Table 1, Fig. 2). MAD values were significantly different between good and poor and intermediate and good quality array CGH profiles ($p < 0.0001$) but not between intermediate and good quality array CGH profiles ($p =$ NS). A significant difference from zero was observed between the MAD value and the total DNA yield after isothermal amplification ($p = 0.002$) with a standardized slope coefficient of 0.40 (Fig. 3).

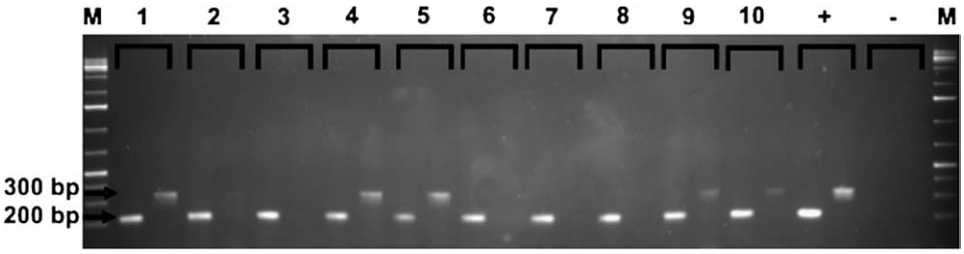

Fig. 1. Gel image of a *β-globin* PCR. DNA samples in lanes 2, 3, 6, 7 and 8 show a PCR product of 200 bp (intermediate DNA quality) and DNA samples in lanes 1, 4, 5, 9 and 10 show both 200 bp and 300 bp products (good DNA quality). M, marker; +, positive control; −, negative control.

Table 1

Representation of the 59 GC samples (FFPE). DNA quality was initially scored according to the largest product size obtained by *β-globin* PCR (PCR). Samples with fragments of at least 300 bp were scored as good DNA quality and samples displaying 209 bp fragments were scored as intermediate (interm) DNA quality. In addition, DNA quality was scored according to the isothermal amplification (DNA yield and DNA quality), and according to array CGH results based on the MAD value (MAD value and CGH result). Scores are good, intermediate (interm) or poor

Sample number	PCR (bp)	DNA yield (μg)	DNA quality	MAD value	CGH result
1	Good	4.98	Good	0.096	Good
2	Good	4.67	Good	0.092	Good
3	Good	5.80	Good	0.102	Good
4	Good	3.89	Good	0.157	Interm
5	Interm	3.41	Good	0.111	Good
6	Good	7.33	Good	0.127	Interm
7	Interm	2.34	Interm	0.118	Good
8	Good	2.44	Interm	0.105	Good
9	Good	1.08	Interm	0.124	Interm
10	Good	1.58	Interm	0.111	Good
11	Good	1.72	Interm	0.117	Good
12	Good	1.83	Interm	0.116	Good
13	Interm	1.45	Interm	0.137	Interm
14	Interm	1.15	Interm	0.171	Interm
15	Good	2.54	Interm	0.104	Good
16	Good	2.00	Interm	0.138	Interm
17	Good	1.28	Interm	0.084	Good
18	Good	1.48	Interm	0.093	Good
19	Good	2.52	Interm	0.105	Good
20	Good	2.28	Interm	0.131	Interm
21	Good	1.87	Interm	0.131	Interm
22	Good	2.00	Interm	0.163	Interm
23	Good	1.91	Interm	0.160	Interm
24	Interm	2.40	Interm	0.121	Interm
25	Interm	2.18	Interm	0.088	Good

Table 1
(Continued)

Sample number	PCR (bp)	DNA yield (μg)	DNA quality	MAD value	CGH result
26	Good	1.94	Interm	0.110	Good
27	Good	1.60	Interm	0.133	Interm
28	Good	1.87	Interm	0.123	Interm
29	Good	1.66	Interm	0.111	Good
30	Good	1.90	Interm	0.095	Good
31	Interm	2.40	Interm	0.128	Interm
32	Interm	2.62	Interm	0.162	Interm
33	Good	1.58	Interm	0.117	Good
34	Good	1.16	Interm	0.125	Interm
35	Interm	2.77	Interm	0.139	Interm
36	Interm	0.45	Poor	0.959	Poor
37	Interm	0.64	Poor	0.511	Poor
38	Interm	0.68	Poor	0.289	Poor
39	Interm	0.56	Poor	0.199	Poor
40	Interm	0.55	Poor	0.154	Interm
41	Interm	0.60	Poor	0.140	Interm
42	Interm	0.69	Poor	0.187	Poor
43	Interm	0.48	Poor	0.167	Interm
44	Interm	0.44	Poor	0.145	Interm
45	Interm	0.51	Poor	0.136	Interm
46	Good	0.25	Poor	0.169	Interm
47	Good	0.21	Poor	0.116	Good
48	Interm	0.15	Poor	0.149	Interm
49	Interm	0.39	Poor	0.168	Interm
50	Interm	0.66	Poor	0.176	Interm
51	Interm	0.46	Poor	0.161	Interm
52	Interm	0.56	Poor	0.115	Good
53	Interm	0.61	Poor	0.092	Good
54	Good	0.93	Poor	0.096	Good
55	Interm	0.62	Poor	0.178	Interm
56	Interm	0.51	Poor	0.183	Poor
57	Interm	0.49	Poor	0.253	Poor
58	Interm	0.24	Poor	0.160	Interm
59	Interm	0.50	Poor	0.184	Poor

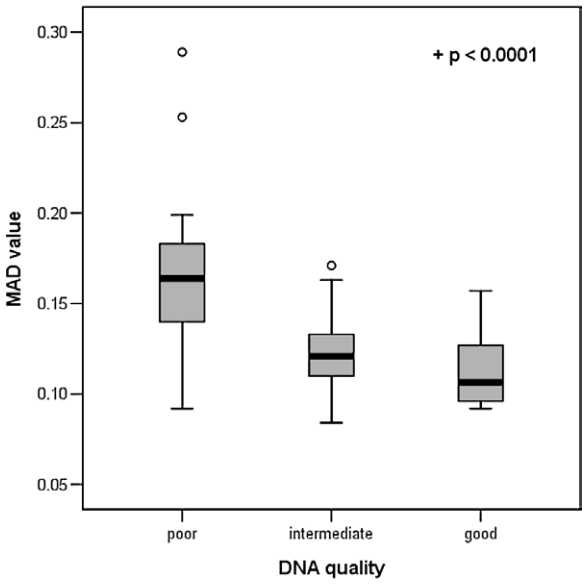

Fig. 2. Box plot of DNA quality measure obtained by isothermal amplification (x-axis; good, intermediate and poor) *versus* array CGH outcome (MAD values). Median MAD values (line across the box) are 0.107, 0.120 and 0.164, respectively. The box represents 50% of the data values and the whiskers represent the maximum and minimum values except for the outliers which are marked with circles. $+$, significant difference between good and poor and between intermediate and poor DNA quality.

According to van Beers et al. [13] DNA samples with PCR products larger than 200 bp yield successful array CGH results. When comparing array CGH results with the results of *β-globin* PCR, DNA with a PCR product $>$ 200 bp did not always yield high quality array CGH results, since DNA of eight of these samples showed poor array CGH profiles (13.6%). When assessing DNA quality based on the result of isothermal amplification, poor quality array CGH profiles were only obtained from poor quality DNAs and good and intermediate DNA quality did not yield poor quality array CGH profiles (Table 1, Fig. 3). Of all 35 DNA samples with good or intermediate quality, 18 (30.5%) and 17 (25.4%) resulted in good and intermediate array CGH profiles, respectively. Four samples (6.8%) still showed good quality array CGH profiles and 12 samples (20%) showed intermediate quality array CGH profiles, when the DNA was of poor quality according to the isothermal amplification scoring system.

The colon carcinoma sample, the seven HNSCC samples and the two uvula samples were tested using the isothermal amplification method only. Results are shown in Table 2. Good correlation between array CGH quality and prediction by isothermal amplification was found.

3.2. *Comparison hybridization of amplified and non-amplified DNA*

To test the linearity of the isothermal amplification procedure, hybridizations before and after DNA amplification were performed on two different array CGH platforms with tumor DNA from three different tissue types. Two GC samples were hybridized on oligonucleotide arrays and BAC arrays. The CRC sample and the HNSCC sample were hybridized on oligonucleotide arrays only. According to isothermal amplification scoring system, DNA from GC and CRC samples (FFPE) were of intermediate quality score, and the DNA of the HNSCC sample (fresh-frozen) was of excellent quality score. Array CGH using DNA before and after amplification showed the same pattern of chromosomal aberrations after segmentation for all samples independently of the platform (Fig. 4 and Suppl. Fig. 1: http://www.qub.ac.uk/isco/JCO). MAD values of chromosome 2p of array CGH results of amplified and unamplified DNA were comparable for 4 out of 6 samples tested. Only the CRC sample and the GC2 sample (oaCGH), showed an increase MAD value when using amplified DNA (0.117 *versus* 0.127 for the CRC sample and 0.159 *versus* 0.239 for the GC2 sample).

4. Discussion

Archival FFPE tissue samples are an important source for studying large clinical sample sets by array CGH, especially of tumors, since genomic information can be correlated to clinical pathological data and patient outcome. A major advantage of array CGH over expression arrays is the possibility to use archival FFPE material. A major limitation of array CGH however remains the high experimental costs. Costs can substantially be reduced by improving the success rate of array CGH experiments through omitting poor quality samples. Methods such as gel electrophoresis and PCR technologies have been used to test DNA quality, but are not always reliable predictors for array CGH quality. In the present study, length of PCR was not a reliable predictor for array CGH success, since within the group of 200 bp products DNA resulting in intermediate and poor quality array CGH results cannot be distinguished. When using only the DNA

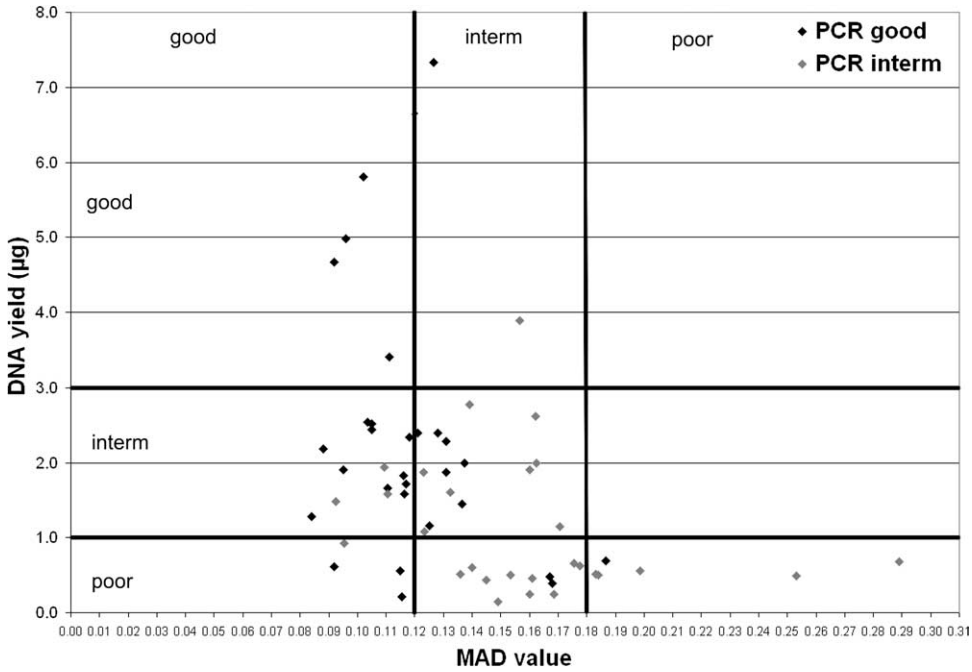

Fig. 3. Correlation of MAD values (x-axis) and total DNA yield (µg) obtained after isothermal amplification (y-axis). Each diamond represents one sample. Color of the diamonds represent DNA quality obtained by *β-globin* PCR; light grey diamonds represent samples with an intermediate (interm) DNA quality (200 bp product) and black diamonds represent samples a good DNA quality (both 200 bp and 300 bp products). The three different categories good, intermediate (interm) and poor are indicated for both the MAD values and the total DNA yield.

Table 2

Results of DNA quality testing using the isothermal amplification (DNA quality) and array CGH results (CGH result) of the CRC, HNSCC and uvula samples. Scores are excellent, good, intermediate and poor

Sample	Material		DNA quality	CGH result
CRC	FFPE	macrodissection	Intermediate	Good
HNSCC	Fresh frozen (biopsy)	microdissection	Excellent	Good
HNSCC	FFPE	microdissection	Poor	Poor
HNSCC	FFPE	microdissection	Poor	Poor
HNSCC	FFPE	microdissection	Poor	Poor
HNSCC	FFPE	microdissection	Poor	Poor
HNSCC	FFPE	microdissection	Poor	Poor
HNSCC	FFPE	microdissection	Poor	Poor
Uvula	FFPE	microdissection	Excellent	Good
Uvula	FFPE	macrodissection	Excellent	Good

samples yielding PCR products of at least 300 bp, 18 samples (30.5%) which gave good array CGH results would have otherwise been missed. Vice versa, 12 out of 59 (20.3%) samples yielding PCR products of 200 bp gave poor array CGH results. Our results show that isothermal amplification is a better and more accurate method to predict array CGH quality. Although the MAD values, reflecting the array CGH quality, between good and intermediate DNA quality obtained by the isothermal amplification scoring system are not different, poor quality CGH profiles were only obtained from intermediate and poor amplifying DNAs and good amplifying DNA never showed poor array CGH profiles. Both intermediate and good DNA quality scores obtained by the isothermal amplification method were suitable for array CGH technology which makes it less important to distinguish these two groups. Only few of the DNA samples ($n = 4$) were not in accordance to what was predicted using the isothermal amplification scoring system, with poor DNA quality

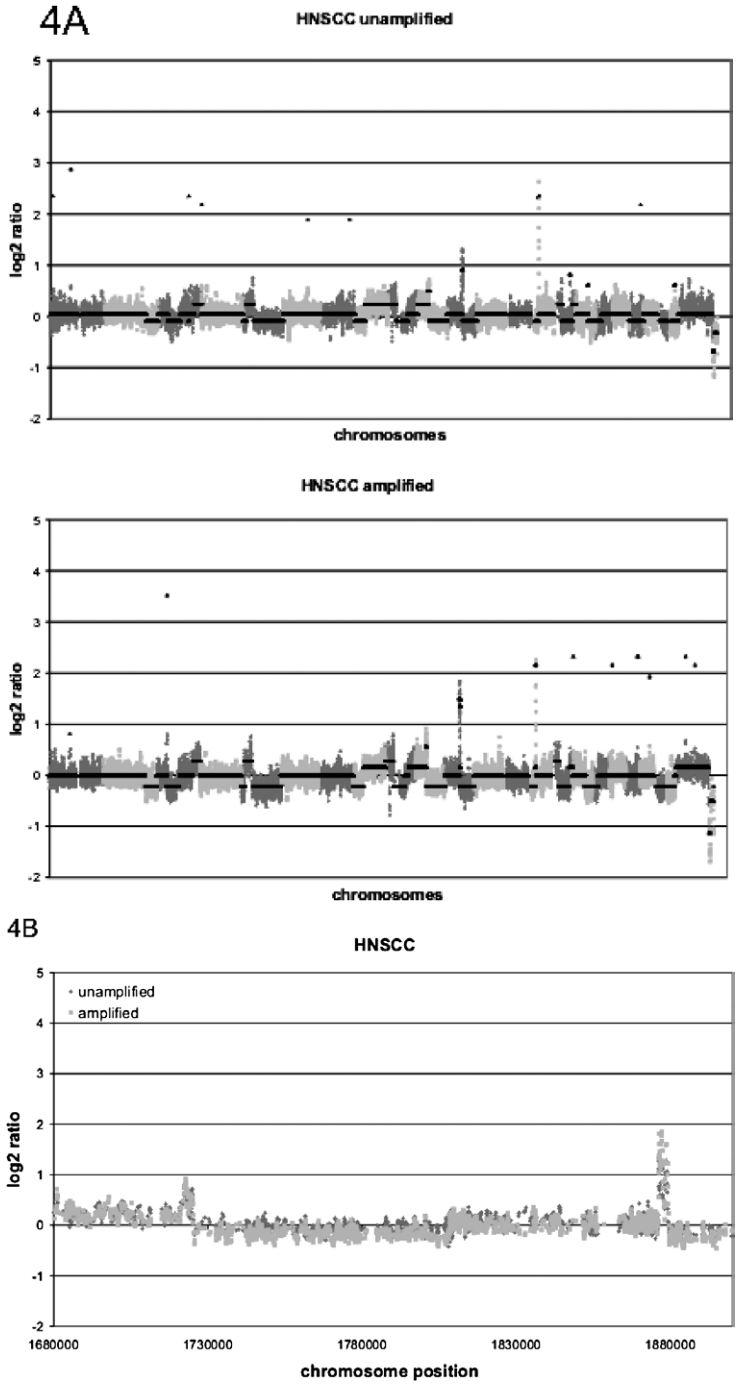

Fig. 4. OaCGH results of the HNSCC sample before and after amplification using the isothermal amplification show the same pattern of chromosomal aberrations (A). A detail of the copy number gain and loss on chromosome 10 and 11 (B). Log_2 ratios were calculated with a weighted moving average [2], using a window of 250 kb, and plotted according to chromosome position (from chromosome 1 to Y).

resulting in good array CGH results. This limitation could not be experimentally explained, since repeating the quality testing gave the same results. DNA quality testing using isothermal amplification yielded reliable prediction of array CGH quality in all HNSCC, uvula and colon cancer tissue samples. Even in difficult FFPE samples, such as gastric cancer tissues, DNA quality testing using isothermal amplification was a more precise predictor compared to PCR-based quality testing. When excluding all poor quality DNA of GC samples, 16 (20%) samples yielding good and intermediate array CGH profiles would be missed, which is superior to PCR-based testing in which 30% would be missed when excluding samples with PCR products shorter than 300 bp, and 12 samples (20%) were unsuitable for analysis when using all samples with PCR products $>$ 200 bp.

Using the isothermal amplification method, factors influencing DNA quality can be quickly and easily tested, without the use of expensive and elaborate array CGH procedures. The method was successfully applied on micro dissected HNSCC samples (results not shown). Another advantage of the isothermal amplification quality test is the resulting amplified DNA. The amplification procedure is linear, resulting in reproducible array CGH profiles, even when using DNA obtained from archival FFPE specimens. Besides little additional noise amplified and unamplified DNA showed the same pattern of copy number aberrations. Thus even if very limited amount of DNA is available, all material can be used for the quality procedure and the amplification product can be used for downstream genomic analysis such as array CGH.

In our hands, the isothermal amplification is a reliable method for identifying genomic DNAs that are suitable for array CGH, even for FFPE tissue samples, and for obtaining amplified material from small samples. Availability of good predictors for successful array CGH can reduce experimental costs and labor thus makes studies of genomic profiling on large series of archival samples sets or large clinical trials more accessible.

Acknowledgements

We thank the mapping core and map finishing groups of the Wellcome Trust Sanger Institute for initial BAC clone supply and verification. We thank M. van de Wiel for his statistical advise. This work was financially supported by the Dutch Cancer Society, grant KWF2004-3051. We furthermore acknowledge funding by the EU-sixth framework project: DISMAL, Contract No.: LSHC-CT-2005-018911.

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