APPLICATION NOTE

CGH Labeling Protocol for Small Volumes of DNA Sample

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CYTAG™ CGH LABELING KIT (ENZ-42671)

INTRODUCTION

Recent advances in genomics have dramatically increased our capacity to analyze both normal and cancer cells, revealing a multitude of changes in genomic DNA, such as mutations and copy number alterations. Array comparative genomic hybridization (aCGH) has become a valuable, genome-wide screening tool for the detection of chromosomal aberrations in the form of copy number variations. The technique is relatively simple involving fluorescent labeling of sample DNA followed by co-hybridization with normal control DNA spotted onto a glass slide or a chip. The major advantage of aCGH is that it offers improved resolution compared to traditional techniques and is more robust than mRNA expression arrays. Recent studies demonstrated the ability of aCGH to detect submicroscopic chromosomal imbalances in the range of a few megabases in 10–15% of patients with mental retardation and multiple congenital defects. Consequently, an increasing number of cytogenetic facilities have implemented this technique not only for postnatal and cancer diagnosis but also for prenatal diagnosis. Since a simple DNA sample is required, much of the laborious steps in traditional karyotyping and cell culture can be circumvented.

The cytogenetics laboratory of the CHU Clermont-Ferrand (France) conducts on a daily basis the cytogenetic analysis of prenatal (e.g. chromosome number variations), postnatal (e.g. gene fusion, mutations) and oncological/hematological (e.g. gene fusion, gene expression and mutations) samples. The laboratory has at its disposal a range of chromosomal and molecular techniques to deliver cytogenetic diagnostics to the whole Auvergne region. Amongst the services offered by this laboratory is included an array CGH platform. Engineers and research technicians working on this platform rely on small amounts of genomic material, which are extracted, purified and eluted in small volumes to carry out subsequent experiments with concentrated DNA samples. The main objective of this study was to look at the compatibility of such a sample type with the CGH labeling kit from Enzo Life Sciences. Upon adjustment of the volumes of the different reagents, satisfactory yield, high specific activity, high signal intensity and low derivative log ratios were achieved with the CYTAG™ CGH Labeling Kit from Enzo Life Sciences whilst demonstrating significantly improved labeling efficiency when compared with Agilent's SureTag® DNA labeling kit.

MATERIALS AND METHODS

DNA was extracted from either blood (n = 2) or chorionic villi (n = 2) using Maxwell® 16 LEV Blood DNA kit and Maxwell® 16 instrument from Promega. CYTAG™ CGH Labeling Kit were sourced from Enzo Life Sciences. It was shipped on dry ice and upon receipt; it was stored at -20°C in a non-frost-free freezer. Kit reagents were aliquoted and protected from light exposure at all times. Reagents provided with this kit were summarized in Table 1. Labeling with CYTAG™ CGH Labeling Kit from Enzo Life Sciences (ENZ-42671) was compared with labeling obtained with SureTag® DNA labeling kit from Agilent Technologies (5190-3400).

Reagents from Enzo Life Sciences	Vial ID	Volume Supplied
Primers/Reaction buffer	1	400 µL
Cyanine 3-dUTP Nucleotide mix	2	2x50 µL
Cyanine 5-dUTP Nucleotide mix	3	2x50 µL
Klenow Exo$^-$ DNA polymerase	4	20 µL
Stop buffer	5	100 µL
Nuclease-free water	W	1 mL

Table 1: Reagents provided with the CYTAG™ CGH Labeling Kit from Enzo Life Sciences

Labeled DNA was prepared by the incorporation of Cyanine-labeled nucleotides according to the procedure described below and summarized in **Table 2**. For each pair of genomic DNAs to be compared, one sample was labeled with Cyanine 3 and the other with Cyanine 5. If further validation is desired, the labels can be swapped in a parallel or subsequent experiment.

	Step	Component/Condition	Amount
1.	Add	DNA (300 ng)	up to 7.6 µL
2.	Add	Primers/Reaction buffer (Vial 1)	8 µL
3.	Add	Nuclease-free water (Vial W)	to 15.6 µL
4.	Incubate	99°C, 10 minutes	
5.	Incubate	Ice, 5 minutes	
6.	Add	Nucleotide mix (Vial 2 or 3)	
7.	Add	Klenow Exo$^-$ DNA polymerase (Vial 4)	0.4 µL
8.	Incubate	37°C, 4 hours	
9.	Add	Stop buffer (Vial 5)	5 µL
10.	Incubate	65°C, 10 minutes and 4°C hold	

Table 2: Procedure overview for small DNA volume reaction

Denaturing DNA and annealing random primers

300 ng of genomic DNA were combined with 8 µL of Primers/Reaction buffer (Vial 1). The reaction mixture was then brought to 15.6 µL with Nuclease-free water (Vial W). The DNA was denatured at 99°C for 10 minutes and placed on ice for 5 minutes. After a brief centrifugation, samples were returned on ice.

Extending primers with Klenow Exo$^-$ DNA polymerase

While on ice, 4 µL of the appropriate Cyanine dye-labeled Nucleotide mix (Vial 2 or 3) and 0.4 µL of Klenow Exo$^-$ DNA polymerase (Vial 4) were added to the primer-annealed DNA sample. Tubes were flicked gently to mix contents, briefly centrifuge and incubated at 37°C for 4 hours. 5 µL of Stop Buffer was added to the DNA samples. Tubes were mixed gently, briefly centrifuge and incubated at 65°C for 10 minutes and hold temperature at 4°C.

Purifying the labeled DNA

Each labeling reaction should be purified separately. MinElute® purification columns from Qiagen were used and manufacturer's protocol was followed. Samples were applied to the MinElute® columns and centrifuge for one minute. Columns were then washed twice with 700 µL of Buffer PE. Flow-through was discarded and the columns placed in clean 1.5 mL micro-centrifuge tubes. DNA was eluted twice using 15 µL of sterile water for a total volume of 30 µL.

Depending on the requirements of the hybridization platform, volume reduction may be required. The DNA 12230 concentrator from Thermo Scientific was used to bring the sample volume down to 9.5 µL, which is the volume recommended for 8x60K arrays from Agilent Technologies. Genomic DNA should not be excessively dried or pellets might become difficult to resuspend.

Determining DNA yield and dye incorporation

A NanoDrop® ND-1000 UV-VIS Spectrophotometer from Thermo was used in the Microarray Measurement Mode to determine yield and dye incorporation in 1.5 µL from each sample. DNA yield and dye incorporation obtained with the CYTAG™ CGH Labeling Kit from Enzo Life Sciences were compared with DNA yield and dye incorporation achieved with SureTag® DNA labeling kit from Agilent Technologies.

Preparing the labeled DNA for hybridization on Agilent Oligonucleotide Array

Cyanine 3- and Cyanine 5-labeled DNA eluates were combined and the volume brought up to the volume specified by Agilent for SurePrint® G3 human CGH 8x60K microarrays. DNA preparation, pre-hybridization, hybridization and array washing were performed as indicated for 8x60K microarrays in the Agilent genomic DNA analysis protocol for oligonucleotide array-based CGH (version 7.3, March 2014). Arrays were scanned with Agilent C microarray scanner and analyzed with Agilent Feature Extraction 10.5.1.1 software. Genomic copy number aberrations were identified using the ADM-II algorithm of Agilent Workbench 7.0.4.0.

RESULTS

Comparative analysis of DNA yield and specific activity

Measurement of DNA yield and specific activity allows the evaluation of the quality of the DNA labeling for CGH analysis. High DNA yield and high specific activity also correlate with increased accuracy of variant detection and minimized manual data analysis. Using either Enzo's CYTAG™ CGH Labeling Kit or Agilent's DNA labeling kit, about 500 ng of DNA were generated with an input of 300 ng of genomic material **(Fig. 1A)**. Specific activities were, however, significantly different ($p<0.001$). Each microgram of DNA labeled with Enzo's CYTAG™ CGH Labeling Kit contained about 54 pmol of incorporated Cyanine 3 and 39 pmol of incorporated Cyanine 5. In comparison, each microgram of DNA labeled with Agilent's DNA labeling kit only contained about 20 pmol of incorporated Cyanine 3 and 20 pmol of incorporated Cyanine 5 **(Fig. 1B)**.

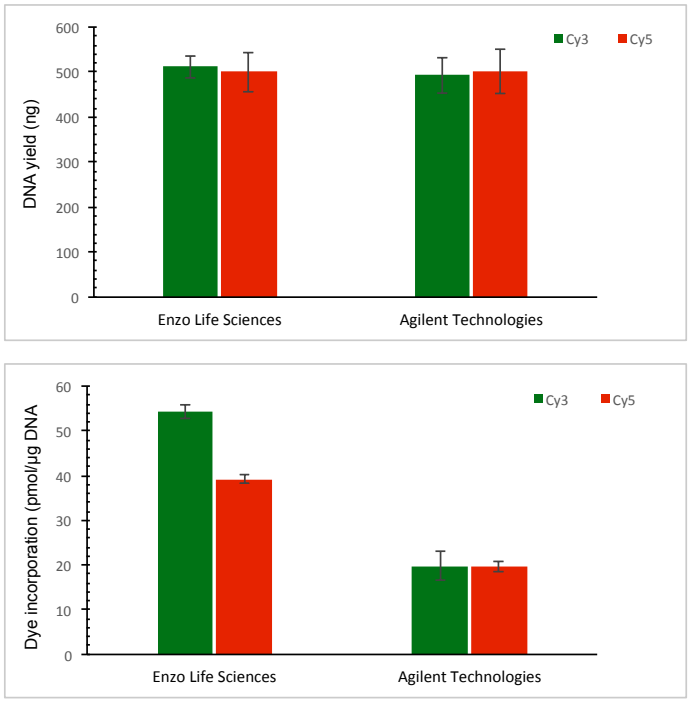

Figure 1. DNA yield and specific activity comparative analysis. DNA yield (A) and specific activity (B) after labeling with the CYTAG™ CGH Labeling Kit from Enzo Life Sciences or SureTag® DNA labeling kit from Agilent Technologies and measurement using NanoDrop ND-1000 spectrophotometer. Statistical significance was determined using the Mann-Whitney U-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$ with $n = 4$ patients).

Comparative analysis of derivative log ratio (DLRs) and signal intensity

Low derivative log ratios (DLRs) and high signal intensities reduce the need for experimental repeats. Derivative log ratios were significantly lower ($p<0.05$) using Enzo's CYTAG™ CGH Labeling Kit with DLRs around 0.12 when compared with Agilent's DNA labeling kit and DLRs around 0.18 **(Fig. 2A)**, regardless of whether the DNA samples were labeled with Cyanine-5 (DLRs1) or Cyanine-3 (DLRs2). Signal intensities were also significantly different ($p<0.01$). Enzo's CYTAG™ CGH Labeling Kit generated Cyanine 3- and Cyanine 5-labeled DNA samples with fluorescence signals of 740 and 1110 RFU, respectively. In comparison, signal intensities were about 400 RFU for DNA samples labeled with Cyanine 3 and Cyanine 5 from Agilent's DNA labeling kit **(Fig. 2B)**.

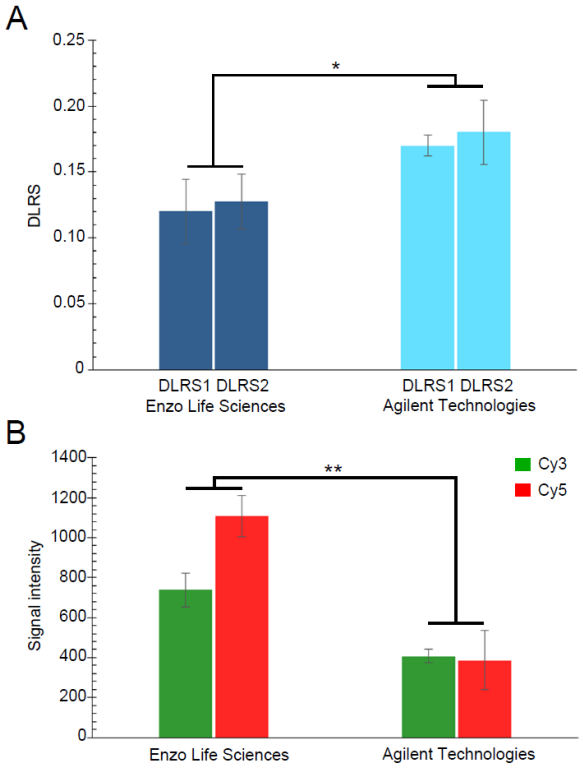

Figure 2. Derivative log ratio and signal intensity comparative analysis. Measurement of derivative log ratio (DLRs) (A) and signal intensity (B) after labeling with CYTAG™ CGH Labeling Kit from Enzo Life Sciences or SureTag® DNA labeling kit from Agilent Technologies, hybridization on a SurePrint® G3 human CGH 8x60K microarray and scanning. Statistical significance was determined using the Mann-Whitney U-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$ with $n = 4$ patients).

Superior labeling technology results in more uniform dye incorporation so that comparisons between genomes are done at higher resolution and with improved signal-to-noise ratios. High quality data provides fewer errors (false positive or false negative) and less time with manual analysis of the data, thereby increasing efficiency. Analysis of segments of chromosomes 1 and 6 of prenatal DNA samples extracted from chorionic villi and labeled with Enzo's CYTAG™ CGH Labeling Kit (**Fig. 3A** and **3B**, respectively) or Agilent's DNA labeling kit (**Fig. 3C** and **3D**, respectively) demonstrated dramatically improved signal-to-noise ratio, higher efficiency with fewer false positives and false negatives as well as a higher resolution when using Enzo's CYTAG™ CGH Labeling Kit.

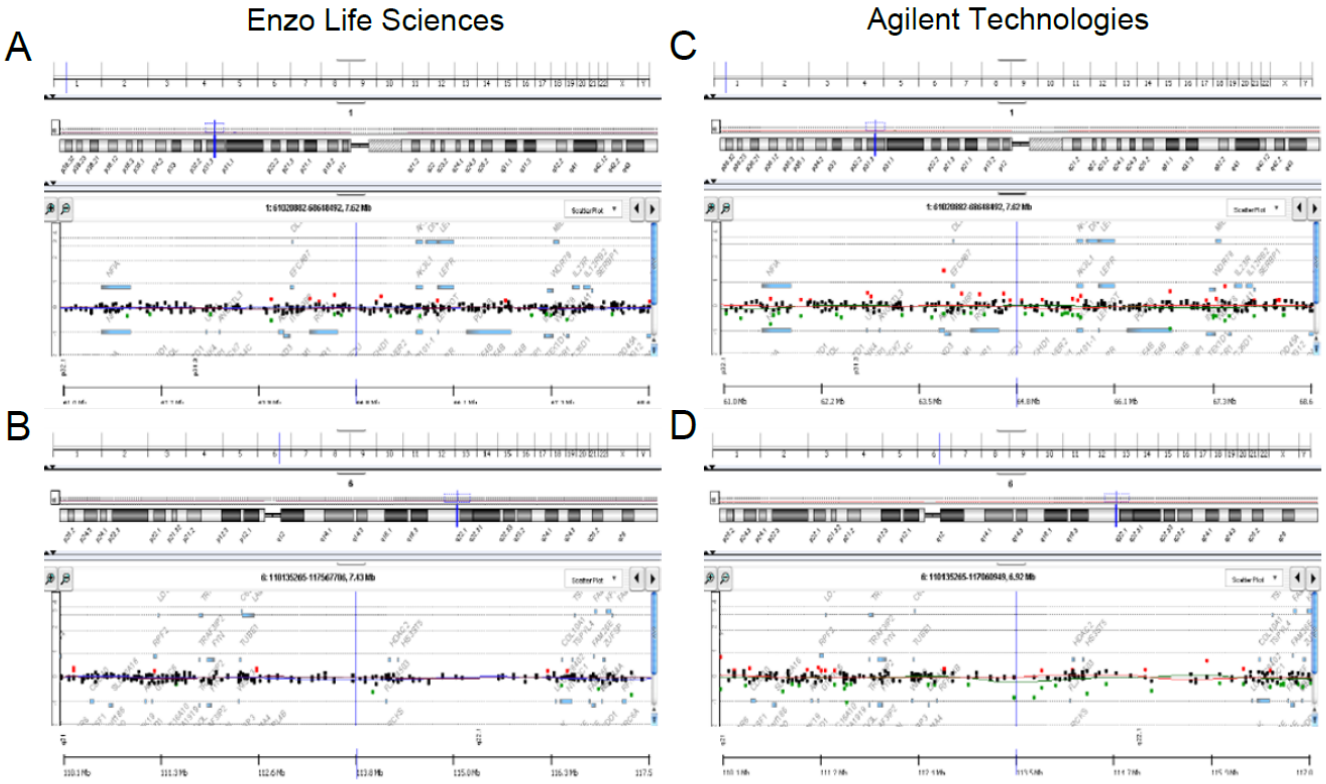

Figure 3. Comparative analysis of segments of chromosomes 1 and 6 in prenatal DNA samples. Upon scanning, the quality of the labeling in prenatal DNA samples was visually inspected. Segments of chromosomes 1 and 6 were selected to demonstrate the superior labeling obtained with the CYTAG™ CGH Labeling Kit from Enzo Life Sciences (A and B, respectively) when compared with the Agilent's kit (C and D, respectively).

CONCLUSION

The aim of this work was to establish the compatibility of Enzo Life Sciences' CYTAG™ CGH Labeling Kit with small volumes of DNA samples and determine the quality of the labeling in terms of specificity and intensity when compared with Agilent's SureTag® DNA labeling kit. Volumes of primer/reaction buffer, nuclease-free water, nucleotide mix, Klenow Exo⁻DNA polymerase and stop buffer were adjusted accordingly. The use of Enzo's CYTAG™ CGH Labeling Kit resulted in the successful labeling of small volumes of DNA samples (less than 7.6 µL) and small amounts of DNA (300 ng). The specific activity and the signal intensity were both maximized whilst minimizing derivative log ratios, thereby validating its use as ideal CGH labeling kit for small volumes of DNA samples.

Visit www.enzolifesciences.com/cgh for more information about our CYTAG™ CGH Labeling Kit including:

- References
- Cited Samples
- Other Application Notes

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