Applications

Note 188 | June 2008

Use of Cy™ labeled cDNA in microarray analyses after determination of the incorporation rate with the Eppendorf BioPhotometer plus and UVettes®

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Abstract

Photometric measurements of fluorescence-labeled cDNA samples were easily performed using the BioPhotometer plus and the Eppendorf UVette. Here, all absorption readings for the chosen 10 mm light path were well within the reproducible range for such measurements (> 50 mE). Good correlation between the measured FOI and signal intensity on the microarray was observed. It could therefore be assumed that loss of sample quality due to the measurements themselves or due to absorption inside the UVette was negligible.
Thus, the system of BioPhotometer plus and UVette is optimally suited for reproducible quality control of fluorescence-labeled cDNA probes.

Introduction

The complete decoding of an organism's genome sequence is only the beginning of the difficult task of understanding the function of all genes of an organism. Novel tools, such as „DNA Microarrays", have revolutionized molecular biology by their ability to utilize all available sequence information for functional analysis. DNA microarrays facilitate the understanding of complex regulatory networks of a living organism, for example those activated in response to changing environmental conditions [1, 2].

However, the manufacturing process of a DNA microarray is both cost- and labor-intensive due to choice and production of thousands of DNA probes representing the organism's entire genome. These DNA probes are printed onto a glass slide in a high density arrangement using robotics. These glass slides are then available for hybridization with labeled cDNA probes. Also, RNA isolation, reverse transcription into cDNA and fluorescent labeling, up to the final hybridization of the cDNA probes with the microarray, are labor-intensive and costly. Furthermore, the fact that the obtained raw data are only the basis for further analyses needs to be considered. For this reason, quality control of the various steps is of critical importance. In order to decide whether to go ahead with a given experiment, we quantified incorporation of fluorescent dyes into cDNA using the Eppendorf UVette and the BioPhotometer plus, and subsequently used these measured probes in microarray analyses. The results show reliable determination of rate of incorporation and demonstrated that the samples can be used for subsequent hybridizations without quality loss.
The rate of incorporation, i. e. "molecules of dye per number of nucleotides", is termed FOI (Frequency of Incorporation) and expressed in the units "molecules/kb or „pmol/µg". As a rule, only probes with an FOI value of at least 15 molecules/kb should be considered for use in microarrays. The new Eppendorf BioPhotometer plus was used to determine the FOI values of Cy-labeled cDNA probes. At the same time it was of critical importance to determine whether the individual photometric readings correlated with the microarray signals. The BioPhotometer plus is especially well suited for quality control of cDNA probes: directy after measuring, all important quality parameters such as dye- and cDNA concentration, as well as the resulting FOI value, are calculated automatically, followed by a clearly laid out results display.

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Often, fluorescence-labeled biomolecules are measured in very small volumes as well as in low concentrations, using microliter cells or spectrophotometers such as the ND-1000 by Nanodrop® (ThermoScientific®). These are capable of directly measuring volumes of 1 µl or less. In these cases, samples of very low concentration are measured below the detection limit of the photometer. These measurements are not always reproducible, and no reliable results can be issued regarding the rate of incorporation of the dye, as a light path of 1 mm or less, used for these measurements, is not suitable for samples of low concentration.

In the present study we are proposing an alternative procedure: labeled probes are evaluated in a larger volume, using a cuvette with a longer light path (10 mm) in order to measure samples of low concentration in a reproducible fashion. The use of a 10 times longer light path results in 10 times higher absorbance. For this reason all measurements were performed in the Eppendorf UVette. The Eppendorf UVette is a UV-transparent micro cuvette, capable of measuring volumes as small as 50 µl while retaining a 10 mm light path. The single-use cuvettes made from plastic are individually packaged and free of DNases, RNases, DNA, RNA and proteins. Undiluted samples were measured in the UVette and used directly for subsequent microarray experiments. Here, the question arose whether measurements in the UV range using the BioPhotometer plus and/or possible absorption in the UVette could result in loss of sample quality.

In the experiments presented in this report a cDNA microarray of the organism *Dictyostelium discoideum* was used. *D. discoideum* is an amoeba residing in forest soil, which displays many biological processes similar to mammalian cells. *D. discoideum* allows for the investigation of pivotal biological questions via the application of methods from the fields of molecular biology, cell biology, biophysics and biochemistry. Apart from investigations into the processes of development and differentiation, *D. discoideum* is used especially for analysis of amoeboid cell motility and cytoskeletal organization [3]. The organism's 34 Mb genome is allocated on six chromosomes, coding for approximately 12,000 genes [4].

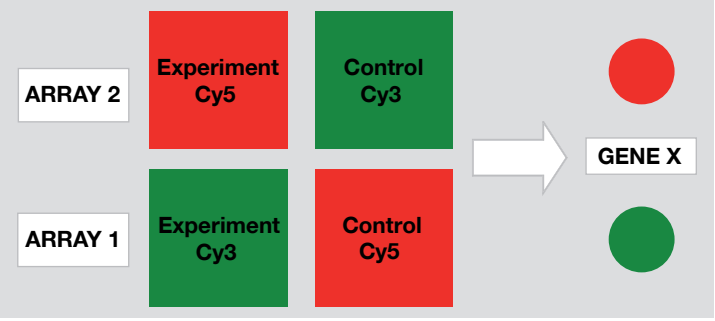

Figure 1: Dye-Swap diagram.
cDNA from untreated and treated cells is labeled with Cy3 and Cy5, respectively. In this example, upregulated genes appear red in one display (Array 1) and green in the other (Array 2).

Materials and Methods

D. discoideum RNA was isolated with the Qiagen RNeasy® Mini kit (Qiagen, Hilden, Germany) using the protocol for cytoplasmatic RNA isolation. The FairPlay™ Microarray Labeling Kit (Stratagene, La Jolla, CA, USA) was used to generate the target probes. 20 µg of total RNA were reverse transcribed into cDNA in the presence of aminoallyl-dUTP. The cDNA was subsequently labeled with activated Cy™3 and Cy™5 (GE Healthcare Europe Ltd., Freiburg, Germany) [5]. The Eppendorf BioPhotometer plus was employed for photometric determination of the FOI, using the methods Dye550-ssDNA (Cy3) and Dye650-ssDNA (Cy5), respectively. Absorbance values for the fluorescent dyes Cy3 and Cy5 were measured at 550 nm and 650 nm, respectively, while cDNA concentration was measured at 260 nm. The FOI was calculated *via* programmed methods, while simultaneously determining the amount of cDNA and fluorescent dye. Table 1 shows the calculation factors used in the BioPhotometer plus. All measurements were performed in the Eppendorf UVette.

The hybridizations included so called "dye swaps" to rule out dye artefacts. RNA from treated and untreated cells was isolated and both were labeled with Cy3 and Cy5, respectively. The corresponding Cy3 and Cy5 labeled probes were then mixed, and two microarrays were hybridized. One microarray experiment shows an upregulated gene displaying the Cy3 label, whereas the other combination shows the same upregulated gene with a Cy5 fluorescent label (see diagram in fig. 1).

Table 1: Calculation factors for the concentrations of ssDNA, Cy3 and Cy5

Extinction	ssDNA	Cy3	Cy5
1	40 ng/µl	6.7 pmol/µl	4 pmol/µl

Microarrays were read with a ScanArray® 4000XL and data analysis was performed with ScanArray® Express v3.0 (PerkinElmer Life Sciences, Waltham, MA USA). The *D. discoideum* microarray used in these experiments contains partial sequences of 450 characterized genes and approximately 5,400 non-redundant ESTs (Expressed Sequence Tags) from the Dictyostelium cDNA project [6]. A complete description of this microarray is available on the GEO website (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo; accession number GPL1972).

Results and Discussion

cDNA processing for microarrays is time consuming and manufacturing of microarrays is costly. It is important to avoid the problem of valuable arrays not being relevant post hybridization due to insufficient labeling density. Therefore, it is recommended to subject the probes to photometric analyses prior to hybridization. Usually, only small amounts of labeled probe are available, hence little material can be put aside for measurement and subsequent disposal. Typically, no more than 3 µl of sample volume are available for quality control of the probe. In the mean time certain spectrophotometers are on the market (ND-1000, Nanophotometer in combination with a microliter measuring cell), in which small amounts of liquid will suffice for measurement. However, the fact that these instruments utilize a light path of 0.2 mm or 1 mm, respectively, might result in a problem as the cDNA probes, after labeling with fluorescent dye, are present in very low concentrations of a few nanogram per microliter. If these samples were measured in a 10 mm light path, the range would be between 50 and 200 mE. This corresponds to the lower range of reproducibility in customary spectrophotometers where measurements with good reproducibility are just possible. Measurement of probes labeled with a fluorescent dye using light paths of 0.2 mm or 1 mm would theoretically result in absorption values of 1 to 4 mE or 5 to 20 mE, respectively. These values are too low to guarantee reproducible determination of incorporation rates. In order to achieve a photometrically reliable range in our experiments, the entire reaction of labeled cDNA was measured in the Eppendorf UVette, and these probes were subsequently used directly for microarray hybridization. The advantage of this procedure is that absorbance is measured in a 10 mm light path, thus enabling reproducible measurement of incorporation rates, even in low concentration cDNA. Table 2 shows the results of photometric analyses of labeled cDNA from untreated (control) and treated (experiment) cells. The Cy3 and Cy5 concentrations, as well as the cDNA concentration, were determined from the measured absorbances at 550, 650 and 260 nm. Additional measurements at 280 nm enabled a quality control (ratios A260/280 of the sample DNA. Furthermore table 2 shows the absorbance values (theoretically expected) for 0.2 mm and 1 mm light paths, respectively. These results demonstrate that all measurements would result in readings below 50 mE using 0.2 mm or 1 mm light paths. In contrast, all measurements in the UVette (10 mm light path) are in the correct range. All measurements in table 2a) were performed in the Eppendorf BioPhotometer plus. The absorbance values of fluorescent dye and cDNA can be determined in one step.

Table 2: Photometric determination of absorbance of Cy3- and Cy5-labeled cDNA

a) Results of a measurement with 10 mm light path (real measurement in the Eppendorf BioPhotometer plus and UVette)								
cDNA probe	Extinction 260 nm mE	Extinction 280 nm mE	Quotient E260/E280	Extinction 550 nm mE	Extinction 650 nm mE	Concentration cDNA [ng/µl]	Concentration Dye [pmol/µl]	FOI [Dye/kb]
Experiment Cy3	125	66	1.88	92	-	4.6	0.616	43
Control Cy3	121	62	1.96	84	-	4.5	0.562	41
Experiment Cy5	138	71	1.95	-	56	5.1	0.548	35
Control Cy5	112	56	2.00	-	71	4.2	0.408	32
b) Calculated absorbances for 1 mm light path (theoretical)								
Experiment Cy3	12.5	6.6		9.2	-			
Control Cy3	12.1	6.2		8.4	-			
Experiment Cy5	13.8	7.1		-	5.6			
Control Cy5	11.2	5.6		-	7.1			
c) Calculated absorbances for 0.2 mm light path (theoretical)								
Experiment Cy3	2.5	1.32		1.84	-			
Control Cy3	2.42	1.24		1.68	-			
Experiment Cy5	2.76	1.42		-	1.12			
Control Cy5	2.24	1.12		-	1.42			

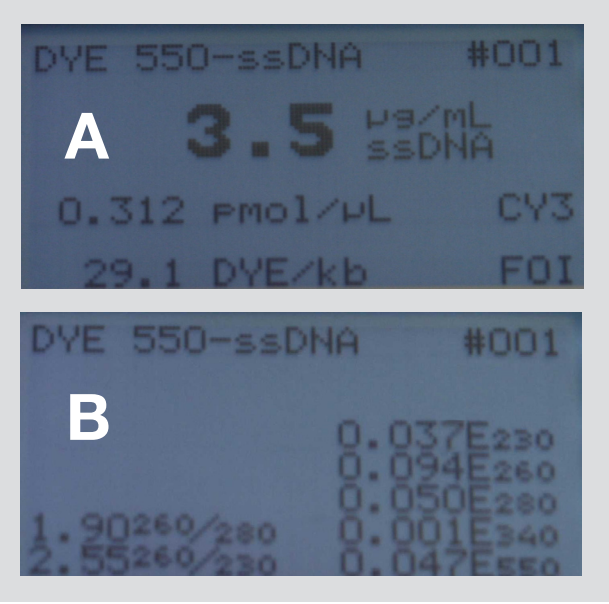

Figure 2: Typical results from measurements of a labeled cDNA probe.
A) The concentrations of cDNA, dye and FOI are calculated from the measured results shown in the display.
B) In addition, the absorbance values at 230, 260, 280, 340, and 550 or 650 nm, respectively, are shown in a subsequent display. Furthermore, the 260/230 nm and 260/280 nm ratios, indicating sample purity, are displayed. These ratios are important for the evaluation of sample quality prior to further processing.

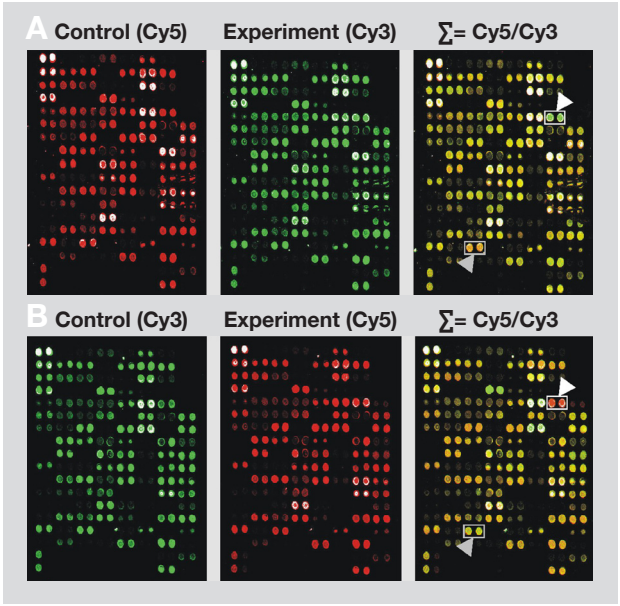

Figure 3: Results from the hybridization of the *D. discoideum* microarray with labeled probes after measuring the concentration of cDNA and dye using the BioPhotometer plus. Corresponding regions of two microarrays, resulting from two independent hybridizations (dye swap) are shown.
A) Labeling of control cDNA with Cy5 and experimental cDNA with Cy3. The white and gray arrowheads point towards one upregulated gene (green) and one downregulated gene (red), respectively.
B) Labeling of control cDNA with Cy3 and experimental cDNA with Cy5. The white and gray arrowheads indicate the same genes as in A, which are also shown as upregulated (red) or downregulated (green), respectively.

As shown in figure 2, the concentrations of both components and the resulting FOI are determined via pre-programmed parameters and displayed clearly. Following determination of incorporation rates, the probes were used for microarray hybridization. The critical questions were whether the labeled probes would be suitable for microarray analyses even after being subjected to measurement, and whether the microarray analysis would yield the expected signal-to-noise ratio. The signals of the fluorescent dyes were analyzed as described in materials and methods. Here, images were obtained for Cy3 and Cy5, respectively. Figure 3 shows the corresponding section of two different slides: the Cy3 and Cy5 signals, respectively, as well as the combination overlay of both signals. Further, figure 3 shows cross-over labeling (dye swap) of the respective probes:

cDNA from untreated cells (control) was labeled with Cy5 (upper panel) as well as with Cy3 (lower panel). The “dye swap” shows that the differentially regulated genes identified in the merged images A and B, respectively (white and gray arrowheads), are not due to dye artefacts: Therefore, upregulated genes appear green in A and red in B (white arrow), whereas downregulated genes appear red in A and green in B (gray arrow). Our results also confirm that for the same slide, signal intensities of the dyes correlate well with their previously measured rates of incorporation (data not shown).

Conclusions

The experiments presented in this report demonstrate that cDNA probes labeled with fluorescent dye can be measured in the BioPhotometer plus using the 10 mm light path of the Eppendorf UVette. The measured absorbance values are clearly within the recommended range for highly reproducible measurements (> 50 mE). If the same samples were to be measured photometrically in a 1 or 0.2 mm light path, the absorbance values would fall in the range of very few mE, leading to low reproducibility.
In order to obtain sufficient sample material for measurement in a 10 mm light path, the entire reaction volume (50 µl) of labeled cDNA was measured.

The excellent signal-to-noise ratio of the subsequent microarray experiments confirmed that sample quality was not compromised by the measurement, and that possible absorption by the plastic material of the UVette either did not occur or was negligible.
Evaluation of the labeled cDNA samples in the BioPhotometer plus proved to be especially user-friendly, since not only cDNA concentration and dye concentration are calculated simultaneously, but additionally the incorporation rate is determined. All results are clearly outlined in a display window. Thus, the system of BioPhotometer plus and UVette is optimally suited for reproducible quality control of cDNA probes labeled with fluorescent dyes.

Literature

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Ordering Information

Product	Description	Order no. international	Order no. North America
BioPhotometer plus	230 V / 50-60 Hz Adapter for Europe; further adapter variants are available 120 V / 50-60 Hz, power plug North America	6132 000.008	952000006
Thermo printer DPU 414	Including adapter and printer cable 230 V 120 V, North America	6131 011.006	952010140
Thermo paper	5 rolls	0013 021.566	952010409
UVette®	Original Eppendorf plastic cuvette, individually wrapped, to be used directly in the BioPhotometer, certified *RNase-, DNA-* and protein free; pack of 80.	0030 106.300	952010051
UVette® routine pack	Eppendorf Quality purity grade, re-sealable box; Box of 200	0030 106.318	952010069
Cuvette rack	For 16 cuvettes	4308 078.006	930001066

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