

Genisphere Kits incubated with the Advalytix ArrayBooster

Sensitivity increase of microarray experiments by an additional hybridisation step with multiple labelled detection molecules.

Introduction

The labelling kits from Genisphere have been designed to increase the sensitivity of microarray experiments by an additional hybridisation step with multiple labelled detection molecules. A specific oligonucleotide sequence is attached to the primer used for cDNA synthesis.

After the cDNA hybridisation a fluorescence labelled molecule (3DNA Capture reagent) is hybridised to the specific sequence introduced during the cDNA synthesis. These 3DNA molecules are dendrimers containing a well defined number of dye labels (45, 375 depending on the kit). The large number of dye molecules per cDNA and the fact that this number is well defined leads to high signal intensities and low variation coefficients in microarray experiments. (Refer to <http://www.genisphere.com> for further details)

The Advalytix ArrayBooster (refer to <http://www.advalytix.de> for further details) has been designed to increase the homogeneity and minimise cross hybridisations of microarray experiments by using a highly efficient agitation mechanism based on surface acoustic waves. The active agitation overcomes the diffusion limitation of the hybridisation process and ensures that the system reaches equilibrium more rapidly than in a non agitated reaction.

Experimental Conditions

Experimental results with Genisphere Array 50 and Array 350 kits (45 and 375 dye labels per dendrimer) were carried out on a rat oligonucleotide chip consisting of 50 genes. Hybridisations were compared for four experimental conditions: With and without mixing using the ArrayBooster and for a one step hybridisation and a two step hybridisation as recommended in the Genisphere protocol.

As a model system for gene expression profiling we used Wistar-Han rat liver (Charles River Laboratories) as a sample. The cDNA was prepared from 50 µg of rat liver total RNA as described in the Genisphere kit protocol (2x 25 µg) with the provided RT primer for Cy5 labelling (vial 2). After the neutralisation step the cDNA was concentrated and purified using the Qiagen (Hilden/Germany) LabelStar Array Kit eluting it in 40 µL of water (2x 20 µL) according to the enclosed manual. 5 µL of the eluted solution was used per hybridisation in a total volume of 40 µL (AdvaCard AC1A).

A total of eight slides were hybridised with two different kits (Array 50 Kit and Array 350 Kit) using two different protocols (one step and two step reaction) with and without agitation.

A Table A: Details of experiment components

Name	Description
Array 50 Kit	45 dye labels per 3DNA molecule
Array 350 Kit	375 dye labels per 3DNA molecule
one step reaction	mixing of cDNA and 3DNA, incubation of the mixed solution for 16 hours
two step reaction	hybridisation of cDNA of 16 hours, subsequent washing followed by incubation with 3DNA for 3 hours

One set of slides was hybridised as described in the Genisphere protocol using the formamide buffer at 45°C (vial 7) without the optional ethanol washing step after the cDNA hybridisation and with the CY5 3DNA Capture Reagent (3DNA, vial 1). For another set of slides the cDNA hybridisation and the 3DNA hybridisation was done in one step, with all other conditions as described above.

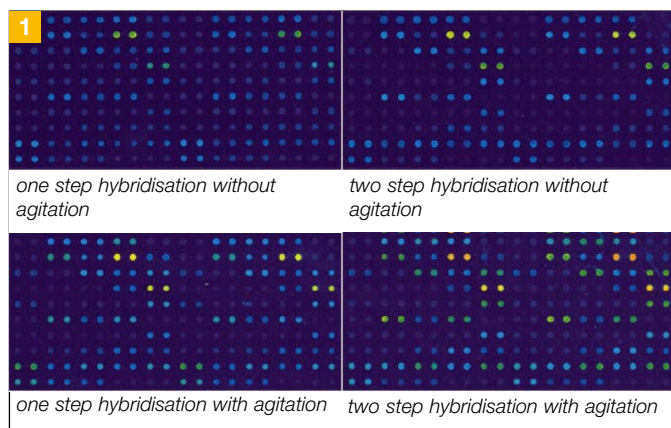
Here the cDNA and the 3DNA were mixed with the hybridisation buffer and incubated for five minutes at hybridisation temperature prior to pipetting the solution on the microarray. All cDNA hybridisations were incubated for 16 hours, the 3DNA hybridisations in the two step experiments were incubated for 3 hours.

The microarrays were spotted with 50 different 50mer oligonucleotides with four replicates each on epoxysilane-coated slides. For microarray scanning and image analysis we used the GenePix 4000B scanner and the GenePix Pro 4.0 software (Axon Instruments, Union City/USA). All data shown in this application note are raw signal intensities with local background correction.

Hybridisation Results

Array 50 Kit

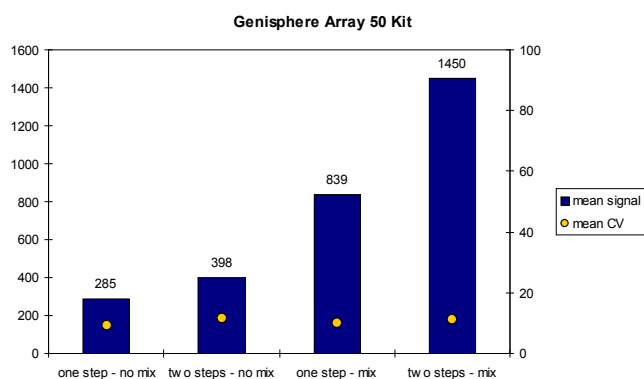
The results of the hybridisations with and without mixing, in a one step and a two step reaction each are shown in figure 1.



Pseudocolour images of microarrays incubated with different protocols. Each array contains four replicates, two adjacent in each of the two subarrays.

The two step reaction with agitation yields the highest signal intensities followed by the one step reaction with agitation. The one step reaction without mixing gives the weakest signals. The CVs for all incubations are on the order of 10% based on the four replicate spots (see fig. 2). Mixing increases the signal intensities by a factor of three for the one step reaction and a factor of 3.5 for the two step reaction.

2 Mean signal intensities and coefficients of variation (CV) of spots with signal to background ratios larger than 2. Whereas the mean CV values of the four replicates are quite similar (about 11%) in all experiments, the mean signal intensities are much higher in the mixed experiments (factor 3 in the one step experiment and factor 3.5 in the two step experiment). The highest signal intensities are achieved with the agitated two step protocol.

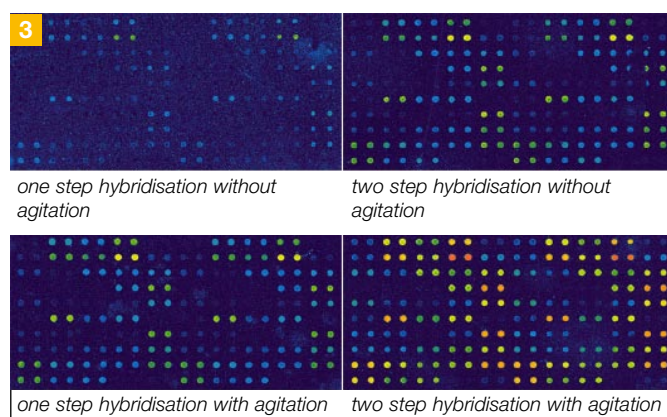


The signal intensities can be significantly enhanced by agitation as both reactions (cDNA hybridisation and 3DNA hybridisation) are diffusion limited. We believe that the lower signal intensities of the one step reaction are caused by the large size of the dendrimers. The dendrimer-cDNA complex does not bind as effectively to the spots as the cDNA molecule alone because of steric hindrance. Furthermore the diffusion rate of the complex is considerably lower than

that of the cDNA molecule. In a two step hybridisation, as recommended in the Genisphere protocol, these effects are overcome by using a very high excess of dendrimers in the second reaction step. Overall, the best results are achieved if both steps of the protocol are done separately in the ArrayBooster with agitation.

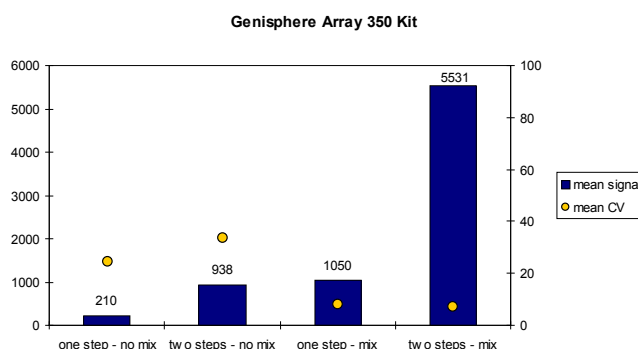
Array 350 Kit

The scans for the four different protocols are shown in figure 3 and the corresponding data in figure 4. The strongest signals are observed in the two step reaction with agitation followed by the agitated one step reaction and the two non mixed experiments.



Pseudocolour images of microarrays incubated with different protocols. Each array contains four replicates, two adjacent in each of the two subarrays.

4 Mean signal intensities and coefficients of variation (CV) of spots with signal to background ratios greater than 2. Whereas the mean CV values of the 4 replicates are quite similar (about 8%) in the mixed experiments, they differ significantly in the non mixed experiments and are much higher (24% and 34%). The signal intensity is increased by agitation (factor 5 in one step experiment and factor 6 in two step experiment). The best signals are achieved with the agitated two step protocol.

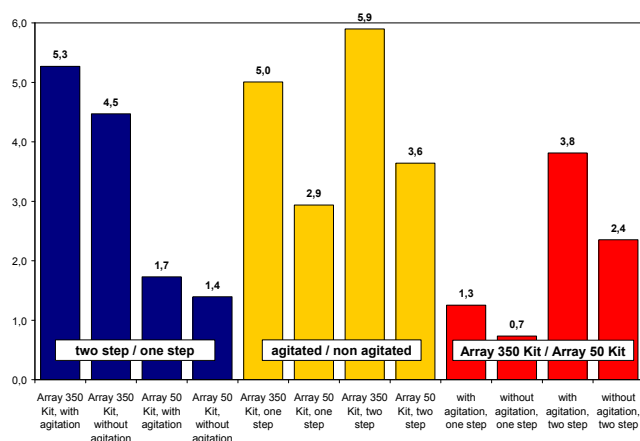


The amplification factors achieved by mixing are higher than in the case of the Array 50 Kit and amount to a factor of five for the one step experiment and six for the two step experiment. The CVs of the agitated hybridisations are about 10% whereas the CVs for the static experiments have values of well above 20%.

Discussion

In the case of the two step experiments the Array 350 Kit gives considerably higher signal intensities than the Array 50 Kit (ratios of 3.8 and 2.4) whereas the signals of both kits are comparable in the one step reactions (ratios of 1.3 and 0.7), i.e. the larger number of dye molecules per dendrimer does not lead to higher signal intensities in the one step reactions. We conclude that the dendrimers containing 375 dye molecules (Array 350 Kit) are substantially larger than those containing 45 molecules (Array 50 Kit). Increased steric hindrance and slower diffusion seems to compensate the effect of the increased number of dye molecules.

5 Figure 5: Summary of the results described in this application report. Ratios between the signal intensities shown in figures 2 and 4 are compared. Values shown in blue are the ratios between the two step and the one step reactions (two step/one step), values shown in yellow are the ratios between agitated and non agitated experiments (agitated/non agitated), and the numbers shown in red are the ratios between the Array 350 Kit and the Array 50 Kit (Array 350/Array 50).



Agitation significantly enhances the signal intensity in all experiments. This indicates that at least one of the reaction steps is limited by diffusion. The amplification factors are lower in the Array 50 Kit (2.9 and 3.6) compared to the Array 350 Kit (5.0 and 5.9). We attribute the larger amplification factor to the larger size of the dendrimers in the Array 350 Kit resulting in slower diffusion and a higher signal amplification factor. The comparison between the one step reactions and the two step reactions can also be explained along the same lines. The Array 50 Kit ratios (1.4 and 1.7) are much lower than those of the Array 350 Kit (5.3 and 4.5) indicating that larger molecules have poorer hybridisation efficiency.

In summary the best results are achieved with a two step protocol and agitation in the ArrayBooster. The fact that the agitated two step hybridisations give the highest signal intensities and the lowest CVs shows the importance of agitation in microarray hybridisations also for the Genisphere Kits.