1. Summary

Oligonucleotide fingerprinting (OFP) is a high-throughput alternative to tag sequencing methods to determine the spectrum and abundance of genes in genomic DNA or cDNA libraries. This method currently relies on the sequential hybridisations of at least 200 short (8-12 mer), radioactively labeled DNA oligonucleotides to arrayed dsDNAs. After image analysis, a sequence fingerprint is generated for each clone based on the hybridisation signals. Then according to the fingerprint similarity, the clones from the same gene can be grouped together using clustering algorithms.

The main problems of the classic OFP method include high oligoprobe number and the use of radioactivity. To resolve these problems, here we have exploited the high affinity and good mismatch discrimination of oligoprobes modified with locked nucleic acid (LNA). In our method, short (hexamer or heptamer) LNA-modified oligoprobes are labelled with fluorescent dyes (e.g. Cy5). Hybridisation results are recorded with a CCD-camera developed for this purpose. The sensitivity of the fluorescence detection permits the convenient use of homogenous liquid assay as well as nylon membrane support. Thus hybridisation data quality is improved, and the process accelerated, simplified, and less expensive.

1.1 Oligonucleotide fingerprinting using iFRET technology

A method for in-solution analysis of DNA libraries by means of hybridisation with short (6- or 7-mer nucleotides long) fluorescent oligoprobes with real-time optical detection by induced fluorescence resonance energy transfer (iFRET) has been established. Only hybridised probe is detected as its fluorescent signal is generated through the excitation of the intercalating dye when a hybridisation event takes place. Thus, elimination of unreacted oligoprobe excess is not necessary. Furthermore immobilisation of the analysed library is avoided. It is essentially independent from the surface binding capacity or DNA immobilisation chemistry, requiring only accurate dispensing of both oligoprobe and target. Therefore, the assay can be performed entirely in solution for conventional OFP analysis, in micro-droplets or nano-well-array format.

We have reduced the length of the hybridisation oligoprobes to 6-mers, which allow the use of smaller total number of oligoprobes and offer better mismatch discrimination. Furthermore, we dramatically increased the throughput of the system

in terms of the possible number of clones analysed in a single experiment, by miniaturization of the hybridisation process in a nanowell format, and by optical detection of the hybridisation through iFRET. Miniaturisation of the system and optical signal detection also led to significant reduction of analysis cost per sample. Multiplexing of the assay can be achieved using differentially labelled specific oligoprobes in the same solution. The method is homogeneous, and the generation of a universal set of genotyping reagents is possible because of the use of extremely short LNA-modified oligoprobes.

1.2. Oligonucleotide fingerprinting using nanoporous nylon membrane

Along with further exploitation of non-radioactive technique, we developed a robust method for the detection of hybridisation events using a microarray-based assay on a nanoporous membrane platform. Inherent limitations of standard arrays on glass slides, such as low binding capacity, low signal level, limited re-utilisation of the arrays and long processing time, could be overcome. The application of LNA-modified oligoprobes improved the hybridisation sensitivity, thereby allowing the use of shorter, and therefore reduced total number of oligoprobes for dsDNA characterisation.

The technique is characterized by a hybridisation time of less than 1 hour and the use of Cy5-labelled, 7-mer oligoprobes modified with LNA nucleotides. The volume of the DNA spotted onto a nanoporous membrane could be reduced to ~4 nl with detectable signal intensity. Moreover, the amount of the DNA target could be reduced to 4 fmol. The slides could be successfully stripped and re-probed for at least 19 times without significant loss of signal intensity.

In a proof of principle study 26 different LNA-modified 7-mer oligoprobes were hybridised with a set of 66 randomly selected human genomic DNA clones spotted on a nanoporous membrane slide. The performance of the approach was evaluated by sensitivity analysis using Receiver Operating Characteristic (ROC) curves. The hybridisation performances of LNA-modified and non-modified heptamers were compared. It was shown that the LNA modification clearly improves sensitivity and specificity of the hybridisation. In order to test the clustering performance of LNA-modified oligoprobes de novo clustering based on hybridisation patterns was performed and compared with in silico clustering. It was demonstrated that the

application of LNA-modified oligoprobes allows for reliable clustering of DNA sequences.

The described approach might dramatically increase the throughput of techniques based on OFP, by decreasing the total number of oligoprobes requested for analysis of large clone sets and reduction of the sample/reagent consumption. The method is particularly advantageous when numerous hybridisation-based assays must be performed for characterisation of sample sets of 100,000 or more.