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A Comprehensive Analysis of the Microarray Gene Expression Protocol and Results Using the Mouse T-cell Gene Expression Profile

Onoriode Oyiborhoro[1*], Oriakhi Kelly[2], Esosa S. Uhunmwangho[1], Kingsley A. Iteire[3] and Enoh F. Akpojotor[4]

[1]Department of Biochemistry, Faculty of Basic Medical Sciences, University of Medical Sciences, Ondo State, Nigeria.
[2]Department of Medical Biochemistry, School of Basic Medical Sciences, University of Benin, Benin City, Edo State, Nigeria.
[3]Department of Anatomy, Faculty of Basic Medical Sciences, University of Medical Sciences, Ondo State, Nigeria.
[4]Department of Computer Engineering, School of Engineering, Delta State Polytechnic, Otefe, Oghara, Delta State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. Author OO performed most of the experiments, performed wrote the protocol and wrote the first draft of the manuscript. Authors OK and ESU managed the analyses of the study. Authors KAI and EFA managed the literature searches and statistical analysis, respectively. All authors read and approved the final manuscript.

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ABSTRACT

The microarray technology is a very powerful technology that combines molecular biology and computer technology to analyze the gene expression levels for most or all of the genes in a whole genome simultaneously, at very high resolutions. This technology has wide applications, including gene interaction studies for discovery of genes responsible for different diseases; classification of

**Corresponding author: E-mail: doconos2013@gmail.com, ooyiborhoro@unimed.edu.ng;*

cancers and other diseases; prediction of clinical outcomes or prognosis for different diseases; response to therapy and development of new therapeutic agents, including gene therapy. It is therefore a very potent, unbiased and sensitive technology for the discovery of novel genes involved in the pathogenesis or control of diseases including cancers and autoimmune diseases. In the present study, we seek to give a clear and detailed account of the microarray gene expression protocol using the mouse T-cell gene expression profile, including challenges involved and how to overcome them, as well as detailed analysis of results obtained.

Keywords: cDNA; GeneChip microarray; single stranded oligonucleotide.

1. INTRODUCTION

In 1983, Kary Banks Mullis invented the polymerase chain reaction (PCR), a technique which uses specific primers to amplify total RNA in order to allow easy detection of such specifically amplified gene, which can then be visualized by gel electrophoresis. The possibility of analyzing up to 40 genes simultaneously using the microarray technology was first made public in 1995. During the initial stages of its introduction, the cDNA microarrays analyzed several hundreds to few thousands of cDNA, hybridized to extracted mRNAs of interest. Later on however, it became possible to chemically synthesize short single-stranded DNA fragments called oligonucleotides on microarray platforms to form a GeneChip unto which mRNA of interest hybridizes via its cDNA. The level of expression of such mRNA can therefore be analyzed.

With the complete human genome sequence readily available (provided by the human genome project completed in 2003) and using this oligonucleotide microarray technology, it is now possible to analyze more than 50,000 genes, including the entire 20,000-25,000 genes of the human genome simultaneously in a single experiment [1].

1.1 Basic Principles of Microarray

The human genome is believed to contain between 22,000 to 25,000 genes and each human cell has some of these genes activated and highly expressed, while others are turned off or poorly expressed. Microarray experiments seek to answer the questions: what genes are highly expressed and what genes are poorly expressed or turned off? Answers to the above questions can be provided for any cell or tissue sample, through gene expression profiling, using microarray. A particular cell or tissue sample of interest is collected; mRNA is then extracted from the sample and DNA copies called complementary DNA (cDNA) are synthesized from extracted mRNA, using the reverse transcriptase enzyme. Next, the cDNA copies are purified and labeled with fluorescent dyes, after which they are applied onto a microarray chip, where they hybridize to complementary gene sequences present on the gene chip. The microarray chip is then scanned to reveal data which can be analyzed to reveal genes that are highly expressed (up-regulated) or down-regulated.

The above basic principle can be applied in the detection of genes involved in the pathogenesis of a disease condition, for example, breast cancer. In such studies, samples are taken from both cancerous and normal breast tissues called test and control samples respectively. After tissue preparation, mRNA is extracted from both samples and converted to cDNA, using reverse transcriptase enzyme. cDNA from both samples are then purified, labeled with fluorescent dyes and placed onto a microarray slide; for competitive hybridization to synthetic complementary sequences of the short single-stranded DNA called oligonucleotides, present on the microarray chip, leaving its (cDNA's) fluorescent tag. Scanning of the chip produces red signals for genes that are highly expressed or up-regulated in cancerous tissue compared to normal tissue, while green signals are produced for genes highly expressed or up-regulated in normal tissues compared to disease tissue. Genes with equal expression levels for both normal and disease tissues produce yellow signals.

1.2 Microarray Technology as a Basic Platform for Other Technologies

Besides providing basic information concerning the level of gene expression and therefore genes likely to be involved in a particular disease condition, the microarray technology can also function as a basic platform upon which other technologies or researches are built. For example, after using microarray to identify a potential candidate gene that may be involved in a disease process due to its high expression

level (up-regulation), researchers can then proceed to carry out transgenic experiment, whereby the candidate gene is introduced into an animal model e.g mouse and observed for development of disease, including cancer. This way, the likely clinical manifestations (signs and symptoms) and anatomical changes associated with the disease can also be studied in animal models. Also, a gene suspected to be tumor suppressor gene (probably due to its high expression levels in normal tissue and down-regulation in cancerous tissue), can be tested for its tumor-suppressive effects, using gene knockout experiments, whereby the gene is knocked out or replaced in normal tissues, which are then observed for possible tumor growth in the absence on the gene.

Data obtained from microarray experiments has also been found to be very useful for the development of novel therapeutic agents for treatment of both rare and common diseases. It is hoped that such therapeutic agents will help to modify the level of expression of genes of interest and yield favorable phenotypic expressions for such gene modifications.

Using a T-cell gene in autoimmune disease as an example, a microarray experiment can be used as a platform to determine whether the T-lymphocyte is highly expressed in normal healthy individuals without autoimmune disease (control samples) and down-regulated in autoimmune disease patients (test samples). With this knowledge, researchers will then proceed to investigate the specific role of T-cells, with the question: is the T-cell gene an immune suppressor gene? To provide an answer for this question, the T-cell gene is knocked out of animal models and the effect of this knockout is observed, to see whether such knockout models would eventually develop autoimmune disease.

In a similar way, if a gene (e.g. BRCA2) is found to be highly expressed (up-regulated) in a breast cancer tissue and poorly expressed (down-regulated) in normal breast tissue as detected in a microarray experiment, BRCA2 becomes a candidate gene for the development of breast cancer. This (microarray result) will then serve as a platform for further investigation, to answer the question: is BRCA2 a cancer-causing gene? To provide an answer for this question, BRCA2 is injected into animal models using transgenic technology and such study models are then observed for development of cancer, which will confirm the role of BRCA2 as a cancer-causing gene. The above two illustrations are examples of how powerful the microarray technology can be, both as a basic research tool and a platform for other research technologies.

1.3 Heterologous Hybridization

While most conventional homogenous DNA-based microarray platforms are developed for gene expression analysis in specific species, the heterologous hybridization method allows the use of microarray platforms designed for one organism to be applied for the study of gene expression in other species within the same phylogenetic group. Such cross-species application of a single microarray platform is made possible because of the significant sequence identity or similarity that exists among different species of a phylogenetic group [2]. The immediate advantage of heterologous microarray is that there is no longer need for development of microarray platforms for every single non-traditional genetic model organism before such organisms can be studied using microarray gene expression techniques. The gene expression profiles of non-traditional models can now assessed on existing platforms for traditional genetic models, so long the organisms involved are ancestrally related. The emergence of heterologous microarray techniques has therefore helped to conserve the cost and time required for synthesis of cDNA clones, expressed sequence tags (ESTs) or oligonucleotide sequences necessary for fabricating new specie-specific microarray platforms.

However, it is worthy of note that the greater the phylogenetic distance or sequence diversity between the species in question, the lesser the reliability of results obtained. Similarly, the length of the DNA probe present on the microarray platform for the traditional genetic model may be significantly different from that of the non-traditional specie and this may impair experimental outcomes. Overcoming most of the challenges of heterologous microarray is largely dependent on the choice of experimental design [2].

1.4 Other Challenges and Pitfalls of Microarray

Despite the wide range of application and usefulness of the microarray technology, several challenges are encountered in its application. Firstly, analysis and interpretation of the huge amount of raw data obtained from microarray experiments seem to be very challenging.

Selecting useful data from not too relevant ones therefore poses some problems. A microarray experiment is also characterised by several procedures and protocols, making quality control and standardization of protocols a bit difficult. Similarly, samples and other materials used in microarray experiments are often complex and need to be in high quantity and of high quality. Very stringent planning, implementation and control of microarray experiments are therefore necessary, if meaningful results are to be obtained. Despite these observations, the microarray technology continues to evolve rapidly as a very powerful tool for scientific research.

Apart from the DNA microarray technology which involves binding of fluorescently labeled target genes to complementary probes on an array chip, other current methods of gene expression profiling include Northern blotting, Real time Polymerase Chain Reaction (RT-PCR), Serial Analysis of Gene Expression (SAGE), comparative Expressed Sequence Tag (EST), Massively Parallel Signature Sequencing (MPSS) and RNA-Seq [3]. Each of these techniques has its own challenges, advantages and disadvantages, in terms of cost or economic feasibility, technicalities involved, flexibility, genome coverage, number of samples required and complexity of bioinformatics analysis (Table 1). Although the sequence-based analytical methods such as SAGE, comparative EST and MPSS are highly specific for gene identification, they are however, more expensive to execute, when compared to other techniques that are based on gene hybridization [3]. Detailed analysis of individual technique is outside the scope of this article.

2. MATERIALS AND METHODS

As a first step in a microarray experiment to analyze the expression profile of an unknown (Ukn-1) gene in a sample containing mouse T-cells, the following equipment/materials were assembled: micro-centrifuge, micro-pipettes, eppendorf tubes and racks, stop clock, rotator evaporator, vortex mixer, water bath, spectrophotometer, corvettes, heating blocks, R100- Luckham shaker, Igloo, cy-3 mix, cy-5 mix, TRI reagent, chloroform, isopropanol, 75% ethanol, DEPC-treated water, agarose gel, oligo-DT, 2.5 M NaOH, 2M HEPES-free acid, capture buffer, 1GFX column, wash buffers, mouse array gene chip, cover slip, blocking mix, hyb mix, pre-hybridization buffers and hybridization buffers. Previously isolated mouse T-cell samples were obtained from a health facility for the purpose of this study.

2.1 Extraction of mRNA

500 ul of TRI reagent was added to the sample in order to break down the cells into their molecular components (DNA, RNA and proteins). 100 ul of chloroform was then added to the sample and vortexed for 15 secs, in order to partition molecules into different layers. Sample was then centrifuged for 15 mins and the upper-most colorless aqueous layer/phase which contains the RNA component collected into a fresh eppendorf tube, using a 250 ul pipette. 250 ul of isopropanol was added to this sample (RNA), vortexed to mix, allowed to stand for 10 mins and then centrifuged for another 10mins in order to precipitate the RNA, thus purifying it of any chloroform contamination.

Table 1. Operational features (Advantages and disadvantages) of different methods of gene expression profiling

Technique	Cost	Technical requirement	Flexibility	Genome coverage	Number of samples	Complexity of bioinformatics data analysis	Specificity
Microarray	**	**	**	***	**	***	**
Macroarray	**	*	***	**	**	**	**
RNA-Seq	***	***	**	***	*	***	***
Northern Blotting	*	*	***	*	*	*	***
RT-PCR	*	*	**	*	***	*	**
SAGE	***	***	**	***	*	***	***
EST	***	**	**	***	*	***	***
MPSS	***	***	**	***	*	***	***

*Key: * (Low) ** (Moderate) *** (High)*

The supernatant (chloroform) was removed using a pipette and 500ul of 75% ethanol added to the RNA pellet, mixed and then centrifuged at 7500rpm for 5mins, in order to wash the RNA pellet. The supernatant (ethanol) was again removed as much as possible using a pipette and the RNA pellet air-dried for 10mins, with care being taken not to over-dry. Finally, 100ul of Di-Ethyl-Pyro-Carbonate (DEPC) was added to the pellet and mixed repeatedly for 5mins, in order to dissolve the pellet. 5ul of the RNA sample was taken for running of agarose gel **(**Fig. 1) and another 10 ul taken, to which 990ul of water was added and taken to the spectrophotometer for determination of the purity/concentration of extracted RNA.

2.2 Conversion of Extracted mRNA to Single Stranded cDNA (sscDNA) and Labeling of sscDNA

3ul of oligo-DT primers was added to 8 ul of extracted mRNA sample and mixed gently. Sample was incubated at 70ºC for 5mins and left to cool at room temperature for 10 mins, to allow annealing of primer and mRNA in order to initiate synthesis of sscDNA. Sample was centrifuged for 15 secs, after which 9ul of cy-5 was added to it and mixed gently, centrifuged for 30secs and incubated at 42ºC for 1 hr,50 mins. The function of cy-5 was to label the sscDNA in the presence of cyscribe reverse transcriptase (RT) enzyme which synthesizes the new sscDNA strand. Any residual unlabeled RNA is then degraded by addition of 2.5 M NaOH, after which 10ul 2M HEPES-free acid was added, in order to neutralize the effects of the base (NaOH) and return pH back to normal. Sample was mixed by vortexing and centrifuged for 15secs in preparation for purification stage.

2.3 Purification of Labeled cDNA

500ul of capture buffer was added to a 1GFX column in order to precipitate the cDNA and get it stuck to the membrane of the column. Labeled cDNA sample was then added into the column and mixed. Following centrifugation of the column (with its content) at 13,000 rpm for 30 secs, 500ul of wash buffer was added (to wash away excess dye and contaminants) and the mixture centrifuged again at 13,000 rpm for 30secs. Next, we discarded the liquid in the collection tube, placed column back into the collection tube and centrifuged at 13,000 rpm for 30 secs. We then transferred the column into a fresh eppendorf tube, added 30ul of elution buffer to the tip of the column and centrifuged at 13,000 rpm. This was to allow dissolution of the cDNA from the membrane of the column. About 30ul of eluent was obtained, from which we took 1ul to check for labeling efficiency. The remaining eluent was dried down to less than 1ul, using a rotator evaporator. In checking for labeling efficiency, we placed 0.5ul of labeled cDNA onto one end of a slide , diluted the remaining 0.5 labeled cDNA with 10ul of water and then placed 1ul of this diluted cDNA sample onto the other end of the slide. We then allowed the slide to dry at room temperature before taking it for scanning, to check the labeling efficiency (Fig. 2).

2.4 Hybridization of cDNA to Synthetic Single Stranded Oligonucleotide DNA

In this stage of our experiments, 500 ul of pre-hybridization buffer was added to the middle of a GeneChip, taking care not to allow formation of air bubbles. A cover slip was then carefully placed over the chip, ensuring that it covers the chip part of the slide. The chip (with cover slip) was then placed in a cassette and incubated at 42ºC for 1hour. The aim of this was to reduce the background and denature the DNA contained in the GeneChip.

The cDNA sample was collected and re-suspend in the pre-hybridization blocking mix, in order to block out any non-specific binding and remove all oligo-DTs. Re-suspended cDNA was placed in a heat block at 95ºC for 2mins in order to denature it, after which it was centrifuged for 10 secs. Again, we incubated at 75ºC for 45 mins, after which hyb-mix (containing 7.5ul of hybridization buffer + 15ul of 100% formamide) was added to the cDNA, mixed very well and centrifuged for 15 secs. We thereafter proceeded to retrieve our pre-hybridization chip from the cassette and took off the cover slip, without touching the chip. The whole sample (hyb-mix + denatured cDNA) contained in the eppendorf tube was then added to the middle of the chip and a fresh cover slip carefully placed over the chip and incubated in a chamber at 42ºC in a water bath, overnight. This was to allow hybridization to take place between the synthetic oligonucleotide DNA (gene) sequences on the chip and our cDNA.

2.5 Washing and Scanning of Hybridized DNA/cDNA

Chip was placed in a 2XSSC wash buffer and allowed the cover slip to slip away into the buffer.

We then transferred the chip into a 1XSSC+0.2% SDS wash buffer and placed the container on a R100-Luckham shaker for 5 mins. Next, we placed the chip in a 0.1XSSC+0.1% SDS buffer container and again, placed the container on the R100-Luckham shaker for another 5mins. We also did the same with a 0.1XSS buffer container, after which we emptied the 2XSSC wash buffer container, replaced it with a fresh 2XSSC wash buffer and placed the chip into it for few seconds. The chip was thereafter transferred into a 5 ml tube and centrifuged at 1000 rpm for 20 secs. Finally, we covered the 5 ml tube containing the chip with aluminum foil, in readiness for scanning. All of the above procedure was to wash off the background as much as possible (while taking care not to wash off the signals as well), by maintaining strict adherence to concentration of wash buffer, temperature and timing. GoMiner analysis of Array Chip was carried out to reveal specific up-regulated and down-regulated genes (Table 2).

3. RESULTS

3.1 RNA Quality Check

The quality of RNA extracted can be evaluated either by calculating the absorbance at 260 nm/280 nm ratio (from spectrophotometer results), or by visualizing the extracted RNA on an agarose gel. Absorbance at 260nm = 0.284; Absorbance At 280 nm = 0.124; Absorbance At 320nm = -0.026. Using: Abs(260)–Abs(320)/ Abs(280)-Abs(320); we have: 0.284-(-0.026) / 0.124-(-0.026) = 0.284 + 0.026 / 0.124 + 0.026 = 0.31/0.15 = 2.06667.

Therefore, our spectrophotometer absorbance ratio obtained was 2.06667. The agarose gel electrophoresis result obtained (using a horizontal set up, used for nucleic acids) is shown below (Fig.1).

3.2 Concentration of RNA Obtained

Concentration of extracted RNA by calculation, using spectrophotometer absorbance at 260 nm (0.284), with an absorbance of 1 unit at 260 corresponding to 40 ug of RNA per ml (A260nm = 1 = 40ug/ml). This relationship being valid only for measurements in water, which was the medium used in our experiment, with a dilution factor of 100 (10 ul in 1000ul of water: 1000/10 = 100). The following results were obtained.

Using RNA (ng/ul) = Absorbance At 260nm x Factor 40 x Dilution Factor, we have: 0.284 x 40 x 100 = 1136ng/ul.

From ug/ul = RNA (ng/ul from step 1 above)/100, we have:

1136ng/ul/1000 = 1.136ug/ul.

From Total RNA = ug/ul (1.136ug/ul) x Amount of RNA Left in Tube (85ul), we have: 1.136ug/ul x 85 ul = 96.56 ug.

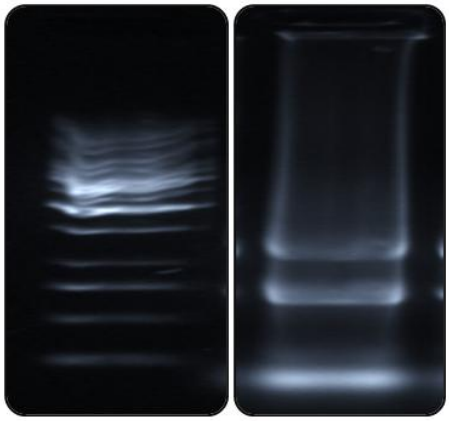

Fig. 1. Agarose gel electrophoresis result showing two bright discrete bands representing the 28S (upper band) and 18S (lower band) subunits of rRNA. The lowermost band-like areas represent small light particles, debris and other products of degradation. The uppermost parts of the result also show some tiny bright spots, representing a protein layer. The ladder-like structure at the left is the DNA size marker, a commercial 1kbp. The agarose gel picture appears to be slightly elongated, with very bright areas of degradation

Labeling Efficiency Check

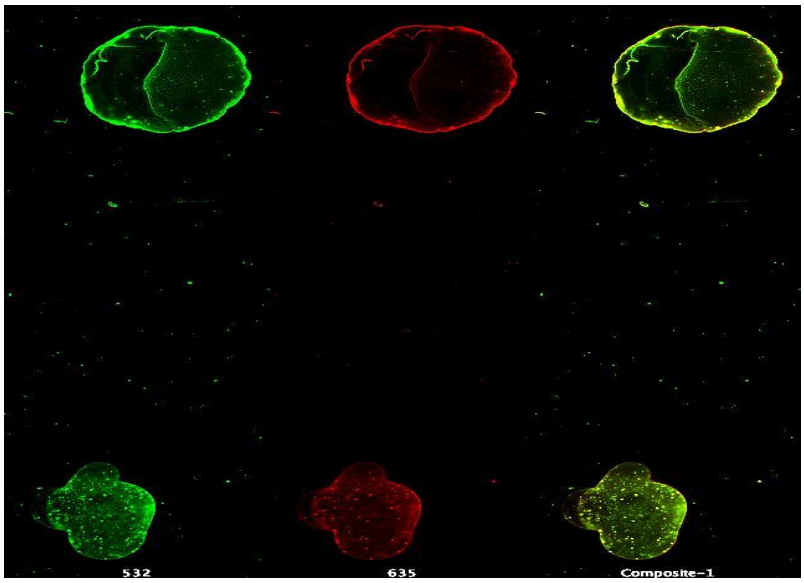

Fig. 2. cDNA labeling using the direct labeling via a reverse transcriptase enzyme procedure. Results obtained showed that the intensities of the green, red and yellow colors were not very bright and literature review suggests both technical and inherent problems as possible causes of poor labeling

Array Chip Hybridization Result

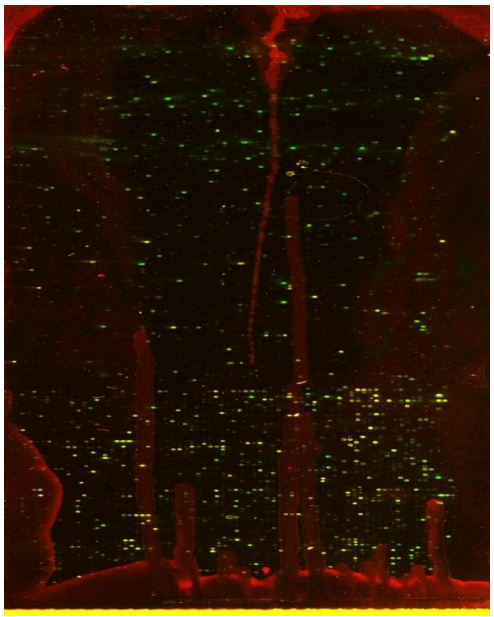

Fig. 3. Array chip hybridization result showing few red signals, some yellow signals and numerous green signals. Some streaks of background running vertically from top to bottom and other thick background around the lower aspects and upper edges can be seen. This is most likely due to insufficient washing to reduce background and allowing some wash solution to dry on the slide. Signal intensity is poor in some areas

Table 2. Up-regulated and down-regulated genes

Up-regulated genes
Zfp385,Zfp278,Xist,Wbp12,Vrk1,Vil2,Vdac3,Tnnt3,Tm4sf9,Tbx21,Surf6,Slc7a9,Shd,Scd2,ScapRny 1,Rgs5,Rec8L1,Rds,Rab5c,Ptma,Prodh2,PltpPem,Pecr,Pdcd1lg2,PAN2,Olfr24,Odf2,Myb,Mitf,Lilrb 4,Krtap8-2,Klra22Kdt1,Kcnk4,Kcnb1,Il10,Ikbke,Ik,Ifnar1,Hdac6,H2-M9,Gzmb,Galnt1,Fpr1 Fgf7,Ensa,Egr2,Edg6,Ech1,D19Ertd678e,Cxcl14,Cope,Chka,Cdh2,Cd4,Ccl4Cacnb3,Bteb1,Axl,Axi n1,Atp4a,Ap4s1,Ap1s1,Acadl.
Down-regulated genes
2900016C05Rik,3110050O07Rik,6330408G06Rik,Anxa5,Arhgef1,Arl6ip4Atp6v0d1,Cntn2,CtseCtss ,D12Bwg1266e,D4Wsu53e,Dnajc7,Dntt,Epb4.1l3Fcna,Foxo1,Ggtla1,Gstp1,Gtf2i,Hmox1,Lef1,Lmo4 ,Mdk,Meg3,Mpg,NischNlk,Phka1,Ppnr,Ptprk,Rbpsuhl,Rgs14,Sepp1,Sfmbt1,Sh2d3c,Slc2a8 Slc40a1,Smad7,Sod1,Tbxa2r,Tcstv1,Thy1,Tmem2,Tnk2,Za20d3andZan.

4. DISCUSSION

4.1 Quality of RNA Extracted

From our results, in a microarray experiment to analyze the expression profile of an unknown gene in mouse T-cell samples, we were able to extract an RNA sample with absorbance ratio of 2.0667.

Generally, this ratio gives an idea of the quality or purity of the RNA and should be as close to 2.0 as possible. Our value of 2.0667 however, shows that the quality of our RNA extracted was good. Ratios less than 1.7 would therefore suggest that extracted RNA is contaminated with other materials and should be purified. Such poor quality RNA are possibly due to high level of contamination of the RNA extracted, and could result from either or all of the following: lack of adequate TRI reagent (500 ul) or inadequate vortexing which could result in cells not being completely broken down into their molecular components, leaving RNA that is contaminated by other components like DNA and proteins. It is also possible that cells were completely broken down but molecules (DNA, RNA, proteins and lipids) were not properly separated or partitioned, due to insufficient chloroform and inadequate centrifuging.

Furthermore, failure to carefully transfer only the uppermost colourless aqueous RNA phase would also mean that some contaminants are also introduced, while errors during addition of isopropanol (which precipitates RNA), inadequate mixing, centrifuging and improper washing with 500ul of 75% ethanol can all lead to very low or negative absorbance ratio and poor RNA quality. Finally and very importantly, excessive air drying after washing could reduce the solubility of the RNA in DEPC, thus leaving a high concentration of RNA un-dissolved and consequently unavailable for spectrophotometer measurement. We particularly took note of this step and ensured that our RNA pellet was not completely dried and are strongly of the opinion that this must have contributed to the good quality of RNA obtained.

The second method of evaluating the quality of RNA extracted is by visualizing it on an agarose gel (Fig. 1). Nucleic acids (DNA and RNA) can be separated by gel electrophoresis using a horizontal gel set-up, as opposed to proteins which use a vertical set-up. The difference between DNA and RNA gel electrophoresis however, is that in RNA electrophoresis, the RNA must first be pre-treated in order to disrupt any internal base-pairing (i.e. RNA's secondary structure must be destroyed). This is necessary because extensive base-pairing in RNA molecules results in a wide range of structural conformations that affects the mobility of molecules in the agarose gel. Pre-treatment of RNA sample is therefore done either by heating or by use of formamide and both processes disrupts RNA's hydrogen bonds and denatures it. In our experiment, we achieved this purpose (denaturing) by incubating our RNA sample at 70ºC for 5 mins, using a heat block.

Also important is the fact that RNA electrophoresis must be performed under conditions that neutralize alkalinity, since RNA is easily hydrolyzed under alkaline conditions. This was provided in our experiment by the use of 5Xcyscribe buffer, which adjusted the pH prior to running our agarose gel electrophoresis.

Coming back to the results, the RNA agarose gel electrophoresis results appear as two bright discrete bands separated by an interrupting dark area. Above the upper bright band, is an elongated dark area, while the uppermost parts of the results show some very thin bright spots.

Below the lower band is another elongated dark area, below which we could see another bright band-like area. Finally and to the left of the results, is a step or ladder-like bright structure with thin horizontal lines interrupted by some wider dark areas. This is a normal RNA gel electrophoresis result.

The upper bright discrete band represents the 28S ribosomal subunit of RNA (rRNA), 4.5Kb, which is usually brighter and more conspicuous than the lower bright discrete band which represents the 18S rRNA (1.9Kb). Presence of any sharp bands above the 28S rRNA band indicates the presence of excess DNA in a sample and can be eliminated by treating such samples with RNase-free DNase to degrade such residual DNA contaminants. Although our results did not present with any obvious bands above the 28S rRNA, this however does not necessarily translate to total absence of contamination, especially by low level residual genomic DNA. Consequently, it is usually good practice to always treat samples with RNase-free DNase as noted previously. The tiny bright spots at the upper-most parts of the result is the protein layer, while the other bright band-like areas below represent the degradation, forming light particles or debris and some DNA particles. The step-like structure on the extreme left of the results is the DNA size marker which actually, is a commercial 1Kbp ladder.

Close examination of the agarose gel results would reveal some form of elongation. This may be due to 'Tailing' of the major bands down the gel, suggesting some degree of degradation of RNA sample, despite its high quality. Finally, we also observed that the lower-most band-like areas representing degraded particles, debris and some DNA particles, was very bright for tube, suggesting that lots of contaminants and debris were isolated from RNA sample in tube during extraction and this must have contributed to the high quality of RNA obtained (2.0667).

4.2 Concentration of RNA Obtained

The concentration of RNA is usually determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer and an absorbance of 1 unit at 260 nm corresponds to 40 ug of RNA/ml (A260=1=40ug/ml). However, this relationship is valid only for measurements in water, which was the medium used in our experiment, with a dilution factor of 100 (10ul in 1000ul of water: 1000/10=100).

In the experiment, the concentration of RNA expected was at least 50 ug. Our experiment however, obtained an RNA concentration far above this value (96.56 ug). This again, was consistent with the high quality of RNA earlier obtained (although a high concentration does not always mean high quality). Abnormally low RNA concentrations could result from contamination of cuvettes with RNase, which degrade RNA. This problem can be prevented by making sure that cuvettes are RNase-free, by washing with RNase-free water. The use of the buffer in which the RNA is diluted for zeroing the spectrophotometer is also advised.

4.3 cDNA Labeling Efficiency Check

In our experiment, we used the direct labeling via a reverse transcriptase procedure. It was therefore important that our labeling efficiency is good because the reasonably high quality and concentration of RNA obtained may not yield a reliable hybridization data if labeling efficiency is poor. The cy-5 fluorescent dye was used for labeling our RNA sample and the intensity of the colour of labeling indicated how well the sample was labeled.

From results obtained (Fig. 2), the intensities of the green, red and yellow signals in our experiment were not very bright. Review of literature on labeling efficiencies with cy-3 and cy-5 fluorescent dyes suggests technical and inherent problems, some of which are beyond the scope of this report, as possible causes of poor labeling. They were however, unanimous in their opinion that the very first step towards an efficient cDNA labeling was to obtain as much quantitative and qualitative RNA sample as possible, during RNA extraction and purification procedures. That is, the higher the concentration and quality of the RNA sample obtained, the more efficient the labeling is likely to be.

4.4 Array Chip Hybridization Results

In experiments to analyze an unknown mouse T-cell gene expression profile, we obtained an array chip hybridization result with very few red signals (corresponding to the Cy5 labels with emission wavelength of 670 nm), moderate amount of yellow signals and numerous green signals (corresponding to the Cy3 labels with emission wavelength of 570 nm). The relative intensity of these signals provides some ratio-based information on genes that are up-regulated (represented by the green signals) and

those that are down-regulated (represented by the red signals). GoMiner analysis of Array Chip hybridization results revealed specific up-regulated genes such as *Sirt 1, Egr 2* Cxcl14, Ccl4 *and CD4* and down-regulated genes such as *Foxo 1* and *Sod 1*.

4.5 Type III Histone Deacetylase Sirtium 1 (sirt 1) - Silent Information Regulator 1

The type III histone de-acetylase sirtium 1 (sirt 1) is a known suppressor of both adaptive and innate immunity. Up-regulation of sirt 1 is therefore required for the negative regulation of T-cell immune response, by suppressing the production of IL-2, a promoter of T-cell proliferation. Also, T-cell receptor mediated recognition of membrane histocompatibility (MHC)/antigen complex by self-reactive T-cells in lymphoid organs, often leads to programed cell death (apoptosis) or cellular silencing through a process known as “ANERGY”.

Anergy is therefore a crucial pathway for the prevention of autoimmunity in mammals and failure of this pathway often leads to increased risk of development of autoimmune diseases like diabetes, SLE, rheumatoid arthritis and multiple sclerosis. What then is the role of Egr2 in all of these? This very important biological pathway called anergy is known to be initiated and maintained by a group of suppressive genes including Egr2, Egr3, Itch, Cbl-b and sirt1, through a self-antigen/TCR coupling activity. Up-regulation of the suppressive genes/proteins listed above including Egr2, is therefore very important and crucial for the maintenance of T-cell tolerance and consequent prevention of autoimmune disease.

This up-regulation of Egr2 and other suppressive proteins is usually mediated by the nuclear factor of activated T-cell (NFAT), which in turn, is activated by the TCR-mediated calcium/ calcineurin pathway [4].

4.6 Early Growth Response 2 (Egr2) Gene

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disease caused by both genetic and environmental contributory factors (Vaishali M et al., 2017). The early growth response 2 (Egr2) gene, a zinc finger transcription factor, has become a central focus in the search for active key players in the SLE pathogenesis pathway. While it is generally believed that knockout of Egr2 in mouse CD2+ T-cells leads to excessive proliferation of T-cells and consequent development of lupus-like autoimmune disease, Keiko Myouzen et al. [5] was able to show that enhanced expression of Egr2 may in fact, increase susceptibility to SLE in humans – an opposite of the situation in mouse.

In their study, Keiko and colleagues observed that single nucleotide polymorphisms (SNPs) in the Egr2 region of the human genome could have a significant positive effect on the expression level of Egr2. Specifically, they identified an SNP situated at the 5’ flanking region of Egr2, the rs10761670 SNP, to be involved in up-regulation of Egr2 expression levels and consequently increased susceptibility to SLE and other autoimmune diseases in humans. Besides the rs10761670SNP, in vitro studies also revealed that two other SNPs, the rs1412554 and rs1509957, both in complete linkage disequilibrium (LD) with rs10761670, enhance the binding of transcription factors, thereby increasing the expression levels of Egr2. This observation suggested a causal regulatory variant role for rs1412554 and rs1509957 SNPs in the up-regulation of Egr2 and consequently, increased susceptibility to SLE in humans but not in mice. Other SLE-susceptibility genes identified through the use of candidate gene study approach include HLA-DRBI, FCGR2B/3A/3B, PTPN22, STAT4 and IRF5 (Vaishali M et al., 2017); while the genome-wide association study (GWAS) approach identified TNFAIP3, BANK1, ITGAM, PXK, KIAH1542 and C8orf13-BLK genes (Paula R et al., 2014; Armstrong D et al., 2014).

In mice, the early growth response (Egr) family including Egr2 is usually expressed during thymus T-cell differentiation and Egr2 plays a vital role in the myelination of peripheral nervous system and development of the hindbrain (Okamura T et al., 2018). In this organism, increased expression of Egr2 has been linked to T-cell receptor (TCR) engagement. Interestingly, counter-effects have been observed in members of the Egr family, in which Egr1 potentiates T-cell proliferation by up-regulating IL-2 while Egr2 and Egr3 suppress T-cells [5].

4.7 CCL4 and CXCL14 Genes

The chemokine family comprising the CC chemokine (CCL1-28), CXC chemokine (CXCL1-6), C chemokine (XCL1 and 2) and CX3C chemokine (CX3CL) are very important factors in the functioning and regulation of the immune system. These chemokines interact with various

receptor sub-groups to bring about an effective regulation of "cellular traffic" in and around several important cells, tissues and organs that constitute the immune system [6].

In vitro experiments carried out in the past have demonstrated the importance of chemokines in the initiation and regulation of migration of mobile cells including T-cells, through the establishment of appropriate physiological and biochemical gradients both in health and disease. They are therefore believed to play active roles in lymphoid organogenesis, lymphocyte generation, differentiation and proliferation and apoptosis. They are also involved in leukocyte adhesion, regulation of cytokines and degranulation. For example, infiltration of tumors by T lymphocytes, especially CD8+ and regulatory T (Treg) cells, is known to be influenced by levels of expression of chemokines, including CCL4 and CCL20 [7].

Using reverse transcriptase-PCR and immunohistochemistry techniques, Liu and colleagues were able to demonstrate that up-regulation of CCL4 and CCL20 in Squamous Cell Carcinoma (Scc) correlated with an increase in expression of CD8+ and regulatory T cells, respectively. Such CCL4-induced expression of CD8+ T cells was further observed to enhance survival rates in Scc patients, while CCL20 induced expression of Treg resulted in poor prognosis. However, in a second pathway, CCL20 has been observed to play a contrary role by mobilizing T helper 17 (Th17), which in turn, activates dendritic cells, with consequent recruitment of CD8+ T cells at tumor sites, with its associated positive prognostic effects. Consequently, CCL20 is believed to have dual opposing effects on natural anticancer immune responses and is therefore not an ideal or reliable prognostic factor [8].

The chemokine CXCL14 is known to be highly expressed in the hippocampus, where it is involved in the regulation of neuronal synapses and hippocampal integrity in neural progenitor cells (NPCs) and mature mammals, respectively [9]. Dysregulation of the normal functioning of chemokines would therefore upset immune homeostasis and pre-dispose to infections, nerve tissue injury and sometimes autoimmune disease including diabetes and SLE. Bradly et al. (1992), demonstrated that pancreas-infiltrating CD4+ T-cells produced a wide range of chemokine and that a high CCL3/CCL4 ratio in pancreatic cells of experimental mice is associated with destructive insulitis, while a low CCL3/CCL4 ratio was observed in diabetes-resistant mice. This observation highlights the importance of the CCL4 gene but not CCL3, in the conferment of resistance against diabetes, an autoimmune disease. Up-regulation of the CCL4 gene is therefore desirable. However, further experimental studies would be necessary to detect a possible role of CCL4 in the establishment of tolerance.

4.8 CD4 Gene

Development of autoimmune disease is usually characterized by a dis-regulation of T-cell differentiation and consequent proliferation. Regulation of this process is therefore crucial to the prevention and treatment of autoimmune disease. Using genome-wide association studies (GWAS), many loci that play a role in T-cell response, including the CD4+ T-cell loci, have been identified. Studies carried out on naive CD4+ T-cells and in vitro differentiated Th1 CD4+, memory Th17-negative CD4+ and Th17-enriched CD4+ T-cells using microarray technology revealed up-regulation of memory Cd4+ T-cells, compared to the naive Cd4+ T-cells of the genes located within the immune disease loci of the human genome. Among the memory T-cells subsets, Th17-enriched CD4+ T-cells were more up-regulated than the Th17-negative cells.

Carrying out studies on the expression signatures in immune-mediated disease-associated genes, Wei Zhang and colleagues sought to elucidate the molecular mechanisms responsible for the difference in expression levels of in vitro differentiated Th1, Th17-enriched and TH17-negative CD4+ memory T-cells. Aim of study was to investigate the role of Th17 CD4+ memory T-cells in health and autoimmune disease. With a background knowledge of the roles of IFN (interferon) gamma, IL-23 and Th1 cells in the development of autoimmune disease through the IL-23 pathway in both humans and mouse models, they were able to demonstrate, through intracellular staining of IL-17 and IFN, that CD161+/CCR6+ and CD161-/CCR6- distinguish Th17-enriched from Th17-negative CD4+ memory subsets respectively. Results obtained showed that a significant fraction of the CD161+/CCR6+ cells expressed IL-17 (up to 52%) in Th17-enriched CD4+ memory T-cells, while the CD161-/CCR6- cells expressed minimal IL-17 in Th17-negative CD4+ memory T-cell.

Furthermore, promoter DNA methylation of the various CD4+ memory T-cells at conserved CpG islands was observed to result in down-regulation of genes. Also, a novel IL23R isoform region on chromosome 1p31 was found to up-regulate gene expression for all CD4+ memory T-cells, with the Th1 subset showing the highest degree of up-regulation.

In conclusion, Wei Zhang et al.[10] were able demonstrate that:

- Enrichment of Th17/Th1 expression up-regulates memory CD4+ genes.
- Presence of isoforms (IL23R) also up-regulates memory CD4+ gene expression levels.
- Post-transcriptional regulation (methy-lation) down-regulates memory CD4+ gene expression.

It is however important to note that these observations do not necessarily establish an autoimmune disease state. More recently, several epigenetic changes such as DNA methylation and non-coding RNAs have been linked to the development of autoimmune disorders, including SLE, rheumatoid arthritis and Sjogren's syndrome, collectively referred to as Systemic Autoimmune Rheumatic Diseases (SARDs) [11]. A key factor that has been identified in the development of SARDs is an alteration in CD4+ T cells and other co-stimulatory molecules which compromise immune regulation and responses [12]. For example, in SLE, treatment of polyclonal human CD4+ T cells with DNA methyltransferase (DNMT) inhibitors have been observed to cause autoreactivity of treated cells and consequent induction of a Lupus-like syndrome.

The autoreactivity of CD4+ T cells was also observed to be associated with up-regulation of adhesion molecules such as CD11a and CD18, with demethylation at the promoter region of CD11a in CD4+ T cells of SLE patients. Furthermore, methylation of microRNAs such as MiR-21, miR-148a and miR126 have been linked to down-regulation of DNMTs in SLE cases, with consequent hypomethylation of CD4+ T cells and overexpression of disease-promoting genes, including CD40L and CD70, especially in the presence of Systemic Sclerosis [13]. Epigenetic profiling of CD4+ and CD8+ cells of patients suffering from Grave's disease has also revealed hypermethylation of genes involved in T-cell receptor signaling, including ICAM1, CD247 and CTLA4 [14]. These findings largely indicate the central role of altered CD4 gene in the dysregulation of natural immune responses and consequent development of SARDs.

4.9 FOXO1 Gene

The forkhead transcription factors (FOXO), belong to family nuclear proteins with a conserved DNA-binding domain. These transcriptional factors regulate a host of biological processes including metabolism, cell growth, development and immune functioning. Members of the FOXO family include FOXO1, FOXO3a and FOXO4. They are actually a sub-group of a larger family known as the forkhead-box factors. The FOXO subfamily is actively involved in oxidative stress response and cell proliferation and is therefore believed to play a role in the pathogenesis of autoimmune diseases like rheumatoid arthritis and SLE.

Using human or mouse primary lymphocytes and lymphocyte cell lines, Chia-Chen Kuo and Shih-Chang Lin [15], were able to demonstrate that down-regulation of FOXO activity resulted in enhanced cell proliferation, a significant feature in SLE and rheumatoid arthritis. Furthermore, FOXO gene knockout in mice resulted in embryonic lethality (Cabrera-Ortega A. et al., 2017), suggesting that FOXO genes (particularly FOXO1) are actively involved in lymphocyte homeostasis, through an immune-suppressive function [16]. Absence of FOXO also resulted in loss of regulatory T-cells' ability to curb excessive immune response [17]. Finally, Kuo and Lin also observed that FOXO1 and FOXO3 genes were dominant at the transcript level in peripheral blood mononuclear cells and that SLE and rheumatoid arthritis patients had significantly lower FOXO1 transcript levels than was seen in healthy controls. Concluding, they suggested that transcriptional dis-regulation of FOXO1 gene could have a link with the pathogenesis or development of SLE and rheumatoid arthritis. An experimental finding (using microarray technology) that suggests down-regulation of FOXO1 gene in autoimmune disease like SLE is therefore consistent with the above conclusion.

4.9.1 SOD1 gene

Amyotrophic lateral sclerosis (ALS) is a severe disease condition characterized by progressive degeneration of the nervous system. Although the cause of ALS is not known, genetic factors have been suggested in both familial and

sporadic ALS, while environmental factors associated with occupational exposures are also believed to be risk factors for development of ALS. Animal models used for the study of ALS also showed inflammatory and immune abnormalities, which were traced to mutations in the superoxide dismutase 1 (SOD1) gene [18]. It was therefore believed in the past, that abnormalities of the immune system may contribute to the development of ALS.

More recent studies however suggest that the immune changes seen in ALS is probably a response to damage to nervous system, rather than a cause of ALS and could therefore be protective in nature [19]. Down-regulation of the SOD1 gene would therefore suggest an abolition of this protective role. However, once activated, this immune response could also worsen an ALS condition. Methods aimed at promoting the beneficial and protective effects of the immune response are therefore targets for development of therapy against ALS and other neurological diseases.

Although the mechanism of action is not fully understood, mutation in SOD1 gene is believed to worsen ALS disease. In humans, post-mortem studies carried out on ALS patients also revealed immune abnormalities [20]. Also, gene expression profile studies of post-mortem human samples revealed up-regulation of the signalling gene TLR4, suggesting a chronic macrophage activation. Mutation in SOD1 gene was however found to account for only a small proportion of subjects in humans and the implication of this is that observations in mouse models cannot be generalized to include humans.

When treated with intra-ventricular injection of the enzyme capase1, SOD1 mutant mice were observed to show reduction in disease severity, suggesting that the capase1 pathway, mediated by inflammatory molecules (e.g. IL-1beta), have a role to play in the pathogenesis of ALS. Finally, vaccination of SOD1 mutant mice with SOD1 protein induced protective immunity and lessened disease, giving credence to the earlier suggestion that down-regulation of SOD1 may be associated with increased risk for ALS and associated inflammatory and immune conditions in mice.

Our results also showed some streaks of background running vertically from top to bottom and some thick background around the lower aspects and upper edges of the chip, all of which effectively obliterated signals around these areas. The signal intensity was also observed to be poor in some areas. Generally speaking, signals can be weakened by low RNA concentration, insufficient labeling and by probe cells having too few molecules to capture adequate signals during hybridization.

In our experiment, we are strongly of the opinion that areas of poor signal intensities resulted from insufficient labeling, rather than low RNA concentration. Problems arising from probe cells having too few molecules to capture adequate signals are usually due to the choice of probe selected and there are high density DNA microarray probes with thousands of probe cells, in a variety of grid styles. The task of managing which clone goes where, among the huge number of probe cells present, can sometimes be intimidating. A number of companies however, perform image analysis by placing a grid over the array, to allow integration of the signals from each probe cell and this helps in solving problems due to probe cells [21].

The streaks of background seen in our array chip hybridization results could be due to several reasons: the hybridization solution might have been too intensely fluorescent and therefore needed to be fully removed prior to scanning; some wash solutions might have been allowed to dry on the slide or the chip (slide) might have been left to sit for too long in the centrifuge (this should ordinarily not be more than a few seconds) before spinning. In our experiment, we suspect that all 3 factors listed above were compromised. That is, our chip was not sufficiently washed to get rid of the background. Also, some amounts of wash solution must have been left behind and allowed to dry on the slide. We also left the chip to sit on the centrifuge for a period longer than necessary, while executing some other experiments. This again, must have contributed to the drying of some amounts of wash solution on our chip.

Other problems that may be associated with array chip hybridization results include irregular spot morphology due to printing irregularities, which can be avoided by regular pin maintenance and careful monitoring of spots; pre-hybridization high background due to poor coating of slides with fluorescence, which can be solved by scanning the slides before printing and comet tails formed by unbound DNA on slides, a problem that is avoided by washing printed slides with 1% SDS prior to hybridization, with a view to eliminating all unbound DNA.

5. CONCLUSION

The microarray technology remains a very powerful tool for gene expression profiling, even in the presence of other rapidly evolving techniques. It has found useful applications in the discovery of disease genes, classification of cancers, disease diagnosis, prediction of clinical outcomes and development of new therapeutic agents and targets. This article therefore brings to the scientific domain, a detailed protocol and result analysis of a sophisticated technology that remains valid in a rapidly developing field of Molecular Biology. As the technology continues to evolve with possible reduction in costs of installation and operation in the future, it is hoped that it will also become readily available and accessible, even in poor economies of the world. A detailed step by step protocol as contained in this article is therefore highly relevant, especially in areas where the microarray technology is still relatively new.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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