

## Molecular Diagnosis of Lymphoma

### *Outcome Prediction by Gene Expression Profiling in Diffuse Large B-Cell Lymphoma*

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#### Summary

This chapter describes the illness diffuse large B-cell lymphoma (DLBCL) and why research has and continues to focus on creating accurate predictors of response to treatment to allow individual risk assessment for a patient and individualization of treatment choice to maximize the chances of cure. Microarray technology has the promise to bring these objectives within reach. The first papers attempting to identify molecular signatures of response and outcome using microarray technology were generated using DLBCL samples and are described. The different types of microarray platform and data analysis tools are reviewed followed by a detailed step-by-step guide to data generation using the Affymetrix chip system from RNA extraction to laser scanning of the hybridized and stained chips.

**Key Words:** Diffuse; lymphoma; microarray; Affymetrix; expression profiling; outcome; prediction.

#### 1. Introduction

##### ***1.1. A Clinical Summary of Diffuse Large B-Cell Lymphoma***

Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin's lymphoma (1) and follows an aggressive course, resulting in the death of patients within months without treatment (2). Researchers, when using anthracycline-based combination chemotherapy, have found that 80 to 85% of patients respond, with more than 50 to 76% achieving a complete response, that is, no disease identifiable 4 or more weeks after the end of treatment (3,4). Unfortunately, in nearly half of responders, their lymphoma returns, with only a minority being cured by more intensive retreatment (5). Thus, at 5 yr from the diagnosis of DLBCL, less than 50% of patients remain alive, disease-free (1). The best, validated predictor of response and outcome to treatment to date is a crude index based upon five clinical variables termed the International

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Prognostic Index (IPI [6]). The IPI stratifies patients into low-, intermediate-, high-intermediate, and high-risk groups, with the respective 5-yr overall survival of each group being 73, 51, 46, and 32%. Because most patients fall into the intermediate group, the IPI fails to define their risk any better than the risk for all patients considered together. Therefore, the ability to predict which patients will respond to treatment and be cured of their lymphoma and which will ultimately die of their DLBCL still cannot be performed accurately for the majority.

The variability in clinical response and course of DLBCL and the recognition of morphological subtypes and nuances has led to the hypothesis that DLBCL is in fact composed of several distinct subtypes with differing outcomes to treatment. Because such subtypes cannot be successfully reproduced on the basis of morphological appearance alone, the search for molecular methods of subdividing DLBCL into meaningful clinical subtypes has long been the goal of lymphoma clinicians and scientists.

## **1.2. An Introduction to Microarray Technology**

The means of discovery of molecular signatures of response, long-term outcome, and subtype definition took a great leap forward with the advent of microarray technology (7,8). By allowing the simultaneous measurement of the mRNA expression of 100s to 1000s of genes from across the genome, array technology offered the potential to screen thousands of genes without the need for preconceived hypotheses.

Microarrays can investigate mitochondrial RNA (mRNA) or genomic deoxyribonucleic acid (DNA). Genomic DNA arrays can reveal large-scale gains and losses or mutations and polymorphisms, whereas mRNA targeted arrays allow gene-expression to be studied. An array is constructed on a solid substrate, often termed the “platform,” made of either glass (microarray) or nitrocellulose or nylon (macroarrays). Two types of array dominate the expression profiling field: “chips” where oligonucleotide probes are grown out from the substrate surface, and “dot-blot” slides or membranes, where tiny dots of probe solution are blotted on to the substrate. A third type “oligonucleotide ink-jet/piezo” arrays, has been developed more recently and will be described briefly. For a list of commercial suppliers of arrays, visit <http://www.lab-on-a-chip.com> and <http://ihome.cuhk.edu.hk/~b400559/array.html>.

On the substrate, 100s (macroarrays) to 1000s (microarrays) of complementary DNA (cDNA) amplicons or oligonucleotides are immobilized in an ordered, grid pattern. Confusingly, the arrayed cDNA amplicons or oligonucleotides are called “targets” in most systems but “probes” in Affymetrix chips. Because this chapter refers to the use of the Affymetrix system, the cDNA amplicons or oligonucleotides will be referred to as “probes” henceforth. To the “probes,”

labeled complementary (c)RNA, cDNA, or genomic DNA are hybridized in a manner similar to that of Southern and Northern blotting. (In most systems, the labeled cRNA, cDNA, or genomic DNA is referred to as the “probe,” whereas in the Affymetrix system, and the remainder of this chapter, it is referred to as the “target.”) Each of the immobilized “probes” acts as an assay for its specific partner strand in the complex nucleic acid mixture of the target. The target labels can be fluorophores, biotin, or radioactive phosphorus. Arrays designed for radioactive labeled target (macroarrays) require as little as 50 ng RNA (5000 cells), compared with the minimum of 5  $\mu$ g (500,000 cells) required for fluorescent systems.  $^{32}$ P-based arrays, however, produce reliable readouts only for highly expressed genes, which are the minority. Fluorescence-dependent arrays allow the detection of low- and medium-abundance genes, which are the majority, as well as those genes highly expressed at the mRNA level. It is the fluorescence-dependent arrays that have been used to great effect in the study of DLBCL and that will now be described in more detail.

### 1.3. “Dot-Blot” Arrays

The probes for “dot-blot” slides are created from cDNA clone libraries using the polymerase chain reaction to amplify part of the cDNA clone insert of each bacteriophage in the library. The “arrayer,” a robot, arrays 0.25–1 nL of probe cDNA solution in the same place on the 20 or more chemically treated slides under production. As many as 40,000 different probes can be arrayed per standard size slide. After the probe solutions have dried, the probes are covalently fixed to the slide using ultraviolet light. Excess probe is then washed off using 0.1% sodium dodecyl sulfate) Depending on the number of probes held, a batch of slides takes anywhere from 1 to 8 h to make. Maintaining the clone libraries required for dot-blot slides makes slide manufacture too expensive for all but the largest noncommercial organizations, unless the number of probes arrayed is compromised, and has sensibly lead to the establishment of core facilities capable of providing arrays to a network of labs. Nonetheless, the resultant slides are cheaper to produce than oligonucleotide arrays. A concern with cDNA arrays is the clone error rate, that is, when a clone transpires not to contain the plasmid sequence it is meant to. In one quality-control study, of the 1189 clones sequenced, only 62% were uncontaminated and contained cDNA inserts that had significant sequence identity to published data for the ordered clones (9).

Because the volume of each probe spot deposited can vary, apparent expression changes could be produced by spot differences alone. To compensate for this artifact, two target samples are used: a test sample and a control or reference RNA, usually created from pooled cell lines in sufficient quantity to be used with all the test samples in a particular experiment. The test sample RNA

is converted into labeled cDNA target using one fluorophore whereas the reference RNA is converted into labeled cDNA target using a different fluorophore. Equal amounts of test and control target are added to each array, allowing the test and reference cDNA fragments to compete to hybridize with the probes. If a test cDNA is in excess the resultant signal will come from its fluorophore, whereas if the control cDNA dominates, the fluorescence detected will come from the control cDNA. Thus, the problem of dot variability is overcome by using a competitive hybridization method. For a comprehensive guide to the manufacture of dot-blot arrays see the protocol pages of <http://www.microarrays.org/index.html>. cDNA arrays can be purchased from Agilent (<http://www.chem.agilent.com/>) as well as other suppliers.

### 1.3.1. Oligonucleotide Ink Jet/Piezo Arrays

A development from dot-blot arrays, oligonucleotide ink jet/piezo arrays use no-contact, less-wasteful piezo electric pulse or ink-jet technology to propel a tiny volume of synthesized oligonucleotide solution on to the platforms. Oligomers of 60 base-pair lengths were found to offer optimal sensitivity and specificity (**10**). Sensitivity down to one transcript per cell can be achieved. Standard and custom-made 25- or 60-mer oligonucleotide arrays are available from Agilent (<http://www.chem.agilent.com/>) and Amersham Biosciences (<http://www4.amershambiosciences.com/>). Whether the additional cost (compared with standard cDNA arrays) is truly outweighed by more informative data production remains to be decided.

## 1.4. Chip Arrays

Chip arrays consist of oligonucleotide probes that are synthesized *in situ* on silica wafers under the control of photolithographic chemistry (**11**). The lengths of both the probes and targets are critical to the sensitivity and specificity of the hybridization reactions. Probe oligomers between 20 and 60 bases in length provide the best balance between discriminant and sensitive hybridization. As well as the correct oligonucleotides, “missense” oligonucleotides are created in parallel for each probe, which allow the specificity of the hybridization reaction to be determined by comparison of the extent of hybridization to the missense and sense probes, as well as background noise reduction by subtraction of missense from sense signal. In the Affymetrix system (see [www.affymetrix.com/](http://www.affymetrix.com/)), the sense probes are 25 mers (high specificity but reduced sensitivity compared with longer oligomers) and are referred to as “perfect matches” (PM), whereas the missense probes are termed “mismatches” (MM), differing from their sense probe by just their middle, 13th base. The lower the value of the missense:sense signal ratio, the better. For each gene, a set of 11–20 PM and MM probe pairs is laid down, thereby increasing the sensitivity for detecting a gene transcript

to, at best, 1 in 300,000 transcripts. Through the reduction in feature size, as many as 54,000 gene probes can now be included on one chip. As with dot-blot slides, the target for oligonucleotide chips is created from test sample RNA. The RNA is reverse transcribed into cDNA followed by transcription back to RNA, as cRNA. Because this two-step procedure results in amplification of the mRNA, less RNA is required for chip arrays compared with dot-blot arrays (5–10  $\mu\text{g}$  vs 30  $\mu\text{g}$ ). To produce the labeled target, biotin-conjugated bases are used in the synthesis of the cRNA. The labeled cRNA requires fragmentation into lengths of 20 to 200 base pairs, followed by denaturing before application to the array. Unlike dot-blot arrays, competitive hybridization with a reference RNA is not required for interchip array expression profile comparison because the oligonucleotide synthesis of each feature is so precisely controlled that interchip variation is minimal. The cost of commercially available generic chips has reduced substantially since their introduction, making the technology affordable to an increasing number of research facilities. Furthermore, customized chips can be ordered that contain only the gene features of interest from previous experiments or known to be critical to a particular illness, tissue, or set of experimental conditions.

### **1.5. Expression Profiling in DLBCL**

The two major platforms for expression profiling have been used in DLBCL and have produced remarkable and reproducible expression signatures that reveal molecularly defined subtypes of DLBCL and predict for response to treatment and long-term outcome (*12–14*).

In an article by Shipp et al. (*14*), diagnostic, pretreatment, frozen biopsy material was studied from 58 patients with DLBCL and 19 patients with follicular lymphoma, a closely related germinal centre malignancy. The expression profile, or “signature,” of each biopsy was created using the Affymetrix 7800 gene chip, which consisted of probe features representing 7817 known and putative genes. The resulting gene expression data was analyzed using supervised learning techniques (*15*). The first distinction attempted, based on expression variance, was between the DLBCL and follicular lymphoma samples. This proved possible, and the resulting 30-gene model’s reproducibility was confirmed by the “leave-one-out” cross-validation technique. Next, subdivision of the DLBCL samples into different subtypes based upon outcome to treatment was examined, again using a weighted-voting algorithm (*16*). A recurring subset of genes was indeed discovered that were highly associated with the distinction between those patients that had a good outcome (i.e., alive and cured yr after treatment) and those with a bad outcome (i.e., dead from lymphoma or DLBCL unresponsive to treatment). A model using just 13 genes could predict outcome for the 58 patients, with 70% of the patients stratified in the good-risk

group being alive at 5 yr vs just 12% of the patients placed in the bad-risk group remaining alive at 5 yr ( $p = 0.00004$ ).

An interesting discovery was that the 13-gene model could subdivide the patients contained within the different IPI subgroups into “cured” and “dead/refractory” subgroups with significantly different outcomes, revealing that the gene expression-based outcome predictor contained additional information to the clinical prognostic model, the IPI. For example, when the 37 patients defined as low/low-intermediate risk according to the IPI were sorted by the 13-gene model, those displaying a “cured” gene-expression signature had a 5-yr overall survival of 75% compared with a 5-yr overall survival of 32% for the patients displaying the “fatal/refractory” signature ( $p = 0.02$ ). The combination of clinical and molecular variables suggests a possible strategy for further individualization of patient treatment decisions. Nevertheless, additional information still remains to be captured because the use of both models in series failed to produce groups with 100% and 0% 5-yr overall survivals. Finally, the 13-gene model suggested novel therapeutic targets and strategies. Whether therapeutic leads suggested by expression profiling will prove useful and result in molecularly determined individualization of treatment remains to be seen.

In the other two articles, the clustering analysis was performed using a different approach termed hierarchical clustering (17). Alizadeh et al. (12) demonstrated that molecular subclassification of DLBCL on the basis of gene expression was possible. The two subtypes had expression profiles similar to those of different putative cells of origin. This finding was confirmed and extended in the much larger series of Rosenwald et al. (13), in which DLBCL was divided into three subtypes termed germinal center-like, activated B-cell-like and type 3 DLBCL. In both articles, the patients with lymphomas subclassified as germinal center-like fared better than the patients with lymphoma subclassified as activated B-cell-like, suggesting clinical relevance to DLBCL subclassification according to the putative cell of origin.

### **1.6. The Stages of a Gene Expression Profile Experiment**

The procedures fall into three stages: (1) sample preparation; (2) array hybridization and image acquisition; and (3) data handling and interpretation. Each of these stages is outlined herein.

#### **1.6.1. Sample Preparation: From Living Tissue to Labeled Target**

Control of RNA quality and quantity extracted from cell-lines is straightforward, as the sample is 100% interesting and fresh. However, from human body tissues guaranteeing excellent quality and quantity of RNA is more difficult. First, obtaining sufficient human tissue of interest is often problematic. Although radioactive systems, using phosphorus-32, can use very small amounts of RNA

(50 ng) retrievable from a 16-gage needle core biopsy, they are reliable only for high-abundance genes, which are the minority. For fluorescent systems, considerably more tissue is required, necessitating an open biopsy. Second, minimizing the time in which RNA degradation can occur from collection to freezing or processing of the material is critical. Considerable resources and co-operation between many individuals, not least the patients who allow the samples to be taken, are required to overcome these obstacles. Once obtained, verification of sufficient tumor infiltration of each sample must be undertaken. If the tumor infiltrate is too low, conventionally taken as less than 50%, the expression profile obtained will relate more to the nonmalignant cell population, such as fibroblasts and nonmalignant lymphocytes than the tumor itself. In such cases, purification of the tumor cells may be required by cell sorting or microdissection (18). Cell sorting may change the tumor's expression profile because of the cells' response to processing. If microdissection is undertaken, additional polymerase chain reaction (PCR) amplification of the RNA will be needed if a fluorescent, as opposed to a radioactive, array system is to be used (19). This in turn carries the risk of differential signal amplification, distorting the expression pattern being sought, a problem that is being actively addressed (20,21).

Once the RNA has been extracted, it requires cleaning of protein and other impurities to maximize the efficiency of the transcription reactions and target hybridization to probe. The cleaned RNA is reverse transcribed into cDNA, followed by transcription back into labeled cRNA using biotinylated ribonucleotides. The two-step procedure of first- and second-strand cDNA/RNA synthesis uses a poly T primer to anneal to the poly-A tail present at the 5' end of all mRNA. Before synthesizing the second cDNA strand, the original RNA is destroyed to prevent its continued replication. As already mentioned, to make an efficient target out of the labeled cRNA, it must be fragmented into small pieces and then denatured.

To summarize, sample preparation consists of the following steps:

1. RNA extraction (2 h)
2. RNA purification (1 h)
3. First-strand cDNA synthesis (1 h)
4. Second-strand cDNA synthesis (2 h)
5. Clean-up of double-stranded complementary DNA (ds-cDNA) (1 h)
6. In vitro transcription: cRNA synthesis (5 h)
7. Fragmentation of biotinylated cRNA (1 h)

### 1.6.2. Array Hybridization and Image Acquisition

Hybridization of the fragmented target to the array requires overnight incubation at 45°C. After removal of the target solution from the chip, unbound target is removed by performing several washes. Staining of the biotin-labeled,

hybridized target fragments is achieved using streptavidin, which strongly binds to biotin. The streptavidin is made visible by virtue of its conjugation to the dye phycoerythrin, which fluoresces under laser light. To amplify the streptavidin–phycoerythrin (SAPE) signal, a biotinylated antibody against streptavidin is incubated with the array after the first exposure to SAPE. A second incubation with SAPE solution is then performed, followed by washing to remove unbound SAPE. The expression profile on the array is read by measuring the fluorescence produced by scanning the array with a laser at 570 nm. Once captured, the fluorescent intensity of each target–probe hybrid is converted into a numerical value to produce quantitative gene expression levels for each feature of the array.

To summarize, array hybridization and image acquisition involves the following steps:

1. Hybridization of the fragmented target to the probe array in the cartridge/chip (overnight).

The “Fluidics” steps: (2 h)

2. Washing to remove nonspecifically bound target.
3. First SAPE staining.
4. Signal enhancement by addition of a biotinylated antibody against streptavidin.
5. Second SAPE staining.

Post-Fluidics: (10 min)

6. Laser scanning.

### 1.6.3. Data Analysis

Data analysis has, for the first time in biomedical science, become the bottleneck in experiment completion, as the acquisition of data outstrips the capacity to find meaning within it. Data analysis involves the following steps:

1. Confirmation of successful, specific hybridization across the chip.
2. Data transformation and normalization.
3. Exclusion of nonvarying genes.
4. Performance of cluster analyses/data mining.
5. Cluster reproducibility testing.

The raw data from each chip require interrogation by Microarray Suite (Affymetrix) to confirm successful hybridization. The sensitivity of an array can be gauged by ascertaining the lowest concentration of control oligonucleotide in the hybridization cocktail and through assessing PM to MM ratios across each probe set.

Precluster analysis, the data require elimination of background signal, filtration, and normalization. Microarray suite subtracts background automatically

by taking the MM from PM signal intensity for each probe and probe set. High background implies that impurities, cell debris, and salts, as well as target are binding to the probe array surface in a nonspecific manner and fluorescing at 570 nm (the scanning wavelength) This nonspecific binding causes a low signal-to-noise ratio (SNR), meaning that genes for transcripts present at very low levels in the sample may incorrectly be called absent and excluded from further analysis. High background creates an overall loss of sensitivity in the experiment. The purer the starting RNA, and later the ds-DNA and cRNA, the less background produced.

In order for the data from each chip in a series to be compared with the data of the others, adjustments need to be made. Low-quality readings are removed, or "filtered." These are predominantly low intensity readings (in which the signal is close to background, causing relative error to increase) and the highest intensity readings (in which saturation of the probe feature occurs) necessitating application of a "floor" and "ceiling" to exclude such observations. For the remaining features, their intensities require adjustment to account for variations in target labeling efficiency and hybridization, producing false expression changes on comparison with other chip intensities. This involves two processes called "scaling" and "normalization." "Scaling" involves the setting of a scaling factor to be applied to all the intensities on a chip. By applying the same scaling factor to each chip in a data set, a source of false expression variation is prevented. "Normalization" involves the removal of systematic variation, such as variable hybridization labeling efficiency and starting quantity of RNA, that affects the measured gene expression levels to differing extents for each array. Generation of "normalization" factor adjusts signal intensities on each chip so that the intensities of each are readily comparable with the rest in the data set. Using the Affymetrix software, "Normalization" involves generation of a factor that allows the average intensity of an experimental array to be adjusted to the average intensity of the baseline array. The normalization factor for a particular array will therefore change when the comparison baseline array is changed. For detailed reviews and methods on "normalization," see <http://www.dnachip.org/mged/normalization.html> (22,23).

A further form of necessary data filtering is the removal of genes with no or minimal variance across the data set. Such filtering greatly aids identification of the most interesting differentially expressed genes by reducing false groupings and clustering on use of the different data mining tools (24). The simplest method to eliminate stable genes is application of an expression fold-difference threshold, usually of twofold. Only those genes with greater than twofold expression changes are taken forward for data analysis. More sophisticated methods of identifying differentially expressed genes involve calculation of intensity-independent and -dependent Z scores for the data set, so allowing the

variably expressed genes to be defined as those lying outside of the 95% confidence intervals derived (25) and use of analysis of variance techniques (26).

After these steps, two principal methods of data analysis are used, unsupervised and supervised. In an unsupervised analysis, no preconditions are applied to the data set under scrutiny. The program used is merely asked to split the data set into varying numbers of groups or clusters using the profiles for all or a selected group of gene probes. This can be a powerful way of discovering unexpected similarities and differences between samples but can also result in groups of little obvious clinical or scientific relevance. In a supervised analysis, gene profiles are sought that associate with a prescribed distinction between the different samples, be it the time points of samples in a drug exposure experiment or the outcome to treatment of the patients the samples came from.

Numerous programs and tools are used to perform unsupervised and supervised analyses. Frequently used analysis methods include hierarchical clustering (17), self-organizing maps (15), k-nearest neighbor (27), principal components analysis (28), support vector machines (29), and weighted voting (16).

Hierarchical clustering is the most commonly used unsupervised analysis tool. It involves the creation of a phylogenetic tree, where the branching lengths represent the degree of similarity between the data sets. Hierarchical clustering can produce groupings with clinical correlation but can also miss many of the ways in which data varies by being too rigid a structuring tool. The resultant cluster pictures are referred to as “dendrograms.”

The self-organizing maps (SOMs) algorithm is a neural-network based method that allows extraction of “executive summaries” from the data by defining the *n* most important groupings. Different numbers of nodes or partitions are suggested to the program by the operator to group the most variable genes. The relationship of individual data points to each node is then computed, and all the data points are shuffled one by one. Eventually, the grouping of the gene data points is maximized, just as if a vase were reconstructed from the millions of shards of glass produced when broken. One of the advantages of SOMs is imposition of partial, rather than complete, structure on the data set. In addition, SOMs can be refined or reorganized as discoveries are made. One of the problems with SOMs is the need to remove all genes with stable expression across the majority of samples in the data set. If these low-variability genes are included in the analysis, false clusters are produced.

K-nearest neighbor organizes the data into similar groupings in *k* dimensional “gene expression” space. The means of defining the vector, or expression distance, between data points distinguishes k-nearest neighbor clustering from that of SOMs. Both techniques are adept at “training” an algorithm using provided data, be it histology type or extent of T-cell infiltration, to recognize biologically meaningful patterns in the data set.

Multidimensional scaling can be used to reduce complex data sets to a few specified dimensions so that relationships between groups can be more effectively visualized, in effect cutting down most of the trees in the forest to see the most interesting. Multidimensional scaling is not a clustering technique (30). Principal component analysis works similarly, revealing the dominant themes within the data set by elimination of less significant variation trends. It is best used in conjunction with a supervised learning technique such as k nearest neighbor or SOMs as it lacks definition when used alone (31,32).

Cluster reproducibility testing is essential after the generation of supervised and unsupervised analyses. Because the expression variance of large numbers of genes has been mined, false positive results can easily be produced. Such testing can be performed using the discriminating gene subset defined by the same analysis tool on a separate group of allied samples, or where further samples are not available, by using the “leave-one-out” method. This method involves re-examining each sample in turn using the analysis tool gene subset to see whether the sample will be reassigned to the same cluster or a different one. Once all samples have been reviewed, the true positive and type 1 and 2 error rates can be calculated. By varying the number of genes used in the assignment process, the optimal number can be defined. Two other means of assessing the robustness of groupings and clustering are by applying different analysis tools to see if the results of the first analysis are reproduced, and by performing permutation testing, whereby the strengths of randomly generated clusters are compared to those derived by the chosen data mining tool (33). If the permuted clusters transpired to have less strength than the observed clusters, the latter are considered to be of statistical significance.

In an ideal world, each sample should be prepared and analyzed more than once to eliminate or contain the variability in the data due to technical factors. Because of the constraints of RNA availability and cost, this is rarely done. Criteria for determining the quality of data sets are still evolving, as are the best methods and practices for data analysis and mining.

It is important to ensure that you are quite clear as to what question(s) you are attempting to answer through expression profiling and whether you are in fact going to be arraying the right material in sufficient quantities to come close to meaningful insights into cancer biology and clinical heterogeneity. The following reference gives guidance on minimal sample size (34). Finally, data storage, which initially is manageable using Excel and Filemaker Pro, rapidly becomes a major logistic issue as your experiment files expand. Guidance on best practice for data storage (required by several journals for article publication) has been created by the MIAME consortium (Minimum information about a microarray experiment) See <http://www.mged.org/Workgroups/MIAME/miame.html> for details.

## 2. Materials

When working with chemicals, always wear a suitable laboratory coat, disposable gloves, and protective goggles. Keep away from food and drink. Avoid contact with skin and with eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. If swallowed, seek medical advice immediately and show the container or label. Use a fume hood when handling chemicals.

### 2.1. Preventing RNA Degradation and Contamination

RNA degradation is one of the principal problems and frustrations of gene expression profiling. To prevent RNase contamination, always wear latex or vinyl gloves and use diethyl pyrocarbonate (DEPC)-treated distilled water (see below), sterile RNase-free disposable pipet tips, and Microfuge tubes throughout the procedure. Avoid sources of dust or other contaminants, which can interfere with the multiple procedures you will be undertaking.

1. 2-, 20-, 200-, and 1000- $\mu$ L pipets.
2. RNase-free tips for 2-, 20-, 200-, and 1000- $\mu$ L pipets.
3. RNase-free 0.5-, 1.5-, and 2-mL Microfuge tubes.

### 2.2. DEPC-Treated Distilled Water

1. Distilled water.
2. DEPC (Sigma-Aldrich; cat. no. 15922). Store at 4–8°C; stable for 2 yr. Safety information: DEPC is a suspected carcinogen and should be handled with great care. Toxic if inhaled, in contact with skin, and if swallowed. Keep away from food and drink. Irritating to eyes, respiratory system. Combustible.)

To prepare DEPC H<sub>2</sub>O, add 0.1 mL of DEPC to 100 mL of distilled water or 1 mL of DEPC to 1000 mL of distilled water. Shake vigorously to bring DEPC into solution, then incubate for 12 h at 37°C. Autoclave for 15 min to remove any trace of DEPC. Store, shielded from light at room temperature (RT). Stable for months.

### 2.3. RNA Extraction, Concentration, and Purification

It is essential to produce highly purified RNA free of proteins, polysaccharide, cell debris, and particularly RNases. The former contaminants can interfere with the efficiency of synthesis of biotinylated cRNA and also can bind nonspecifically to probes, resulting in increased background and the masking of low frequency target signals.

#### 2.3.1. RNA Extraction

1. TRIzol Reagent (Invitrogen; cat. no. 15596) Store at 4°C, stable for 6 mo. Safety information: toxic in contact with skin and if swallowed. TRIzol causes burns (see **Note 1**).

2. Chloroform (BD; cat. no. 10077). Store at RT, stable for minimum of 5 yr. Safety information: harmful by inhalation and if swallowed. Irritating to skin. Possible risk of irreversible effects exists. Danger of serious damage to health by prolonged exposure.
3. DEPC H<sub>2</sub>O (*see Subheading 2.2.*).
4. 100% Ethanol (BD; cat. no. 10107). Store at RT, stable for minimum of 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed.
5. 75% Ethanol (make up using 100% ethanol and DEPC H<sub>2</sub>O).
6. 1.5-mL Microfuge tubes.
7. Heating block or waterbath suitable for 1.5-mL Microfuge tubes.
8. Microfuge.

### 2.3.2. Thawing of Suspended Cryopreserved Cells

Although most lymphoma samples are solid pieces of tissue, usually lymph node, some are derived from body fluids, from which the cells have been concentrated and cryopreserved in suspension.

1. RPMI 1640 media (Invitrogen; cat. no. 21875-034). Store at 4°C.
2. Fetal calf serum (Invitrogen; cat. no. 26140-095). Store at 4°C.
3. Sterile trypan blue solution (0.4%; Sigma; cat. no. T8154). Store at 4°C.
4. Cell counter.
5. Cover slips.
6. Microscope.

### 2.3.3. Concentration and Cleaning of Total RNA by Ethanol Precipitation

1. Isopropanol (BD; cat. no. 10224). Store at room temperature; stable for minimum 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed. May cause harm to the unborn child. Irritating to eyes.
2. 3 M sodium acetate, pH 5.2 (Sigma-Aldrich; cat. no. 57899). Store at RT; stable for 1 yr. Safety information: avoid contact and inhalation.
3. 100% ethanol (BD; cat. no. 10107). Store at RT; stable for minimum 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed.)
4. DEPC H<sub>2</sub>O (*see Subheading 2.2.*)
5. 80% ethanol (make up using 100% Ethanol and DEPC H<sub>2</sub>O)
5. 1.5-mL Microfuge tubes.
6. Microfuge.

### 2.3.4. Concentration and Clean-Up of Total RNA Using the RNeasy Mini Kit

The purpose of the total RNA clean-up is to eliminate protein and polysaccharide contamination that can inhibit the transcription and hybridization reac-

tions and increase background noise on the hybridized array by causing non-specific hybridization to the glass surface.

1. 100% ethanol (BD; cat. no. 10107) Store at RT; stable for minimum 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed.
2. DEPC H<sub>2</sub>O (*see Subheading 2.2.*)
3. Microfuge suitable for 2-mL Microfuge tubes.
4. RNeasy mini kit (X50; Qiagen; cat. no. 74104) Store at RT; stable for 1 yr)

The kit contains:

1. RLT buffer. (Safety information: RLT buffer contains guanidine isothiocyanate, harmful by inhalation, in contact with skin and if swallowed. Contact with acids liberates very toxic gas.)
2. RPE buffer. (Supplied as a concentrate. Before using for the first time add 4 volumes of ethanol [96–100%] as indicated on the bottle. Safety information: Flammable.)
3. RNeasy mini spin columns (silica gel-based membranes, binding up to 100 µg of RNA longer than 200 bases)
4. 1.5-mL collection tubes.
5. 2.0-mL collection tubes.

## **2.4. Quantity and Quality Assessment of Total RNA**

### **2.4.1. Assessing the Quantity of Total RNA by Spectrophotometry**

1. Absorbance spectrometer (e.g., GeneQuant II; Pharmacia Biotech)
2. DEPC H<sub>2</sub>O (*see Subheading 2.2.*)

### **2.4.2. Assessing the Quality of Total RNA by Gel Electrophoresis**

1. Agarose (Invitrogen; cat. no. 15510-027) Store in a cool and dry place at RT; stable for minimum 5 yr.
2. Heating block suitable for 0.5-mL tubes.
3. Gel loading tank.
4. Power pack.
5. Ultraviolet light box.
6. Photographic equipment.
7. Denaturing loading buffer (*see Subheadings 2.4.2.1. and 2.4.2.2.*).
8. DNA ladder/marker 200 base pairs (bp) to 3 kbp:  $\phi$ X174 DNA/*Hae*III (Promega; cat. no. G1761; store at –20°C; stable for 1 yr) and 1-kb DNA ladder (Promega; cat. no. G5711; store at –20°C, stable for 1 yr).
9. Ethidium Bromide tablets (Sigma-Aldrich; cat. no. E-2515) Store at RT; stable for minimum 5 yr. Safety Information: harmful if swallowed; very toxic by inhalation. Irritating to eyes, to respiratory system, and to skin. Keep container tightly closed and in a well-ventilated place.

10. 10X TBE stock solution (*see Subheadings 2.4.2.3. and 2.4.2.4.*). Alternately (Invitrogen; cat. no. 15581-028). Store at RT. Safety information: toxic. Harmful by inhalation and in contact with skin. Irritating to eyes and to skin.

#### 2.4.2.1. DENATURING LOADING BUFFER

1. 10X MOPS (3[*N*-Morpholino]propanesulfonic acid; Sigma-Aldrich; cat. no. M-8899) Store at RT; stable for 3 yr. Safety Information: Irritating to eyes, to respiratory system, and to skin.
2. *N,N*-Dimethylformamide (Sigma-Aldrich; cat. no. D-4551) Store at RT; stable for minimum 5 yr. Safety Information: combustible. Teratogen, target organs: liver and kidneys. May cause harm to the unborn child. Harmful by inhalation and in contact with skin. Irritating to eyes and to skin.
3. Mixed-Bed Resin (Sigma-Aldrich; cat. no. M-8032) Store at RT, stable for minimum 3 yr.
4. Formaldehyde 37% (BDH; cat. no. 43753) Store at RT, stable for minimum 5 yr. Safety Information: toxic by inhalation, in contact with skin, and if swallowed. Causes burns. Danger of very serious irreversible effects. May cause sensitization by skin contact.
5. Glycerol (BDH; cat. no. 10118) Store at RT, stable for minimum 5 yr.
6. Bromophenol Blue (Sigma-Aldrich; cat. no. B-8026) Store at RT, stable for minimum 5 yr. Safety Information: avoid contact and inhalation.
7. DEPC H<sub>2</sub>O (*see Subheading 2.2.*).

#### 2.4.2.2. METHOD FOR PREPARING DENATURING LOADING BUFFER

1. Prepare 10X loading buffer (50 % glycerol, 0.1 mg/mL bromophenol blue sodium salt) as follows:
2. Add 0.2 mg of bromophenol blue sodium salt to 500  $\mu$ L of DEPC H<sub>2</sub>O and mix.
3. Add 500  $\mu$ L of glycerol and mix thoroughly.
4. Store at RT.
5. Prepare deionized formamide as follows:
6. Add 1 g of mixed bed resin to 50 mL of formamide.
7. Stir for 1 h.
8. Filter through filter paper.
9. Store at  $-20^{\circ}\text{C}$ .
10. Prepare in bulk 10  $\mu$ L of loading buffer for each 1–1.5  $\mu\text{g}/\mu\text{L}$  sample of total RNA, cRNA, or fragmented cRNA as follows:

DEPC H <sub>2</sub> O	1.75 $\mu\text{L}$
10X MOPS	0.50 $\mu\text{L}$
Deionized formamide	5.00 $\mu\text{L}$
Formaldehyde 37%	1.75 $\mu\text{L}$
10X loading buffer (50% glycerol, 0.1 mg/mL Bromophenol Blue)	1.00 $\mu\text{L}$
Final volume	10.0 $\mu\text{L}$

11. Freeze in 100- to 200- $\mu$ L aliquots and keep at  $-20^{\circ}\text{C}$ .
12. Make a 10mg/mL ethidium bromide solution when ready to add buffer to samples.
13. Add 0.5  $\mu$ L of ethidium bromide (10 mg/mL)/100  $\mu$ L defrosted denaturing loading buffer just before adding the buffer to the samples.

#### 2.4.2.3. 10X TBE STOCK SOLUTION

1. DEPC  $\text{H}_2\text{O}$  (*see Subheadings 2.2. and 3.*).
2. TRIS base (Invitrogen; cat. no. 15504-020). Store at RT; stable for minimum of 2 yr. Safety Information: toxic by inhalation, in contact with skin, and if swallowed. Irritating to eyes, respiratory system.
3. Boric acid (BD; cat. no. 100583R). Store at  $15\text{--}30^{\circ}\text{C}$ ; stable for minimum of 2 yr. Safety Information: irritating by inhalation, in contact with skin, and if swallowed.
4. Ethylenediamine tetra-acetic acid (EDTA) di-Sodium Salt, pH 8.0 (Sigma-Aldrich; cat. no., E-7889). Store at RT, stable for 1 yr.

#### 2.4.2.4. METHOD FOR PREPARING 10X TBE STOCK SOLUTION

1. Make a solution of: (1) 108 g TRIS, (2) 55 g boric acid, (3) 40 mL 0.5 M EDTA, and (4) DEPC  $\text{H}_2\text{O}$  to 1000 mL.
2. Filter through a 0.2- $\mu$ m vacuum filter unit. Store at  $4^{\circ}\text{C}$  and shield from light.

#### 2.4.3. Assessing the Quality of Total RNA

##### *Using the 2100 Agilent Bioanalyzer*

1. Agilent 2100 Bioanalyser System (Agilent Technologies; cat. no. G2940CA). Includes 2100 bioanalyzer equipped with bayonet electrode cartridge, HP Compaq EvoD510 SFF (Pentium IV, Windows 2000), 17" monitor, HP DeskJet color printer, bioanalyzer system software, vortex mixer with chip adapter, and startup accessories.
2. RA 6000 Nano LabChip Kit (cat. no. 5065-4476).

The kit contains:

1. RNA chips.
2. Electrode cleaners.
3. RNA dye concentrate. (Molecular Probes; Shield from light to prevent degradation by light exposure. Store at  $4^{\circ}\text{C}$ . Stable for 3 mo. Safety Information: toxicity unknown, as it contains dimethyl sulfoxide, which facilitates organic molecule entry through the skin, wear double gloves.)
4. RNA 6000 Nano Marker. (Store at  $4^{\circ}\text{C}$  and shield from light.)
5. RNA Gel Matrix. (Store at  $4^{\circ}\text{C}$  and shield from light.)
6. 1-mL syringe.
7. Chip Priming Station (Agilent Technologies; cat. no. 5065-4401).
8. Vortex Mixer IKA.
9. RNA 6000 ladder (Ambion; cat. no. 7152) Aliquot and store at  $-80^{\circ}\text{C}$ .

10. RNaseZAP (Ambion; cat. no. 9780) Store at RT, stable for minimum 5 yr. Safety Information: avoid contact and inhalation.
11. DEPC H<sub>2</sub>O (*see Subheading 2.2.*).
12. 0.5- and 1.5-mL Microfuge tubes.
13. Microfuge.
14. Timer.
15. Heating block suitable for 0.5-mL Microfuge tubes.

#### 2.4.3.1. METHOD FOR PREPARING GEL-DYE MIX

This resultant 132  $\mu$ L of gel-dye mix is stable for 1 wk if kept at 4°C and shielded from light. This volume is sufficient for 4 RNA chips.

1. Pipet 400  $\mu$ L of gel matrix (red dot on lid) into a spin filter receptacle.
2. Place the spin filter into a 1.5-mL Microfuge tube.
3. Centrifuge at RT, 1500g for 10 min.
4. Pipet 130  $\mu$ L of the filtered gel matrix into a fresh 1.5-mL Microfuge tube.
5. Add 2  $\mu$ L of RNA Dye Concentrate (blue dot on lid) and vortex for 1 min to mix.
6. Store at 4°C, shield from light, and use within 1 wk.
7. Store the remaining filtered gel matrix at 4°C and use within 1 mo.

## 2.5. *ds-cDNA Synthesis*

### 2.5.1. *First-Strand cDNA Synthesis*

1. Oligo (dT)23 (Sigma, cat. no. 04387). Store at -20°C; stable for 1 yr).
2. 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH; Invitrogen; cat. no. 18427-013). Store at -20°C; stable for 6 mo.
3. 500- $\mu$ L Microfuge tubes.
4. Microfuge.
5. Two heating blocks suitable for 500- $\mu$ L Microfuge tubes, one with a heated lid or a water bath.
6. SuperScript TM II RNase H- Reverse Transcriptase Kit (Invitrogen; cat. no. 18064-014). Store at -20°C; stable for 6 mo.

The kit contains:

1. 5X First Strand Buffer: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>.
2. 0.1 M dithiothreitol (DTT). Safety information: irritating to eyes, skin, and respiratory system.
3. SuperScript TM II Reverse Transcriptase (200 U/ $\mu$ L).

### 2.5.2. *Second-Strand cDNA Synthesis*

1. 5X Second-Strand Buffer (100 mM Tris-HCl, pH 6.9; 450 mM KCl; 23 mM MgCl<sub>2</sub>; 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.75 mM  $\beta$ -NAD<sup>+</sup>; (Invitrogen; cat. no. 10812-014). Store at -20°C; stable for 6 mo.
2. 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH; Invitrogen; cat. no. 18427-013). Store at -20°C; stable for 6 mo.

3. *E. coli* DNA Ligase (10 U/ $\mu$ L; Invitrogen; cat. no. 18052-019). Store at  $-20^{\circ}\text{C}$ ; stable for 6 mo.
4. *E. coli* DNA Pol I (10 U/ $\mu$ L; Invitrogen; cat. no. 18010-025). Store at  $-20^{\circ}\text{C}$ ; stable for 6 mo.
5. *E. coli* RNase H (2 U/ $\mu$ L; Invitrogen; cat. no. 18021-071). Store at  $-20^{\circ}\text{C}$ ; stable for 6 mo.
6. T4 DNA Polymerase (5 U/ $\mu$ L; Invitrogen; cat. no. 18005-025). Store at  $-20^{\circ}\text{C}$ ; stable for 6 mo.
7. Ethylenediamine tetra-acetic acid (EDTA) di-Sodium Salt, pH 8.0 (Sigma-Aldrich; cat. no. E-7889). Store at RT, stable for 1 yr.
8. 500- $\mu$ L Microfuge tubes.
9. Microfuge.
10. Refrigerated water bath.

Or

11. Refrigerated PCR block with heated lid and wells for 500- $\mu$ L Microfuge tubes.

## **2.6. Clean-Up and Concentration of ds-cDNA**

### **2.6.1. Clean-Up of ds-cDNA**

1. Phase Lock Gel tubes (2 mL, light; Eppendorf; cat. no. 0032 005101). Store at RT, stable for 1 yr.
2. Phenol:chloroform:isoamyl alcohol, 25:24:1, pH 6.6, buffered pH 7.9 (Ambion; cat. no. 9732). Store at  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$ ; stable for 6 mo. Accompanied by a separate pre-made Alkaline Buffer solution to be added just prior to use (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Safety Information: toxic in contact with skin and if swallowed; causes burns.)
3. 1.5-mL Microfuge tubes.
4. Microfuge suitable for 2-mL tubes.

### **2.6.2. Precipitation of ds-cDNA**

1. 7.5 M ammonium acetate (Sigma-Aldrich; cat. no. A-2706). Store at RT, stable for 1 yr. Safety Information: irritant to eyes, skin and respiratory system.
2. Glycogen (5 mg/mL; Ambion; cat. no. 9510). Store at  $-20^{\circ}\text{C}$ ; stable for 1 yr.
3. 100 % Ethanol (BD; cat. no. 10107). Store at RT, stable for minimum 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed.

## **2.7. In Vitro Transcription–Synthesis of Biotinylated cRNA**

1. DEPC  $\text{H}_2\text{O}$  (see Subheading 2.2.).
2. BioArray High Yield RNA Transcript Labeling Kit (Enzo Biochem; cat. no. 900182). Store at  $-20^{\circ}\text{C}$ ; stable for 3 mo.

The kit contains:

- 10X HY reaction buffer.
- 10X Biotin-labeled ribonucleotides (ATP, GTP, CTP, UTP with Bio-UTP and Bio-CTP).
- 10X DTT.
- 10X RNase inhibitor mix.
- 20X T7 RNA Polymerase (*see Note 2*).

### 2.7.1. Clean-Up of cRNA

This step is performed to eliminate unincorporated biotin-labeled ribonucleotides.

1. 100 % ethanol (BD; cat. no. 10107). Store at RT, stable for minimum 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed.
2. DEPC H<sub>2</sub>O (*see Subheading 2.2.*).
3. RNeasy Mini Kit (X50; Qiagen; cat. no. 74104). Store at RT, stable for 1 yr.

The kit contains:

- RLT buffer. (Safety Information: RLT Buffer contains guanidine isothiocyanate, harmful by inhalation, in contact with skin and if swallowed. Contact with acids liberates very toxic gas.)
  - RPE buffer. (The buffer is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle. Safety information: flammable.)
4. RNeasy mini spin columns. (The columns contain a silica-gel-based membrane, which binds up to 100 mg of RNA longer than 200 base pairs.)
  5. 1.5-mL collection tubes.
  6. 2.0-mL collection tubes.
  7. Microfuge suitable for 2-mL tubes.

## 2.8. Assessing the Quantity and Quality of the Purified cRNA

### 2.8.1. Assessing the Quality of Purified cRNA by Spectrophotometry

*See Subheading 2.4.1.*

### 2.8.2. Assessing the Quality of the Purified cRNA by Gel Electrophoresis

*See Subheading 2.4.2.*

### 2.8.3. Assessing the Quality of the Purified cRNA Using the Agilent Bioanalyzer

*See Subheading 2.4.3.*

## 2.9. Increasing cRNA Concentration by Ethanol Precipitation

1. 7.5 M ammonium acetate (Sigma-Aldrich; cat. no. A-2706). Store at RT, stable for 1 yr. Safety information: irritant to eyes, skin and respiratory system.
2. 100% ethanol (BD; cat. no. 10107). Store at RT, stable for minimum 5 yr. Safety information: highly flammable. Keep away from sources of ignition. Keep container tightly closed.
3. DEPC H<sub>2</sub>O.
4. 80% ethanol (made from 100% ethanol and DEPC H<sub>2</sub>O).

## 2.10. cRNA Fragmentation

1. TRIZMA Base (Sigma-Aldrich; cat. no. T-1503). Store at RT, stable for 3 yr. Safety Information: Irritating to eyes, to respiratory system and to skin.
2. Magnesium acetate (Sigma-Aldrich; cat. no. M-2545). Store at RT, stable for 3 yr.
3. Potassium Acetate (Sigma-Aldrich; cat. no. P-5708). Store at RT, stable for 3 yr. Safety Information: Irritating to eyes, to respiratory system and to skin.
4. Glacial acetic acid (Sigma-Aldrich; cat. no. A-6283). Store at RT, stable for minimum 5 yr. Safety Information: Flammable. Harmful in contact with skin; causes severe burns. Keep away from sources of ignition.
5. DEPC H<sub>2</sub>O (*see Subheading 2.2.*).
6. pH meter.

### 2.10.1. Method for Preparing 5X Fragmentation Buffer

Composition: 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc.

Adjust an adequate volume of Trizma base to pH 8.1 using glacial acetic acid. Combine the following components to a total volume of 20 mL:

1 M Tris acetate, pH 8.1	4.0 mL
Magnesium acetate	0.64 g
Potassium acetate	0.98 g
DEPC H <sub>2</sub> O to	20 mL

Mix thoroughly and filter through a 0.2-mm vacuum filter unit. Aliquot as 1 mL volumes and store at RT or 4°C (stable for 1 yr)

### 2.10.2. Assessing the Quality of the Fragmented cRNA

It is not possible to meaningfully assess the quantity of fragmented cRNA. Instead, use the concentration of purified cRNA to calculate the amount of fragmented cRNA.

### 2.10.3. Assessing the Quality of the Fragmented cRNA by Gel Electrophoresis

*See Subheading 2.4.2.*

#### 2.10.4. Assessing the Quality of the Fragmented cRNA by 2100 Agilent Bioanalyzer

See **Subheading 2.4.3.**

#### 2.11. Probe Hybridization Cocktail

The eukaryotic hybridization controls are added to allow the sensitivity achieved on each chip to be assessed, whereas the control oligonucleotide B2 is added to allow the demarcation and orientation of the array grid to be determined in the scanned image. If the control oligonucleotide B2 is accidentally left out, the chip(s) in question will be unreadable.

1. Fragmented cRNA (15  $\mu$ g).
2. GeneChip hybridization control kit (Affymetrix cat. no. 9900454). The kit contains: 20X eukaryotic hybridization controls (bioB 30 pM, bioC 100 pM, bioD 500 pM, cre 2000 pM. Store at  $-20^{\circ}\text{C}$ ; stable for 6 mo. 3 nM control oligonucleotide B2.
3. 2X hybridization buffer (see **Subheadings 2.13.3.** and **2.13.4.**).
4. Acetylated bovine serum albumin (50 mg/mL; Invitrogen; cat. no. 15561-020). Store at  $-20^{\circ}\text{C}$ ; stable for 6 mo.
5. Herring sperm DNA (10 mg/mL; Promega; cat. no. D1811). Store at  $-20^{\circ}\text{C}$  aliquotted; stable for 6 mo.
6. DEPC  $\text{H}_2\text{O}$ .
7. 500- $\mu$ L Microfuge tubes.

#### 2.12. Denaturing the Fragmented Target in the Hybridization Cocktail

Two heating blocks suitable for 500- $\mu$ L Microfuge tubes.

#### 2.13. Hybridizing the Target to the Probe Array

1. Probe array chip(s) (see **Note 3**).
2. GeneChip Hybridization Oven 640 (Affymetrix).
3. 12X MES stock solution (see **Subheadings 2.13.1.** and **2.13.2.**).
4. 2X Hybridization buffer (see **Subheadings 2.13.3.** and **2.13.4.**).
5. Analytical-grade water (e.g., from a bench-top unit such as Simplicity<sup>®</sup> Personal Ultrapure Water System, Millipore; cat. no. SIMS 600 00; see **Note 4**).

##### 2.13.1. 12X MES Stock

1. MES free acid monohydrate (4-morpholineethanesulfonic acid monohydrate 2-(N-morpholino)ethanesulfonic acid; Sigma-Aldrich; cat. no. M-5287). Store at RT, shield from light, stable for 1 yr. Safety Information: irritating to eyes, to respiratory system, and to skin.
2. MES sodium salt (2-morpholinoethanesulfonic acid sodium salt; Sigma-Aldrich; cat. no. M3058). Store at RT, shield from light, stable for 1 yr.
3. Analytical-grade water (e.g., from a bench-top unit such as Simplicity<sup>®</sup> Personal Ultrapure Water System, Millipore; cat. no. SIMS 600 00).



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