

## PRACTICE GUIDELINE

### Guidelines for nucleic acid detection and analysis in hematological disorders

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#### 1. GENERAL INTRODUCTION

Nucleic acid detection in hematological disorders has been carried out in a wide range of medical fields or departments. Hence, it is recommended that the hematology laboratory should draw up guidelines or a manual for each disease (group), field or specialty. Nucleic acid detection is defined as processes which are disclosed using depolymerization of a nucleic acid hybrid (deoxyribonucleic acid; DNA or Ribonucleic acid; RNA) to qualitatively or quantitatively analyze for the presence of a predetermined nucleic acid. Applications of these processes include the detection of single nucleotide polymorphisms, identification of single base changes, genotyping and medical marker for diagnostics purposes.

##### 1.1 Scope

The use of nucleic acid diagnostic tools in the management of hematological disorders presents new challenges to the pathologist. Despite the clear benefits of having these methods, issues of sensitivity and “false positives” require stringent laboratory practices. To assure the success of nucleic acid diagnostics, several key areas warrant attention.

The aim of this document is to provide consensus standards and guidelines for using nucleic acid analysis. It is directed to the laboratories that are either using nucleic acid detection techniques in medical diagnosis or research or intending to establish a testing program using these techniques. It is also a supplementary document for the Department of Standard Malaysia, MOSTI to be read in conjunction with

MS/ISO15189 standards,<sup>1</sup> so that laboratories using nucleic acid detection techniques may be assessed for their compliance.

Hematological disorders fall into two major categories:

- (a) inherited disorders (eg Thalassemia, G6PD deficiency)
- (b) acquired disorders (eg Leukemia).

#### 1.2 Categories of hematological disorders

##### 1.2.1 Inherited hematological disorders

Nucleic acid-based testing for inherited disorders are complex compared with other hematology testing because the information generated has relevance not only for the person being tested but also for other family members. Some of these tests require the patient’s verbal consent after the provision of information by a qualified practitioner. For others, formal pre- and post-test professional genetic counseling, as well as formal written consent and additional confidentiality procedures, are required as part of testing process. For the purpose of this document, two different stages are identified to highlight the importance of nucleic acid-based testing for inherited genetic disorders (Table 1). Both of the stages shall require consent from patients and/ or family member.

The distinction between standard test and research purposes would usually be made by the doctor ordering the test, since that individual will be best placed to appreciate the short term and long-term implications of the test for the patient and other family members.<sup>2</sup>

Thus, a test may be standard in one particular circumstance but complex in another. This

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confusion will only be resolved with background knowledge about genetic testing and its implications.

**1.2.2 Acquired hematological disorders**

Nucleic acid-based testing for acquired hematological disorders is usually undertaken to confirm a clinical diagnosis or to follow progress of a disease, and would be considered to be standard nucleic acid testing.

**1.3 Ethical responsibilities of laboratory:  
Informed consent**

Standard nucleic acid-based testing and any relevant testing for research purposes shall require a written consent from patients and/or family member (Table 1). An appropriate pre-test and post-test professional genetic counseling should be given to patients and/or family members. Consent is needed for any type of nucleic acid-based testing to ensure that the patient is fully informed and has indeed consented, and to protect the laboratory and hospital in case of disputes.

**1.4 Advancement of test**

Nucleic acid-based testing is advancing rapidly and, the significance of a result may change with time and experience. Therefore, the laboratory director should determine what action will be taken in respect to the previous results

(consultation with relevant clinical colleagues). Generally, a significance change of a test or result should be reported to the ordering clinician, who will determine the following step.

**2. CATEGORIZATIONS OF NUCLEIC ACID DETECTION & ANALYSIS (Table 2)**

**2.1 Gene rearrangement assays**

Amplification-based gene rearrangement studies are technically demanding and expensive procedures which should have proper indications if performed for patient care. The following indications may be appropriate in gene rearrangement studies:

- to distinguish a reactive from a malignant process in tissue or blood;
- to document organ involvement (e.g., skin);
- as an indicator of B or T lymphoid origin for a histological neoplastic proliferation of lymphocytes, particularly if immunological studies are not available or are inconclusive

**2.2 Translocations assays**

In general, the indications for using molecular tests to find specific chromosomal translocations are similar to the indications for gene rearrangement studies. Chromosomal translocation studies are most commonly performed to:

- assist in classification of neoplasms characterized by a specific chromosomal

**TABLE 1: Stages of nucleic acid-based testing in inherited disorders**

Stage	Condition
Standard	(a) Nucleic acid-based testing for diagnostic purposes/ routine testing (e.g. the patient has clinical indicators or a family history of an established inherited disorder and the test is being used to confirm the disorder). (b) Neonatal screening programs. Standard written consent is required
Research purposes (e.g. prenatal, array-based testing)	Nucleic acid-based testing for which specialized knowledge is needed for the test to be requested, and for which professional genetic counseling should precede and accompany the test.  Predictive or presymptomatic testing for conditions for which there are no simple treatment, would usually be included in this grouping. Specific written consent and counseling issues are associated with this grouping.

**TABLE 2: Categorizations of hematological disorders and nucleic acid detection**

No.	Nucleic acid detection & analysis	Hematological disorders		Specimens	Nucleic acid
		Inherited	Acquired		
1.	Gene rearrangement assays	–	Lymphoma: <ul style="list-style-type: none"> <li>• B-cell Clonality – immunoglobulin heavy chain gene rearrangement</li> <li>• T-cell Clonality – T-cell receptor gamma chain gene rearrangement</li> </ul>	Tissue/bone marrow/ body fluid/blood	DNA
			Leukaemia (AML): <ul style="list-style-type: none"> <li>• Inv 16</li> </ul>	Blood/bone marrow	RNA
2.	Translocations assays	–	Lymphoma: <ul style="list-style-type: none"> <li>• t(11;14) (CyclinD1/BCL1 translocation, Mantle cell lymphoma)</li> <li>• t(14;18) (BCL2 translocation, Follicular and Large B-cell Lymphoma)</li> </ul>	Tissue/bone marrow/ body fluid/blood	RNA
			Leukemia/Myeloproliferative disorders: <ul style="list-style-type: none"> <li>• t(9;22), BCR/ABL</li> <li>• t(8;21), AML1/ETO</li> <li>• t(15;17), PML/RAR<math>\alpha</math></li> <li>• t(12;21), TEL/AML1</li> <li>• t(4;11), MLL/AF4</li> </ul>	Blood/bone marrow	RNA
3.	Gene mutation assays <ul style="list-style-type: none"> <li>• DNA sequencing</li> <li>• AS-PCR</li> <li>• Others</li> </ul>	<ul style="list-style-type: none"> <li>• Prothrombin mutation</li> <li>• Factor V Leiden</li> <li>• Hemophilia A (factor VIII gene)</li> <li>• Hemophilia B (Factor IX gene)</li> <li>• Thalassemia</li> <li>• G6PD deficiency (G6PD gene)</li> <li>• X-linked agammaglobulinemia (XLA) (BTK gene)</li> <li>• von Willebrand Disease (VWF gene)</li> </ul>	Leukemia/ Myeloproliferative disorders: <ul style="list-style-type: none"> <li>• Fms-like tyrosine kinase 3 (<i>FLT3</i>) mutation</li> <li>• JAK2 mutation</li> <li>• NPM1 insertion mutation</li> <li>• KIT</li> <li>• CEBPA</li> <li>• MPL</li> <li>• BCR/ABL</li> </ul> Clonal eosinophilic disorders: <ul style="list-style-type: none"> <li>• PDGFRA &amp; PDGFRB</li> <li>• KIT</li> <li>• FGFR1</li> </ul>	Blood/bone marrow	DNA
4.	Array-based Assays <ul style="list-style-type: none"> <li>• DNA array (e.g: SNP array, CytoScan, array CGH)</li> <li>• RNA array (expression array)</li> </ul>	Any inherited hematological disorder associated with complexity/normal cytogenetic results	Any acquired hematological disorder associated with complexity/normal cytogenetic results	Blood/bone marrow/ tissue/body fluid	DNA
					RNA

rearrangement, such as the t(14; 18) or bcl-2 gene rearrangement of follicular lymphoma (or follicle center cell-derived lymphomas); and

- detect minimal residual or recurrent disease in tissues, blood, and bone marrow or body fluid specimens.

### 2.3 Gene mutations assays

Although the detection of point mutations may eventually prove to be useful in the screening of hematological disorders, the use of gene sequence analysis may be required to confirm the sequence abnormalities corresponding to known genetic causes of hematologic disease, such as *JAK2* (myeloproliferative diseases) and *FLT3* (AML) mutations.

### 2.4 Array-based assays

This advanced molecular analysis may be useful in some complex cases of haematological disorders with normal cytogenetic results. Several types of array-based assays such as array-comparative genomic hybridization (CGH), gene expression array and DNA array have been applied in research purposes but could be essential to improve the diagnosis of certain complex cases.

## 3. LABORATORY SERVICES

### 3.1 Pre-analytical

#### 3.1.1 Specimen identification

The container in which each specimen is received shall be clearly marked with at least two unique patient identifiers.

All specimens should be accompanied by a requisition form which contains, at a minimum as stated in MS ISO/15189:2014 (5.4):-

- Condition in which specimen was submitted (fresh, frozen, ethanol-fixed, etc.);
- Referring medically-qualified personnel

#### 3.1.2 Specimen transport & storage

The condition for preservation of RNA is more stringent than those required to preserve DNA. When amplification-based methods are used for molecular testing, the risk of obtaining false-positive results is high. Great care must be taken in the process of specimen collection. To the extent possible, specimens should be handled only with disposable instruments, which should then be discarded between specimens. When this

is not possible, instruments must be clean after each testing, and/or new, clean, and preferably sterilized instruments employed.

#### 3.1.2.1 Transport and storage of solid tissue

Solid tissue from postmortem or surgical specimens is appropriate for molecular testing. Ideally, tissue should be snap-frozen or fixed immediately. If freezing is not possible, refrigeration may be adequate (if nucleic acid isolation can be begun within an hour). If the specimen is to be frozen, it should be minced and placed in a ribonuclease-inhibiting buffer. If none of these steps can be accomplished, a portion of the tissue should be fixed. Alcohol-based fixatives are preferred for samples from which nucleic acids are to be isolated.

- *Frozen Tissue*  
Snap-frozen tissue can be transported on dry ice and stored at least at  $-70 \pm 10^{\circ}\text{C}$
- *Fresh Tissue*  
Fresh tissue can be transported on wet ice and processed for testing immediately upon arrival in the laboratory.
- *Fixed Tissue*  
Solid tissue fixed in formalin and embedded in paraffin may give acceptable results.

#### 3.1.2.2 Transport and storage of whole blood and bone marrow aspirates

- *Whole blood*  
Whole blood is collected in anticoagulated (EDTA or citrate) container and transported/ stored at room temperature for up to 24 hours. For DNA-based testing, samples can be stored at 2 to 6°C for up to one week while samples for RNA-based testing must be processed immediately.<sup>3</sup> As a specimen ages, RNA and DNA both degrade. RNA degradation proceeds very rapidly.
- *Bone marrow aspirates*  
Anticoagulated (EDTA or citrate) bone marrow aspirates should be transported on ice and stored at 4°C. For RNA-based testing, it is advisable to use a ribonuclease inhibition buffer to ensure a good quality of RNA.

### 3.2 Analytical

The traceability of the specimen shall be maintained throughout the analytical process.

#### 3.2.1 Nucleic acid isolation

The integrity of the nucleic acid has to be

maintained and impurities present in the final sample preparation be diluted sufficiently to eliminate interference with the test.

- *DNA isolation*

DNA isolation from samples generally requires the lysis of cells and addition of a proteolytic enzyme. The extracted DNA should be resuspended in a buffer or deionized water. Excessive drying should be avoided, as it can affect the structural integrity of the extracted DNA.

Several commercial nucleic acid isolation kits are available that may simplify and reduce the time of this procedure. A variety of methods using different components and procedures to perform the above steps are used. Each laboratory shall evaluate its DNA extraction system for yield efficiency and DNA purity before incorporation into the routine workload.

- *RNA isolation*

RNA is very sensitive to degradation by RNases which may contaminate glassware and plastic ware. Extra care is necessary to ensure that all reagents and supplies used in the extraction and storage of RNA are properly treated to destroy or inhibit RNases. Most liquid reagents prepared in the laboratory may be treated with diethylpyrocarbonate (DEPC) followed by autoclaving (exceptions include reagents containing Tris buffer and reagents with heat-sensitive ingredients such as protein and volatile compounds). A variety of commercial preparations are available that either inhibit or destroy this enzyme activity.

### 3.2.2 Quality and quantity of nucleic acid

A good quality and quantity of DNA shall be evaluated by at least 1 method and RNA shall be evaluated by at least 2 methods (gel electrophoresis should be included) as described below;

- *Spectrophotometry*

DNA and RNA can be quantitated using optical density (OD) on a spectrophotometer. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as “pure” for DNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants

that absorb strongly at or near 280 nm. An OD A260/A280 ratio provides a qualitative measurement of the level of RNA with respect to the amount of contaminating protein that exists in the sample. Ratios above 1.6 indicate high levels of RNA purity. Purity may be improved by re-extraction and precipitation when ratios obtained are below 1.6. RNA with a ratio between 1.6 and 1.9 is often adequate from RT-PCR and other amplification approaches.

- *Gel electrophoresis*

The gel electrophoresis method should be performed with an appropriate DNA or specific RNA marker. The presence of a single band for DNA and at least 2 bands for RNA is accepted as a good quality.

- *Fluorometry*

Fluorometric assays should be able to detect double-stranded DNA and should utilize the appropriate human DNA standards. Such assays can be obtained from several commercial vendors and should be used in conjunction with sophisticated instrumentation. It is important that the user carefully follows the manufacturer’s protocol for both the assay and the use of the instrument, and includes proper controls to verify performance of the process.

### 3.2.3 Nucleic acid testing

#### 3.2.3.1 Detection methods

The current detection systems may include:

- (a) PCR (qualitative & quantitative)
- (b) dHPLC
- (c) gel/capillary electrophoresis
- (d) solid phase (blots, *in situ* hybridization, wells, beads)
- (e) Array technologies (DNA and RNA array)

The traceability of the physical appearance (e.g: gel from electrophoresis) shall be maintained for methods which need to be use for result interpretation.

#### 3.2.3.2 Validation of methods

Laboratories shall only offer nucleic acid detection tests as routine tests if their technical validity has been established by the laboratory.

- *Diagnostic performance*

Molecular methods should be evaluated in



comparison to a “gold standard”. A new methodology should be implemented if it provides a significant new advantage, e.g., increased accessibility, improved turnaround time and justifiable cost benefit. In the absence of a “gold standard,” the diagnostic endpoint could be identified by clinical manifestation.

- *Standard*

In-house tests, or commercial tests endorsed by the manufacturer as ‘for research use only’ or ‘not for diagnostic use’, shall be validated. The procedures and methods used in nucleic acid detection techniques for diagnostic purposes should be validated according to the following protocols:

- (a) The used of known positive and negative samples.
- (b) Comparing with proficiency test material, if available.
- (c) Comparing with an existing validated method in the laboratory.
- (d) The sensitivity of a test, or cutoff values, should be set at a level that is relevant to the diagnostic use of the test.

- *Other*

Inter-laboratory exchange of samples among the laboratories participating.

### 3.2.3.3 Positive and negative procedure controls

At least one negative control (saline or water) should be included in each assay run to reveal any carry-over or contamination that could be present. Positive controls should be included, preferably at a reasonable dilution of the target and should also be carried through the sample preparation method to demonstrate the success of the entire procedure.

An internal control target is also important in assessing the success of PCR assays such as amplification of beta-globin, to demonstrate the presence of amplifiable DNA in the prepared sample. A positive signal for this control is necessary, particularly to be certain of a negative result for the target of the assay.

For RT-PCR assays targeting messenger RNA, successful amplification of an endogenous mRNA transcript such as a housekeeping gene can be used to demonstrate both the quantity of mRNA and the absence of inhibitors.

### 3.2.3.4 Limitations

One should identify any limitations and contraindications for use of the test, including factors that impact adversely on accuracy of test interpretation (e.g., allelic mutations that cannot be detected by the test, less than optimal analytic performance, etc.) and any technical limitations of the assay.

## 3.3 Post-analytical

### 3.3.1 Post-examination procedures

Please refer to Malaysian Standard, MS/ISO 15189:2014.<sup>1</sup>

### 3.3.2 Storage of nucleic acids

Long-term storage should be carried out at –20°C (DNA) or –70°C (DNA or RNA) to prevent degradation. Long-term storage of RNA can be improved by storing as an ethanol precipitate. Stability of the sample can be maintained for several months by storing at 4°C in tightly capped containers. Integrity of samples should be reevaluated before use if stored for extended periods of time at any temperature.

### 3.3.3 Retention of specimens

Retention times for entire molecular specimens (blood/RNA/DNA) should fulfill the requirements as mentioned in the “Guidelines on Retention of Pathology Records and Materials, Part 1 (Version 1/2005).”<sup>4</sup>

## 4 REPORT AND RECORDS

### 4.1 Reporting the results

Results shall be written in accordance to MS/ISO15189:2014 requirements.<sup>1</sup>

However, the report shall include test methods used in the analysis (e.g. PCR, RT-PCR, quantitative PCR) and limitations of test methods.

### 4.2 Terminology in reports

**Positive or detected:** Indicates that a particular substance has been identified in accordance with the laboratory protocols. The term “not detected” is preferred, indicating that particular substances were absent within the limitations of the test(s) performed.

**Mutational:** Nomenclature strictly follows international guidelines.<sup>5</sup>

**Array based technique:** The candidate genes need to be individually named as well as clearly described. Type of specimen and preparative

procedures potentially influencing alterations shall be described.

**Quantitative:** PCR with limited availability of standards or control material, is recommended to interpret the results in the context of previous results, preferably performed in the same laboratory with the same test method. Limitations of the performed quantitative test shall be specified in the report.

**Preliminary report:** A report (verbal or written) may be issued before all studies are completed  
*NOTE: Written preliminary (interim) reports shall be clearly marked as such indicating that the results are still preliminary and will be followed by a validated final report.*

#### 4.3 Record keeping, Record retention, and specimen retention

Hard copy record is recommended. It can also be incorporated directly into the laboratory report and a copy is kept in the laboratory.

The band patterns produced on gels or blots may be recorded in several forms, including:

- (a) photographic film
- (b) computer records

Retention times for entire molecular tests should fulfill the requirements mentioned in the "Guidelines on Retention of Pathology Records and Materials, Part 1 (Version 1/2005)."

Documents traceability shall be maintained.

#### 4.4 Procedural quality control

For each specimen, quality assurance records should be generated and kept on record.

- The sample and requisition should be examined and deemed acceptable, and the condition of the sample upon receipt noted.
- Samples being accepted for testing despite failure to meet the written criteria should be accepted with notation explaining the decision by the laboratory director.
- Records of the sample preparation procedure, DNA quality and yield, (if possible), components of the PCR assay, amplification parameters, and product detection methods should be detailed in a procedural log.
- Performance standards should be determined and recorded for each critical step in the PCR assay.

Assay worksheets can include recorded data on the results of sample preparation, temperatures of water baths, times of incubations, results of control amplification, and other such parameters which are crucial

parts of the analytical procedure.

## 5 LABORATORY PERSONNEL

### 5.1 Qualifications

The medical laboratory's management defines the minimum levels of qualification and experience necessary for staff members within the laboratory:

- (a) Ensuring that the responsible persons (Laboratory Director and/or deputy) possess the required relevant qualification(s) enabling them to be officially in-charge of the analyses.
- (b) Ensuring that laboratory personnel possess at least a recognized medical laboratory technology diplomas (MLT).
  - Management personnel: at least a minimum 6 months experience in nucleic acid-based techniques.
  - Technical personnel: at least a minimum 3 months experience in nucleic acid-based techniques.

### 5.2 Job Description

#### 5.2.1 Laboratory Director and /or Deputy

The director or deputy of the laboratory shall demonstrate an appropriate documentation of the procedures used and tests performed are within the scope of the education, training and experience of individual scientific or technical staff members. The person shall also be responsible to review and interpret the laboratory data; and in providing clinical consultation.

#### 5.2.2 Management personnel

The person-in-charge shall be involved in determining procedures, staff training and/or performing the test.

#### 5.2.3 Technical personnel

The person-in-charge shall perform the test and be involve in related laboratory activities.

### 5.3 Training and competency

Personnel shall be competent to manage and perform the nucleic acid-based techniques. Personnel shall be educated and trained to possess expert knowledge of nucleic acid principles, the technologies employed and the limitations of the tests used. Training, effectiveness of it and work experience shall be documented for all members including management of the medical laboratory.

## 6 LABORATORY FACILITIES

### 6.2 Accommodation

Laboratories undertaking nucleic acid amplification should be configured to minimise the risk of contamination of samples and reagents by other samples in the laboratory or by amplified material. The term 'separate area' means laboratory space that is separated by the performance of the test. The term 'contained area' means a laboratory space that can be isolated either by walls and doors or within the working space of the separate area.

#### 6.2.1 Minimum standards for a nucleic acid amplification facility

The standards listed below are the minimum standards for a PCR laboratory;

Three physically separate areas are required in order to reduce the risk of cross-contamination or carry-over contamination:

- (a) for the extraction of nucleic acids from samples
- (b) for the preparation of PCR reagents (including dispensing of the master mix)
- (c) contained area for amplification and product detection.

Where the areas for preparation of reagents and sample preparation are located within a single room, wide separation of these activities shall be maintained and appropriate procedures and controls shall be implemented to detect contamination.

Post-PCR analysis shall not be incorporated into areas where reagent preparation or sample preparation occurs. The post-PCR area shall be positioned so as to minimise the possibility of cross-contamination of preamplification areas. Generally, this can be achieved by positioning the post-PCR area at an appropriate distance from the preamplification area.

Reagents and equipment shall be limited to the appropriate sections. In particular, no nucleic acid samples shall be taken into the reagent preparation area. Samples shall be stored separately from reagents.

Equipment from other areas shall not be taken into the reagent preparation area.

The movement of specimens and equipment shall be unidirectional; that is, from preamplification to postamplification areas. Only sealed PCR amplification tubes and tube racks shall be

carried between the preamplification area and the postamplification area.

Where equipment (such as tube racks) is returned against the flow, it shall first be decontaminated by decontaminating agents.

Laboratory coats and gloves shall be changed before staff move to or from each area.

### 6.3 Equipment

Entire analytical equipments which will interfere the outcome of results (eg: Pipettes' thermal cyclers, ect.) need to be calibrated by service providers who are also certified or has attained ISO standards specific for the industry.

- *Pipette calibration*

Pipettors shall be calibrated to ensure accurate delivery of reagents. Separate pipettors should be used for reagent preparation, specimen preparation, and postamplification analysis. Pipettors used for nucleic acid amplification set-up should always be separated from amplified products and should remain in the area in which they are used. All pipettors should be cleaned at regular intervals.

- *Thermal Cyclers*

Thermal cycler should be tested and maintained to ensure uniform heating throughout the block. Ramping times are a critical aspect of many assays and should be checked by printing the cycle parameters.

- *Centrifuges*

Centrifuges can serve as a major source of aerosols and contamination if samples are not properly loaded and spun in sealed containers. The speed of the centrifuge should be checked. The instrument should be cleaned on a regular basis and specimen holders checked for cracks and wear.

- *Biological safety cabinets*

Contamination is a key source of erroneous results in the nucleic acid amplification laboratory; therefore, it is suggested that separate safety cabinets or hoods be designated for reagent preparation and for sample processing. Hoods should be decontaminated at the start and end of each work day and immediately after a spill or accident. The airflow in all safety cabinets and hoods should be monitored continuously and calibrated. The placement of ultraviolet (UV) lights in hoods



may decrease the contamination of samples by inactivating DNA, but it also poses an additional safety hazard to workers. Therefore, UV lights should be used with caution. Also, the energy efficiency of UV lights should be checked.

5. Nomenclature for the description of sequence variants [Internet]. 2014. Available from: <http://www.hgvs.org/mutnomen/>.

#### 6.4 Reagents

All reagents used in nucleic acid amplification should be prepared, divided into aliquots, and stored in an area that is separate from the specimen preparation or post-amplification area. Dedicated equipment and supplies should be used. Oligonucleotides should be synthesized and purified in a clean, amplification product-free environment. Once reaction conditions have been optimized, reagents can be premixed into master mixes. These master mixes can be divided into aliquots of the volumes required for each reaction run. This will minimize the number of samplings and reduce the potential for contamination. The reagent lot number should be recorded so that if carry-over does occur, the source can be easily identified.

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