FDA Pre-IDE Submission

Lung Metagene Score (LMS)

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I. INTRODUCTION

Lung cancer is the leading cause of cancer death, accounting for more than 1 million deaths in 2000 and about 18% of total cancer deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for almost 80% of lung cancers, of which 40-50% of these are adenocarcinomas. Although patients diagnosed with stage I NSCLC have an overall 5-year survival rate of about 70%, nearly 30-35% will relapse after surgical resection, thus portending a poor prognosis (Naruke 1988, Strauss 1995, Harpole 1995). Pathologic stage IA (40% of clinical stage I; >20,000 patients/year in North America) represent the fastest growing segment of patients with lung cancer due to the increased use of high-resolution CT scans for screening. Staging based on tumor size and invasion (TNM) remains the mainstay for prognosis and treatment decisions. However, clinical studies have demonstrated a 4 to 15% survival advantage for patients treated with adjuvant chemotherapy after resection in patients with pathological stage IB to IIIA. Investigators at Duke have focused on the identification of markers that may predict poor prognosis and detect early recurrence as well as investigating their application in the clinical setting. A molecular-based tumor model was developed based on expressions patterns collected from microarray data to stratify patients for risk of cancer recurrence (Potti et al., 2006). Based on these predicted recurrence risks, patients in the high risk group would benefit from adjuvant therapy, while limiting low risk patients' exposure to potentially harmful treatments with little benefit.

II. ADMINISTRATIVE REQUIREMENTS

A. Contact Information

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B. Device Information

Trade name (proprietary name): Lung Metagene Score
Common name: LMS

III. INTENDED USE AND INDICATIONS FOR USE

A. Intended Use

The Lung Metagene Score will utilize RNA expression levels from a surgically resected tumor sample to generate an individualized prediction of disease recurrence in stage I NSCLC patients. The probability of recurrence, as determined by the Lung Metagene Score, will then be used by the treating physician to inform the administration of adjuvant chemotherapy.

B. Indication for Use

All diagnosed stage I non-small cell lung cancer (NSCLC) patients.

IV. DEVICE DESCRIPTION

A. Reagents and test components

- RNA Integrity Check (Appendix 1)

Equipment:
2100 Agilent Bioanalyzer (Agilent Technologies, Cat# G2940CA, 1-877-424-4536)
Microcentrifuge (Beckman Coulter 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
MiniVortexer (VWR cat#58816-121, 1-800-932-5000)

Supplies:
RNaseZAP® (Ambion, Inc. Cat# 9780, 1 800 888-8804)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550)
1.5 mL microcentrifuge tubes (RNase free) (VWR cat#14231-062, 1-800-932-5000)
Heating block (VWR cat#13259-030, 1-800-932-5000)
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)

Reagents:
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water
RNase Away (VWR, Cat# 17810-491, 1-800-932-5000)

- Cleanup and Quantification of Biotin-Labeled cRNA (Appendix 2)

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)

Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water
80% (v/v) Ethanol in water
RNase free water (VWR, Cat#EM-9610, 1-800-932-5000)

- Cleanup of Double-Stranded cDNA (Appendix 3)

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water

- First Strand cDNA Synthesis Protocol (Appendix 4)

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips (20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)

Reagents:
One-cycle cDNA Synthesis Kit (Affymetrix Cat#900431, 1-888-362-2447)
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water

- Fragmenting the cRNA for Target Preparation (Appendix 5)

Equipment:

MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Sterile, RNAse-free pipette tips (20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water
80% (v/v) Ethanol in water
RNase free water (VWR, Cat#EM-9610, 1-800-932-5000)

- NanoDrop: Determining RNA Concentration (Appendix 6)

Equipment:
NanoDrop® ND-1000 (NanoDrop Technologies, Inc., phone# +1-302-479-7707)

Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Tissue wipes (VWR cat#82003-820, 1-800-932-5000)
Sterile, RNAse-free pipette tips (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)

Reagents:
5.25% solution of sodium hypochlorite (VWR cat#37001-060)
(bleach-freshly prepared) can be used to ensure that no biologically active material is present on the measurement pedestals.

- Qiagen RNeasy Plus RNA Isolation (Appendix 7)

Equipment:
Mini-Beadbeater (Catalog#3110BX (Instructions for the Mini-Beadbeater 3110BX (Biospec Products, Inc. 800-617-3363))
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Microvials, 2mL with caps (Cat# 10831 BioSpec Products Inc.)
Zirconia/silica beads, 2.5mm (Cat# 11079125z BioSpec Products Inc.)

QiaShredder (Cat# 79654 Qiagen, 800-426-8157)
Sterile 15mL polypropylene tube
Sterile, RNAse-free pipette tips (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550)
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)

Reagents:
RNeasy Plus Mini Kit (Cat#74134 Qiagen, 800-426-8157)
2-Mercaptoethanol (B-ME) (Cat# 21985-023 Gibco)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water

- Second Strand cDNA Synthesis Protocol (Appendix 8)

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)

Reagents:
One-cycle cDNA Synthesis Kit (Affymetrix Cat#900431, 1-888-362-2447)
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water

- Synthesis of Biotin-Labeled cRNA (Appendix 9)

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)

Eukaryotic Poly-a RNA Control Kit (Affymetrix Cat#900433, 1-888-362-2447)
One-Cycle cDNA Synthesis Kit (Affymetrix Cat#900431, 1-888-362-2447)
Hybridization Controls (Affymetrix Cat#900454 and 900457, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water
RNase free water (VWR, Cat#EM-9610, 1-800-932-5000)

- Tissue Procurement Protocol (for RNA) (Appendix 10)

Equipment:
Scale (Ohaus Adventurer Scale 0.1 mg – 100gm AR1140 or comprable substitute)
Small liquid nitrogen storage container

Supplies:
5 mM dermal punch biopsy (Miltex, Ref 33-35)
Cryovials (Nalgene VWR cat # 66008-706)
Styrofoam shipping container
Scalpel blade
10 cm stainless steel probe

Reagents:
Liquid nitrogen or alternative freezing media
Dry ice

B. Instruments

1. Instrument Component.

i) Instrument Name: Affymetrix GeneChip Microarray Instrumentation System

ii) System descriptions [Note: Please refer to 510(k) Substantial Equivalence Determination Decision Summary K042279]:

The Affymetrix GeneChip Microarray Instrumentation System is designed to work with microarrays based on Affymetrix GeneChip ® technology.

FS450Dx Fluidics Station. The FS450Dx (Fluidics Station) is an instrument consisting of four modules installed in a single Station or housing. Each module holds a single GeneChip microarray and performs the functions required for hybridization, washing, and staining of that array. Up to 8 stations communicate to a workstation.

Each module contains controls the addition of target and staining fluids to the array cartridge and subsequent washing of the array. The module contains a pump, valve, thermo-electric system, and LCD that are controlled by scripts selected by the system operator and automatically downloaded to each module, then stored in the module's electronic memory.

GCS3000Dx Scanner. The GCC3000Dx Scanner is a wide-field, epifluorescent, confocal, scanning laser microscope which scans the chip after the staining process performed by the Fluidics Station. Array cartridges are loaded into the scanner by an automatic handler (the

Autoloader) prior to scanning, and returned to the handler after scanning is complete.

iii) Mode of operation: Batch (the scanner automatically loads and unloads chips from the autoloader)

2. Software Component [Note: Please refer to 510(k) Substantial Equivalence Determination Decision Summary K042279]:

GCOSDx Software. The GeneChip® Operating Software (GCOSDx) provides the interface between the user and instrument systems. It is the software that provides instrument control and the application for processing arrays and data collection. Upon completion of scanning of the array, data is passed through GCOSDx to the assay specific software component that contains the algorithms and reporting functions to produce a clinical result.

Device Features Controlled by Software

i. The GeneChip® Operating Software (GCOSDx) provides the interface between the user and the instruments. GCOSDx controls the FS450Dx, GCS3000Dx and the AutoLoaderDx. GCOSDx may also be used to monitor the operations being performed by each instrument.

ii. GCOSDx controls the fluidics station using fluidics scripts specific to the assay being performed. Fluidics scripts are written to a directory specified during GCOSDx installation.

iii. GCOSDx aids and controls scanner movement and image capture including grid alignment. GCOSDx displays a picture of the scan image in an image window on the computer workstation. The software represents the fluorescence intensity values from each pixel on the array in a grayscale or pseudocolor mode. This image is saved as a ".dat" file format.

iv. GCOSDx then uses an alignment algorithm to superimpose a grid on the image to delineate probe cells. The alignment algorithm uses a checkerboard image of control probes, located at the corners of the probe array to superimpose the grid on the scanned image. GCOSDx generates cell intensity data from the image data. The cell analysis algorithm analyzes the image data and computes a single intensity value for each probe cell on the array. This data is saved as a ".cel" file. It is the ".cel" file that is handed to the assay specific software for final data analysis and result reporting.

Operational Environment (Off-The-Shelf Software)

GCOSDx is programmed in C++. GCOSDx functions on MicroSoft® Windows® 2000 SP3 or SP4 Operating System. The work stations also include Internet Explorer 6.0, Office XP and MDAC 2.7 SP1. The operating system will be moved to MicroSoft® XP in the near future. Prior to this shift, the appropriate validations will be performed. Dell workstations included with the system must have 750 MHz to 3.0 GHz Processor speed as well as 256 Mbyte to 1 Gbyte Memory and a 10 Gbyte to 80 Gbyte Hard Drive.

Operational Environment (Custom Software)

Description of Predictor software : OS, language(s), software requirements or dependencies, hardware requirements or dependencies. outputs

Description of Predictor software test plan (outline):

Performance testing: Test a set of well-defined use cases (typically the validation set) for performance including accuracy of predictor, accuracy of diagnostic output, and the response time

Reliability testing: Test a set of well-defined use cases, some typical, most atypical to reflect extreme conditions and ensure software does not fail or abnormally terminate

Sensitivity testing: Determine the sensitivity of predictive model to errors/changes in input data. Specifically,

- Determine sensitivity of the predictive model to general random noise
  - Determine the CV distribution of probeset Signal for samples used for training in original study
  - Determine CV of transcripts/probesets used in model for samples used for training in original study (normal and cancerous)
  - Determine CV distribution of individual transcripts/probesets used in model for normal lung samples collected by independent researchers
  - Via Monte Carlo simulation, add additional noise (percentage-wise) to individual transcripts/probesets in the validation set to determine the level of median CV where the model begins to lose discrimination (plot predictive accuracy versus noise level)
- Sensitivity testing: Determine sensitivity of model to large changes in one or a few data inputs (single-point or localized failures)
  - Iterating through each probeset in the predictive model, alter 2-fold to 10-fold up or down and characterize impact on predictor for the validation set
  - For approximately 1000 random samples of a few probeset inputs (2-5), characterize sensitivity of predictive model to large random changes (2-fold to 10-fold up or down) in these inputs

3. Sample Identification.

All samples will be identified via bar code. The operator will enter patient ID information. The barcode is scanned at the level of the fluidics station and the scanner.

4. Specimens sampling/handling in the software code.

Specimens are processed according to assay instructions. Reagents specific to each assay and fluidics protocol are manually loaded onto the fluidics station.

5. Assay types handled by instrument/software.

Molecular biology, hybridization-based assays.

6. Calibration parameters for the system

Please refer to 510(k) system submission K042279

7. Installation parameters and requirements.

Please refer to 510(k) system submission K042279

8. User definable parameters/open or closed system.

Marketed system will be closed. Please refer to 510(k) system submission K042279.

9. Internal process controls related to the system.

Please refer to 510(k) system submission K042279.

10. Storage of reagents and use of temperature regulation.

Reagents are stored off line. Reagent temperature regulation is therefore not used.

11. Previous submission.review of software development.

The software development was reviewed by the FDA in 510(k) system submission K042279.

12. Level of concern for the software (e.g., major, moderate or minor).

For purposes of the lung cancer clinical trial, risk of injury is very low as the standard of care will not be impacted. A portion of stage 1a NSCLC patients will be selected for chemotherapy and monitored to assess survival compared to stage 1a NSCLC patients receiving observation only.

C. Sample/specimen type and procedures – specimen collection, handling (storage and transportation) and processing.

*Tissue procurement and transport***.** The LMS is intended for use on snap frozen lung tumor tissue which is harvested from lung tumor resection specimens at the time of surgery. This is the ONLY acceptable sample type. The tissue should be harvested within 45 minutes of surgical resection of the tumor using a 4.0 mM dermal punch biopsy (Appendix I: LMP Tissue Procurement). This ensures adequate sample quantity. The sample is then weighed. At least 25mg of tissue is required. A typical 4mM long core of tissue weighs approximately 50-100 mg. The core of tissue is then cut into two pieces (4mm and 1mm long). The specimen is then snap-frozen in a 1.5 ml polypropylene cryo-vial in liquid nitrogen and shipped overnight to our processing laboratory on dry ice.

Histologic assesment of sample quality: On arrival at our processing laboratory, each core of tissue is visually inspected for evidence of thawing in transport. It the sample appears intact the smaller piece (1 mm) is embedded in OCT media and an H&E stained frozen section is made using standard techniques. The frozen section will be evaluated for percent tumor content, percent necrosis, tumor type and tumor grade by an AP board certified pathologist. Tumors that are > 25% necrotic or samples that contain < 50% tumor (i.e., tumors that are highly inflamed, that contain a large amount of normal structures such as normal lung epithelium, or that are highly fibrotic) will be excluded from further evaluation.

RNA preparation and quality assurance: For tumors that pass the above histologic criteria, total RNA is prepared using the Qiagen RNeasy Plus Kit using our tissue RNA extraction standard operating procedure (Appendix II: LMP Qiagen RNeasy Plus RNA Isolation). RNA quantity is assessed using a nanodrop spectrophotometer (Appendix III: LMP NanoDrop Determining RNA Concentration). A minimum of10ug of RNA is required for in this assay; samples with less than 10 ug of RNA cannot be analyzed using this assay. Approximately 30ug of high quality RNA can be routinely purified from 50 – 100 ug of lung tumor tissue. In addition, an estimate of RNA purity is determined by measuring the OD260 / OD 280 ratio. Samples with a ratio less than 1.5 or greater than 2.1 are not suitable for subsequent microarray analysis. Sample RNA quality is checked using an Agilent biolanalyzer (Appendix IV: LMP Agilent 2100 Bioanalyzer RNA Integrity Check). An RNA integrity score (RIN score) is derived from the electropherogram. Cut-off values for the RIN score will be determined in the validation procedures outlined below.

cDNA and biotinylated cRNA synthesis: cDNA is then synthesized from RNA that passes initial quality checks using T7- linked oligo-dT primer according to our cDNA synthesis standard operating procedures (Appendix V - VI: LMP First Strand cDNA Synthesis, LMP Second Strand cDNA Synthesis). cRNA is then systhesized through in vitro transcription with biotinylated UTP and CTP (Appendix VII: LMP Synthesis of Biotin-Labeled cRNA). The cRNA is then chemically fragmented at 95oC for 35 min. (Appendix VIII: LMP Fragmenting the cRNA for Target Preparation). This labeled and fragmented cRNA is the analyte that is used on the Affymetrix U133 plus 2.0 human gene expression array.

D. Principle of operation for the methodology.

- Array hybridization
- Array scanning
- Initial array quality assessment
- RNA normalization of array data
- Generating a metagene recurrence risk score

V. Analytical Performance – Specific Performance Parameters

A. Precision

Given the wealth of discoveries generated using expression microarrays over the past decade, it is surprising that microarray technology remains almost exclusively a research tool. Currently, there is not a single expression microarray-based diagnostic or prognostic test, nor is there a single CLIA-approved laboratory that routinely offers this type of testing for clinical decision making. The reason for this is at least two-fold. First, there is a significant lack of collaboration between basic research laboratories using microarrays for discovery and clinical diagnostic laboratories that have expertise in evaluating, validating and implementing high complexity molecular diagnostics tests for routine clinical use. Second, definitive globally accepted criteria for determining precision, accuracy, and robustness in this type of testing has yet to be defined. This lack of consensus has been the major stumbling block for many laboratories with aspirations of clinical testing using this platform.

Simply defined, precision refers to the ability of an assay to produce the same result every time for a single sample. There are multiple factors that have the potential to affect the precision of the

LMP. The first of these is technical variance, defined as variance that is intrinsic to the instrument or technical components of the assay. Fortunately, many groups have studied the effects of technical variance in the Affymetrix expression microarray platform. These studies conclude that there is minimal variance in this system when doing exact technical replicates (i.e., running the same RNA multiple times on different Affymetrix readers and different U133 chips). We plan to reproduce some of these studies to test the effect of technical variance on the precision of the LMP as follows.

a. Determining the Effect of Technical Variance on the Precision of LMP Predictions of Risk. Since this precise topic has been relatively well studied, we will perform a very focused assessment of technical variance. In addition, the effect of technical variance on precision is intrinsic to the studies of biological variance that are described below. In this study, RNA will be used from each of five different lung tumors. These tumors will be selected based on a recurrence risk score that is very near 50% (the cut-off value for high risk vs. low risk) If the RNA samples pass the spectrophotometic and electrophoretic Q/A criteria, they will then each be split equally into 3-4 distinct 10 ug fractions (depending on the quantity of RNA present). Each RNA sample will be labeled and hybridized to an Affymetrix U133 plus 2.0 array. These arrays will be scanned and the data processed accordingly.

For the Affymetrix U133 plus 2.0 microarray, precision can be considered at the level of the 1.3 million individual features on the array. Alternatively, precision can be considered at the level of individual genes, i.e., the normalized composite expression value of the 22 features for each gene on the array after the RNA normalization procedure. Finally, for the LMP, precision can refer to the final risk score that is generated by the assay. We will evaluate variance on all three levels by calculating percent coefficients of variance (%CV) for each. If we identify features or genes that consistently show a high degree of variance across all samples, these will be excluded from the LMP predicting model. This should increase the precision of the LMP risk scores. Preliminary studies show that although technical variance can be appreciated at the feature and gene level, it has almost no impact on LMP risk scores.

b. Determining the Effect of Biological Variance on the Precision of LMP Predictions of Risk. Distinct regions within a single tumor can have different amounts of neoplastic epithelium, desmoplastic fibrosis, inflammation, normal lung tissue and necrosis. As a result, different biopsy samples from a single tumor can produce different microarray mRNA expression profiles. To be clinically useful, however, the LMP should be robust even in the face of significant 'biological variance'. To test this, we will generate LMP recurrence predictions for multiple biopsies from single tumors. We plan on testing 3 biopsies from each of 20 different tumors for these studies. If biological variance significantly impacts precision of LMP predictions, we will identify the genes with the highest degree of biological variance and build LMP predictive algorithms that exclude these genes. Accuracy of the refined LMP will be measured. We will also experimentally generate biological variance that mimics low tumor content, inflammation or necrosis. Data from these samples will be used to refine the LMP model and to generate gene expression signatures that can identify specific types of biological variance that would preclude accurate LMP predictions.

We have already performed precision experiments on four replicates from each of five tumors. In these studies, we have begun to elucidate the impact of biological variance on a gene expression level. Specifically, we have begun to define genes that show significant geographic variance in any one tumor and are in the process of refining the LMP predictor. More importantly, for each tumor, all four samples showed nearly identical predictions of risk, suggesting that in most cases, tumor heterogeneity will not adversely affect precision. However, additional data and analysis are needed to confirm these initial impressions.

c. Determining the Effect of Pre-Analytic Variance on the Precision of LMP Predictions of Risk. Differences in sample quality and processing can significantly affect the results of a clinical assay. This phenomenon is termed pre-analytic variance. For a robust assay, there are often internal measures of sample quality that can control for pre-analytic variance and are used to determine whether or not data from a given experiment can be accurately interpreted. For example, in the case of simple quantitative PCR-based assays, amplification of a control gene is often used as an internal measure of sample quality. If the control PCR fails, the PCR reaction for the gene of interest cannot be interpreted. In the case of expression microarray-based testing, similar objective measures of sample quality have yet to be defined.

To determine the effects of pre-analytic variance, we will begin by experimentally introducing pre-analytical variance through suboptimal sample collection and processing yielding RNA of poor quality. LMP predictions of risk will be generated from these samples and the effects of pre-analytical variance on precision will be determined. In the best case, pre-analytical variance will have little effect on LMP recurrence risk scores. However, it is likely that suboptimal sample processing will lead to a loss of precision. In particular, samples with significant pre-analytic variance will likely produce different LMP risk scores than optimally processed samples. In this case, signatures of poor sample quality will be generated. These will consist of objective microarray findings including 3' bias in signal intensity, overall signal intensity, signal distribution, and the number of array spots with measurable signal. We will also examine more traditional objective measures of RNA quality including OD 260/280 and electrophoretic banding patterns. From this data, we will develop cutoff values for each of these variables that allow precise LMP predictions. In addition, we will attempt to define expression array-based signatures of poor sample quality using the same metagene approach that was used to develop recurrence risk scores. If successful, these signatures could be a universally applicable way to determine whether microarray data is ultimately interpretable.

B. Reproducibility

Reproducibility for the LMP will be determined at two sites: Duke University Medical Center Clinical Molecular Diagnostics Laboratory and Expression Analysis (a Research Triangle Park-based company that specializes in Affymetrix microarray analysis). This will be performed using split lung tumor tissue samples. Reproducibility between sites will be assayed by calculating %CVs for LMP risk scores and normalized gene expression measurement. We expect that the %CVs in these studies should approach those for technical variance determined above.

C. Cut-off Value

A cut-off value for high risk vs. low risk of recurrence was derived from an ROC analysis of 91 patients in a pilot study.

Pilot Study Design. Gene expression patterns of 91 patients were collected and analyzed to determine whether patients at high risk could be distinguished from low-risk patients in order to create initial risk models based on gene expression data. Forty-six squamous cell and 45 adenocarcinoma samples were obtained from an IRB-approved tumor bank maintained by the Duke Lung Cancer Prognostic Laboratory, which contains paired frozen tumor and adjacent non-cancerous lung samples from more than 800 patients with resected clinical stage I NSCLC. The results of this study have led to the development of the LUNG METAGENE PREDICTOR (LMP) that is able to accurately predict the risk of recurrence in NSCLC patients, achieving an accuracy

well beyond the current clinical prognosis factors. Internal and external validation studies were also performed to confirm the accuracy and predictiveness of the LMP.

Patients and tumor samples. (*Duke cohort)* The study used 91 tumor samples from patients with early stage (Ia/Ib, IIa/IIb and IIIa) identified from the Duke Lung Cancer Prognostic Laboratory on an IRB-approved protocol. All samples were reviewed for percentage tumor content and histologic type prior to RNA extraction. Of the 91 patient samples, 89 had adequate RNA quality for gene expression analysis. The cohort of patients with early stage NSCLC was created with an equal mix of the two major histologic subtypes -- squamous cell and adenocarcinoma. Also, each histologic subset had approximately equal number of patients who survived over 5 years and those who died within 2.5 years of initial diagnosis of a documented disease recurrence, resulting in two distinct risk cohorts (referred to hereafter as the Duke Training Cohorts). Forty percent of the patients were female and the median age was 67 years. Extensive clinical data, smoking history, histopathological factors and complete outcomes data, including location of any recurrence was also available for each sample.

(ACOSOG Z0030) A completed prospective, multi-institutional phase III trial of 1100 patients with stage I NSCLC randomized to complete resection with mediastinal lymph node dissection or sampling. A subset of 416 patients had fresh-frozen tumor collected and banked at ACOSOG Central Specimen Bank at Washington University. Forty samples from patients with at least 28 months of follow up were selected obtained for RNA extraction and microarray analysis. Of these, 25 cases were found to have both acceptable tumor cell content and adequate RNA quality for analysis. Approximately half (n = 13) of these patients had died of cancer recurrence.

(CALGB 9761) A completed multi-institutional prospective phase II trial of approximately 500 patients with clinical stage I and II NSCLC designed to assess the prognostic significance of micrometastatic disease using RT-PCR detection of gene expression of MUC-1 and CEA. Patients had fresh-frozen tumor and lymph nodes collected on a rigorous, quality-controlled tissue preparation protocol such that high quality RNA was extracted from >90% of tumors. Gene expression data was generated from primary tumor samples of 84 patients registered to CALGB 9761. Importantly, this was a blinded external validation step wherein the gene expression based predictions of recurrence made without a priori knowledge of the outcome were independently validated with clinical outcome (survival) by a CALGB statistician. The mean follow-up for patients in this group was 5.3 years. There were 34 patients with recurrence, and 50 patients who were disease-free at the time of follow-up. None of the patients in the Duke, ACOSOG, and CALGB cohorts received adjuvant chemotherapy or external beam radiation.

Histopathologic Evaluation. In each of the cohorts, a single pathologist reviewed all slides for histopathologic evaluation according to WHO criteria, including adenocarcinoma subtype, degree of differentiation, lymphatic invasion, and vascular invasion. Only samples with tumor cell content greater 50% were used for the analysis.

RNA Extraction. Approximately 30 mg of lung cancer tissue was added to a chilled BioPulverizer H-tube [Bio101 Systems, Carlsbad, CA]. Lysis buffer from the Qiagen Rneasy Mini kit was added and the tissue homogenized for 20 seconds in a Mini-Beadbeater [Biospec Products, Bartlesville, OK]. Tubes were spun briefly to pellet the garnet mixture and reduce foam. The lysate was transferred to a new 1.5 ml tube using a syringe and 21 gauge needle, followed by passage through the needle 10 times to shear genomic DNA. For the Duke and ACOSOG samples, total RNA was extracted from the tumor tissue using Qiagen RNeasy Mini kits (Qiagen, Nalencia, CA, USA) and assessed for quality with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA).

Gene expression arrays. The samples from the Duke Cohort and ACOSOG Z0030 were prepared and arrayed using Affymetrix U133 plus 2.0 GeneChips at the Duke Microarray Facility, and the samples from CALGB 9761 were prepared and arrayed using Affymetrix U133A GeneChips at the University of Michigan. Hybridization targets (probes for hybridization) were prepared from total RNA according to standard Affymetrix protocols. The amount of starting total RNA for each reaction was 10 μg. Briefly, first strand cDNA synthesis was generated using a T7- linked oligo-dT primer, followed by second strand synthesis. An *in vitro* transcription reaction was performed to generate the cRNA containing biotinylated UTP and CTP, which is subsequently chemically fragmented at 95^{o}C for 35 min.

The fragmented, biotinylated cRNA was hybridized in MES buffer (2-[N-morpholino]ethansulfonic acid) containing 0.5 mg/ml acetylated bovine serum albumin to the Affymetrix GeneChip Human U133 plus 2.0 arrays at 45^{o}C for 16hr, according to the Affymetrix protocol (http://www.affymetrix.com). The arrays contain over 47,000 transcripts. Arrays were washed and stained with streptavidin-phycoerythrin (SAPE, Molecular Probes). Signal amplification was performed using a biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA) at 3μg/ml. This was followed by a second staining with SAPE. Normal goat IgG (2 mg/ml) was used as a blocking agent.

Scans were performed with an Affymetrix GeneChip scanner and the expression value for each gene was calculated using the Affymetrix Microarray Analysis Suite (v5.0), computing the expression intensities in 'signal' units defined by software. Scaling factors were determined for each hybridization based on an arbitrary target intensity of 500. Scans were rejected if the scaling factor exceeded a factor of 30. Expression was calculated using the robust multi-array average (RMA) algorithm implemented in the Bioconductor (http://www.bioconductor.org) extensions to the R statistical programming environment. RMA generates log-2 scaled measures of expression using a linear model robustly fit to background-corrected and quantile-normalized probe-level expression data and has been shown to have a better ability to detect differential expression in spike-in experiments.

The probe sets were screened to remove control genes and those with a small variance and those expressed at low levels. All raw and RMA transformed data for the Duke, ACOSOG, and CALGB datasets can be accessed at the Gene Expression Omnibus (GEO) databases website (http://www.ncbi.nlm.nih.gov/geo). The GEO accession number for the databases is GSE3593 (username: nevin001/password: IGSP).

Statistical analysis. Statistical analysis was performed using the metagene construction and binary prediction tree analysis used previously in our analysis of gene expression patterns predictive of breast cancer outcomes (Huang et al., 2003; West et al., 2001; Pittman et al., 2004). The initial step filtered out genes whose maximum expression did not exceed the median value of expression or did not vary more than 1-fold across the samples to remove genes with extremely low levels of expression or little variance resulting in a 2071 gene list. Thereafter, we input clinical variables with prognostic significance in NSCLC to include age, gender, histologic subtype, clinical stage, and tumor size at surgery. Next, we clustered the samples into groups based on their expression patterns with the notion that related genes share similar variances in expression using K-means clustering. This algorithm randomly places genes into a predetermined number of groups. The genes are then shuffled among the groups in an iterative fashion to maximize the distinction between each group. The number of designated clusters was also then varied iteratively to further maximize differences between the clusters. The resulting clusters contained anywhere from 15 to 50 genes and represented a unique gene expression pattern.

Singular value decomposition was performed on each cluster to generate a single factor, called a metagene. The metagene is the dominant expression pattern of a cluster and represents a group of genes that share a common gene expression signature in the context of a particular experimental condition. The metagenes are then used in binary decision trees to partition the samples into subgroups. In the trees, a metagene or a clinical variable is used at a branch point to partition samples to 1 of 2 classifications based on similarity or dissimilarity of a sample's gene expression pattern to the metagene. Each tree had several of these branches, and almost 100 trees were generated to determine the metagenes that did the best job of partitioning the samples (Figure 1). Within each metagene, we then identified the genes that lend the most weight to the dominant expression pattern. To guard against over-fitting, given the disproportionate number of variables to samples, we initially performed honest, out-of-sample cross validation analysis to test the stability and predictive capability of our model. Each lung cancer specimen was left out of the data set one at a time. The model was refitted (both the metagene factors and the partitions used) using the remaining samples, and the phenotype of the held out case was then predicted and the certainty of the classification was calculated (Figure 2).

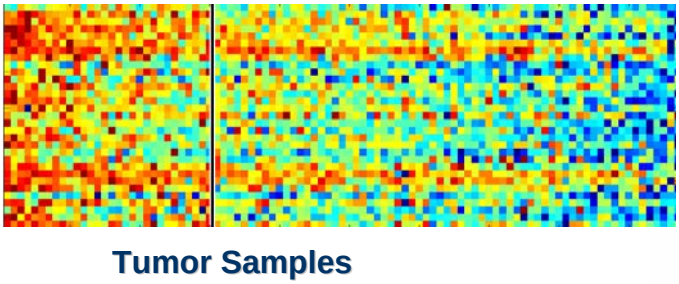
Standard Kaplan-Meier mortality curves and their significance were generated for groups of patients predicted as low or high risk using GraphPad software. For the Kaplan-Meier survival analyses, the survival curves are compared using the log-rank test. This test generates a two-tailed P value testing the null hypothesis, which is that the survival curves are identical in the overall populations. Therefore, the null hypothesis is that the populations have no differences in survival. The importance of the model's accuracy is displayed in Figure 3.

Internal and External Validation. After a model with high predictive ability was generated from the leave-one-out cross validation, the robustness of this genomic model was then tested in an internal validation step using several, random 2/3rd-1/3rd splits of samples within the Duke Cohort (Figure 4). Note that the predictive ability was highest for patients with stage IA disease (n=39).

Further, and more importantly, external validations of our approach were performed using the samples obtained from the ACOSOG Z0030 and CALGB 9761 multi-institutional studies, with blinded patient outcomes (Figures 5). Figure 6 demonstrates the LMS and survival for all stage I patients from the validation cohorts (ACOSOG and CALGB; n=71). The LMS results in a hazards ratio of 8.5. Figure 7 summarizes the results, with no difference observed for either cell type. Importantly, the validation in the CALGB 9761 sample set was performed in a blinded fashion, where the investigators performing the prediction of recurrence did not have *a priori* knowledge of the actual outcome. The predictions of recurrence were then sent to the CALGB statistical center to evaluate and validate the performance of the metagene-based prognostic model.

Simple univariate and multivariate logistic regressions for recurrence (with and without the genomic-based assessment of recurrence risk) were also computed to assess the baseline prognostic value of the individual clinical variables in the Duke (training) and CALGB (validation) cohorts. Standard Kaplan-Meier mortality curves and their significance were generated for groups of patients predicted as low or high risk using GraphPad software. For the Kaplan-Meier survival analyses, the survival curves are compared using the log-rank test. This test generates a two-tailed P value testing the null hypothesis, which is that the survival curves are identical in the overall populations. Therefore, the null hypothesis is that the populations have no differences in survival.

1A.



1B.

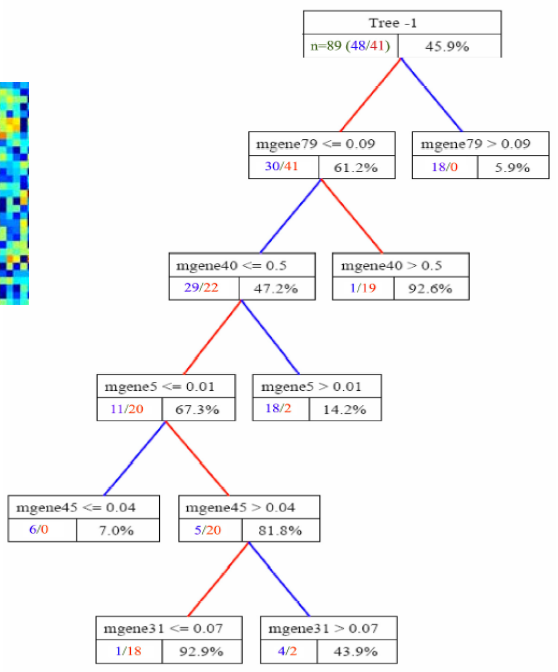


Figure 1. Initial steps in the tree analysis. Figure 1A shows a heat map of the initial step of the decision tree based on outcome. Figure 1B shows the first 5 of 100 steps in the tree (red lines denote cancer death prediction).

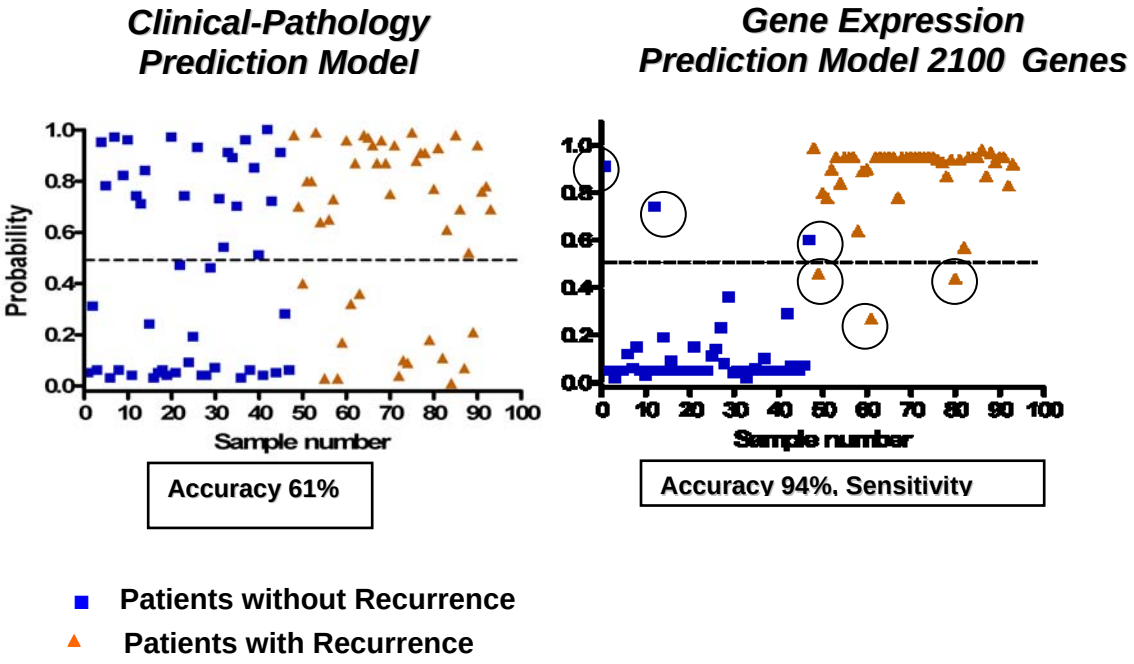


Figure 2. Duke Computational Genomics Model: **Leave-one-out analysis.** The left panel demonstrates the predictive model from clinico-pathologic factors and the right panel demonstrates the predictive model based on gene array data after a leave-one-out validation in the Duke Training Cohort. Individual risk was predicted for each tumor sample (patient). Orange triangles=cancer death and blue squares =cure. The location along the y axis is the probability of cancer recurrence (death) predicted by the model. Although the clinical model had reasonable predictive accuracy, the genomic model was far superior. The 6/91 errors of the gene array model are circled.

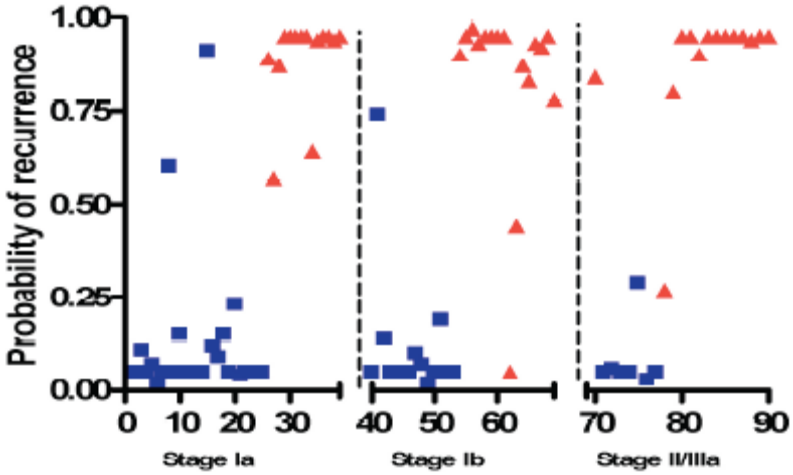


Figure 3. Duke Computational Genomics Model: **By Pathologic Stage.** The left panel demonstrates the results of the predictive model from clinico-pathologic data. The right panel demonstrates the results of the predictive model based on gene array by pathologic stage in the Duke Training Cohorts. Individual risk was predicted for each tumor sample (patient). Green triangles=cancer death and blue squares =cure. The location along the y axis is the probability of cancer recurrence (or death) predicted by the model. Note that the genomic model's accuracy is constant irregardless of tumor size or stage.

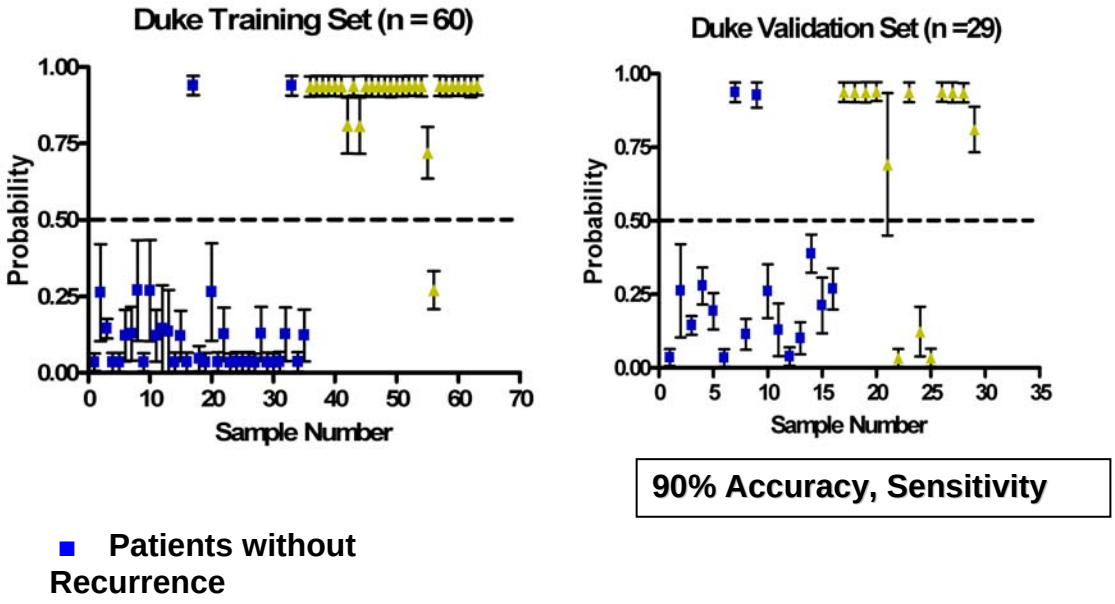
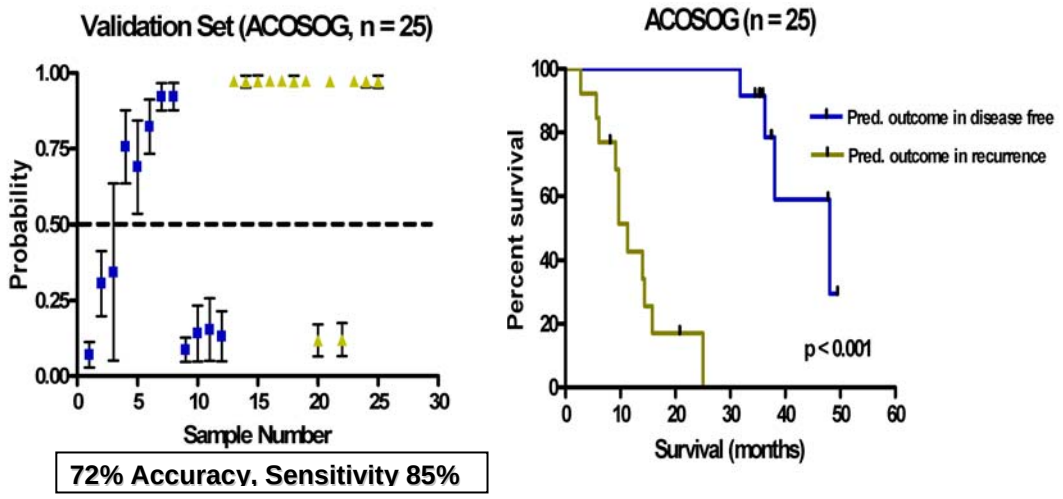


Figure 4. Internal Validation: Randomized 2/3 Training with 1/3 Validation Cohorts. Internal validation analysis where 67% of the Duke Training Cohorts were randomly selected for the training set and 33% for the validation set. This process was repeated 20 times. Individual risk was predicted for each tumor sample (patient). Yellow triangles=cancer death and blue squares =cure. The location along the y axis is the probability of cancer death predicted by the model. The accuracy remained high at 90%.

A. ACOSOG Z0030



B. CALGB 9761: (n=49)

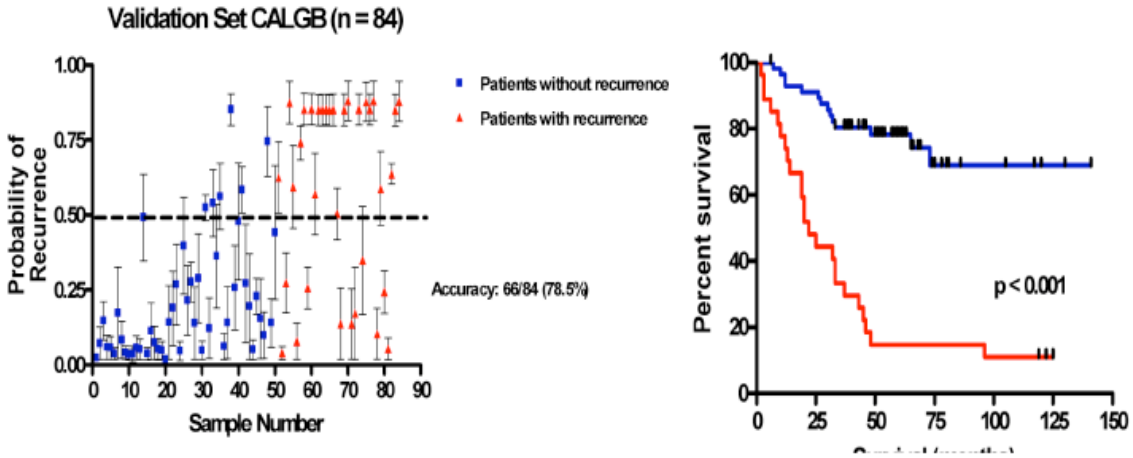


Figure 5. External Validation. 5a) The first external validation set consisted of randomly selected patient tumor samples from the ACOSOG NCI-Z0030 trial (a prospective, randomized multi-institutional clinical trial). The sample included 25 stage I patients with all cell types of NSCLC who were resected without adjuvant therapy. The results predicted by the model were compared to patient outcomes in a blinded fashion. Individual risk was predicted for each tumor sample (patient). Yellow triangles=cancer death and blue squares =cure. The location along the y axis is the probability of cancer death predicted by the model. The accuracy and sensitivity of the genomics model were 72% and 85% respectively. The model predicted cancer death in 11/13 patients, median follow-up of 24 mos.(intermediate). **5b)** The second external validation analysis using randomly selected tumor samples from CALGB 9761 (a prospective multi-institutional NCI trial). The set included 49 NSCLC patients resected without adjuvant therapy. The results predicted by the LMP were compared to patient outcomes in a blinded fashion. Individual risk was predicted for each tumor sample (patient). Red triangles=cancer death and blue squares =cure. The location along the y axis is the probability of cancer death predicted by the model. The accuracy and sensitivity of the genomics model were 90% and 100% respectively (There were no false negatives). The right hand panel has the Kaplan Meier survival analysis by the prediction model, tic marks are censored data p<0.0001, median follow-up was 49 months (adequate). Tumors samples from both validation were banked outside of Duke University.

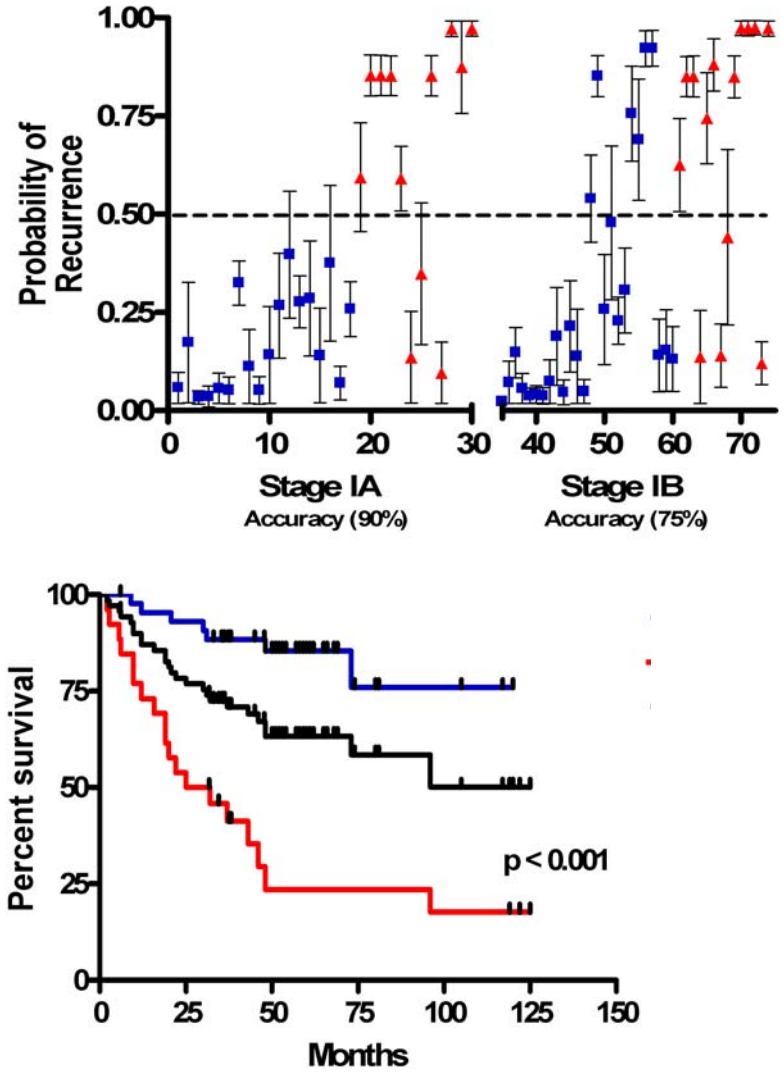


Figure 6. Individualized prognosis based on the LUNG METAGENE SCORE for pathologic stage I patients. Based on the two validation sets (ACOSOG and CALGB; n=71). The location along the y axis is the probability of cancer death predicted by the model, the dotted line denotes the cut-off for the proposed trial. Errors in prediction based on a 50% cancer recurrence (death) risk cut off are circled (LMS> or = 0.5). Note the model is accurate for all stage I patients. The bottom panel has the survival curves for the patients with LMS <0.5 (blue line) and LMS >=0.5 red line) and the overall survival (black line. The hazards ratio for the LMS is 8.4. Red triangles=cancer death and blu squares =cure.

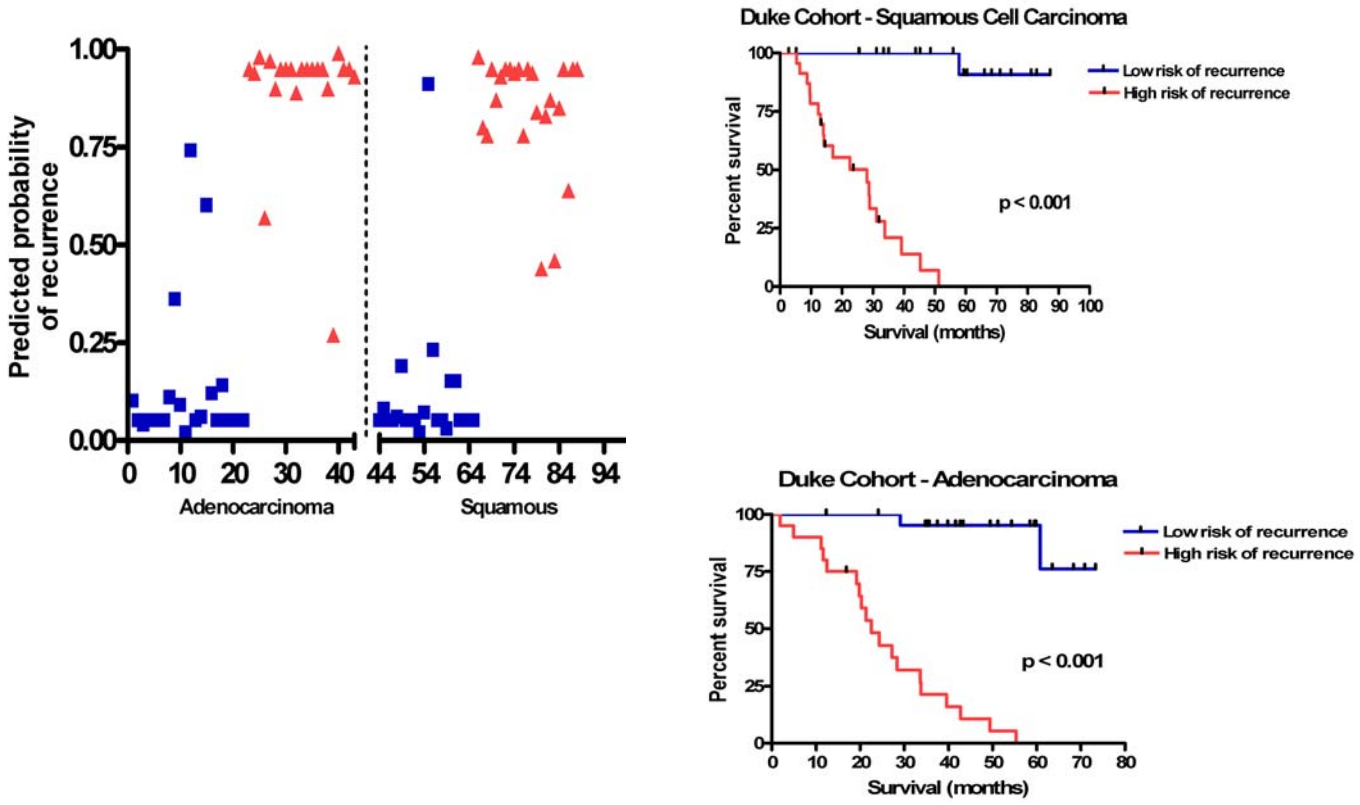


Figure 7. Subset analysis by histology. The LUNG METAGENE SCORE is shown to work equally as well with adeno and squamous histology. Red triangles=cancer death and blue squares =cure. The location along the y-axis is the probability of cancer death predicted by the model.

D. Analytical sensitivity

Analytical sensitivity does not specifically apply to the LMS recurrence risk. However, sensitivity can be considered on a gene expression or feature level for the Affymetrix U133 plus 2.0 array. Measuring the sensitivity of each of the 1.3 million features on this array using spike-in controls would be impossible though. Fortunately, we do not believe this is necessary to generate a precise and accurate LMS recurrence risk. However, it will be important to assess the ability to determine whether or not a particular gene is definitively present or absent. This can be determined through the inclusion of a perfect mismatch for every specific oligonucleotide on the array. Raw data will be filtered using this information to make a 'present' or 'absent' determination for each gene. This will be performed using the MAS5 normalization algorithm from Affymetrix. Genes that are determined to be 'absent' will not be included in the recurrence risk score determination.

E. Analytical Specificity

Similar to sensitivity, analytical specificity does not specifically apply to the LMS recurrence risk. Specificity can, however, be considered on a gene expression or feature level for the Affymetrix U133 plus 2.0 array. Measuring the specificity of each of the 1.3 million features for their target RNAs on this array using spike in controls is also impossible. However, the precise identity of the RNA that is being measured for each spot on the array is technically irrelevant. The important factor is whether the measurements can be made in a precise and reproducible manner. If so, the LMS will be consistent and comparable between different samples.

F. Accuracy

Accuracy of the LMS can be measured against actual clinical outcomes. This is the strategy that is outlined in the clinical performance section of this submission.

H. Linearity

At the basis of the LMS assay is the presumption that microarray-based gene expression measurements using the Affymetrix U133 plus 2.0 arrays are linear. Typically, linearity for quantitative assays is determined through spike-in experiments using defined concentrations of analyte. As this is not possible for each gene on the array, we can instead approximate this measurement by using defined mixtures of RNA derived from various relevant tissues, including lung tumors and normal lung. Using this data we can identify features on the array which do not behave in a linear fashion, or show minimal variance between different tissue types. These data points will likely provide minimal information and will be excluded from the LMS predictive model to produce a simplified predictive algorithm. The accuracy of this refined LMS algorithm will be tested.

VI. Clinical Performance

A. Principal study investigators

Anil Potti, M.D. is an Assistant Professor in the Department of Medicine at Duke University. He contributed to the concept and performed the analytic component required to build the LMS. He

was also directly involved in the independent validations of the LMS and in the initiation of the prospectively planned CALGB trial. Dr. Potti previously completed comprehensive studies in genomic applications under the mentorship Dr. Joseph Nevins (Director of the Center for Genome Technology) at Duke University and is also an oncologist in a high volume Thoracic Oncology Program at Duke. Dr. Potti has served as a PI or Co-PI on several investigator initiated trials and is on the basic science committee for the Cancer and Leukemia Group B (CALGB).

Joseph R. Nevins, Ph.D. is Barbara Levine Professor of Breast Cancer Genomics at Duke University. He is also the Director of the Center for Applied Genomics and Technology in the Duke Institute for Genome Sciences and Policy. Dr. Nevins and colleagues were responsible for the development of the lung metagene predictor as described in the recent New England Journal of Medicine article. This work made use of the collection of a series of lung cancer samples that were used for DNA microarray analysis. Gene expression profiles were generated to predict disease recurrence based on methods developed in previous studies of Dr. Nevins together with Dr. Mike West. These profiles were then demonstrated to accurately predict disease recurrence in three independent datasets and to have the capacity to identify a sub-population of Stage 1A patients at risk for recurrence. This latter result formed the basis for a Phase III clinical trial (approved by the CALGB cooperative group) that will evaluate the ability of this predictor to select patients who might benefit from adjuvant chemotherapy. Dr. Nevins' role in this work was in the design and implementation of the study followed by analysis of the data and preparation of the manuscript and clinical trial description.

David H. Harpole, Jr., M.D. is Professor of Surgery and Vice-chairman for Faculty Affairs and Education, Department of Surgery at Duke University Medical Center. Dr. Harpole directs the Duke Lung Cancer Research Program and is a national leader in prognostic markers in lung cancer and lung cancer clinical trials. The basis of the LMS came from 15 years of research in designing a test that will select patients for chemotherapy after resection of the lung cancer. Dr.Harpole is the PI of the NCI-R01 and the clinical trial to validate the LMS.

Geoffrey S. Ginsburg, M.D., Ph.D. is Professor of Medicine and Professor of Pathology at Duke University and Director of the Center for Genomic Medicine in the Institute for Genome Sciences & Policy. In 2006, he was appointed as Co-Director, Duke Clinical Translational Science Institute. Dr. Ginsburg has built and leads several cross-disciplinary programs that focus on the development of multidimensional predictive biomarkers for cancer, cardiovascular disease and infectious disease and the design and implementation of clinical studies to demonstrate their utility in clinical decision-making. Dr. Ginsburg is responsible for oversight of the development of the device and the clinical trials to define its clinical utility.

Michael B. Datto, M.D., Ph.D. is Assistant Professor of Pathology at Duke University Medical Center. Dr. Datto will be directing the validation of the LMS with regard to biological variance, pre-analytic variance and technical variance. In addition, Dr. Datto will help coordinate sample acquisition and processing, and microarray analysis and interpretation for the clinical validation of the LMP. Dr. Datto is certified by the American Board of Pathology in both anatomic and clinical pathology. He is also the Associate Director of the CAP-certified Clinical Molecular Diagnostics Laboratory at Duke University Medical Center. As such, he has substantial experience in developing, validating and implementing high complexity molecular diagnostics tests.

Xiaofei Wang, Ph.D. is Assistant Professor in the Department of Biostatistics and Bioinformatics at Duke University and Faculty Statistician of the CALGB Statistical Center. Dr. Wang's will be responsible for the overall statistical analysis of the clinical trial. His expertise includes the design

and analysis of clinical trials, survival analysis, semiparametric inference, categorial data analysis, and missing data methods.

B. Study sites

Duke University Medical Center
Durham, NC

VA Medical Center
Durham, NC

VA Medical Center
Minneapolis, MN.

C. Study Design

a. Background. Lung cancer is the leading cause of cancer death in the world. In 2000, more than 1 million peopled died of lung cancer, accounting for about 18% of total cancer deaths worldwide (Parker 1997). Non-small cell lung cancer (NSCLC) accounts for almost 80% of lung cancers, of which 40-50% of these are adenocarcinomas. Although patients diagnosed with stage I NSCLC have an overall 5-year survival rate of about 70%, nearly 30-35% will relapse after surgical resection, thus portending a poor prognosis (Naruke 1988, Strauss 1995, Harpole 1995). Pathologic stage IA (40% of clinical stage I; >20,000 patients/year in North America) represent the fastest growing segment of patients with lung cancer due to the increased use of high-resolution CT scans for screening.

Since the initial description by Mountain et al., the clinical staging system in NSCLC has been the standard for determining lung cancer prognosis. Staging based on tumor size and invasion (TNM) remains the mainstay for prognosis and treatment decisions. Nevertheless, while the TNM approach is useful in defining broad categories of patient risk, it clearly fails to properly classify many individual patients. The opportunities afforded by genomic data, particularly gene expression profiles, to refine and improve the relatively imprecise prognosis afforded by TNM has been demonstrated in many instances. But it is the opportunity to utilize an improved and refined prognosis to change a clinical decision that is critical.

We believe the example presented by the current guidelines for treatment of Stage I NSCLC patients provides an opportunity to employ an improved prognostic model to achieve personalized cancer treatment. Based on the results of JBR-10, IALT and CALGB 9633 (10,13,14), the current standard of care in North America is adjuvant chemotherapy for those early stage NSCLC patients classified as Stage IB and above (www.nccn.org). In contrast, there is no indication for therapeutic benefit for Stage IA patients as a group and observation is the standard of care. In practical terms, a patient with a tumor size of 2.9 cm does not receive chemotherapy whereas a patient with a tumor size of 3.1 cm does. Given the imprecision of a classification based on tumor size, which defines the Stage IA/B classification, an improved prognostic tool based on tumor biology could provide an opportunity to refine the current assessment of risk and thus the decision of who to treat. While the overall survival for Stage IA patients is quite good, approximately 25% will suffer a relapse, indicating that a subset of these patients might benefit from adjuvant chemotherapy. Likewise, while most patients with clinical stages Ib, IIa/IIb, and IIIa NSCLC will be candidates for definitive surgery and adjuvant chemotherapy (as described below), some of these patients will unnecessarily receive potentially toxic chemotherapy. Thus, the ability to more accurately stratify patients within

the current classification scheme for lung cancer may have a clear benefit on health outcomes across the entire spectrum of disease.

Four recent randomized phase III trials have demonstrated a survival advantage (4 to 15%) for patients treated with adjuvant chemotherapy after resection in patients with pathological stage IB to IIIA (Kato 2004, Arriagada 2004, Strauss PASCO 2004, Winton 2005). Results of these studies are summarized in Table 1. These trials were conducted with active regimen and had >80% of randomized patients completing the proscribed number of chemotherapy cycles (55-65% at full dose). The **NCI-C JBR-10** randomized 482 patients with completely resected T2N0, T1N1, or T2N1 NSCLC to either an observation arm or adjuvant chemotherapy with cisplatin and vinorelbine (see trial schema section 5.1). The median follow-up was 5 years and results revealed the adjuvant chemotherapy cohort to have significantly prolonged overall survival as compared to the observation cohort, with an absolute survival advantage of 15% at 5 years for adjuvant chemotherapy (Winton, 2005). The **CALGB 9633** randomized 344 patients with completelyresected T2N0, Stage IB NSCLC were randomized to either observation or adjuvant chemotherapy with carboplatin and paclitaxel (see trial schema). Preliminary results (median follow-up of 34 months) revealed an overall survival advantage of 12% at 4-years for Stage IB patients undergoing adjuvant chemotherapy as compared to those patients in the observation group (Strauss, 2004). However, after long-term follow-up, the survival advantage has disappeared for all patients, resulting in a negative trial. Guidelines for adjuvant therapy in stage IB patients were based on these results alone and the conclusion of the presentation by Strauss et al. at the 2006 American Society of Clinical Oncology meeting was adjuvant chemotherapy is no longer the standard of care for stage IB (European and Canadian centers had already reached this conclusion).

Trial and Analysis	**Adjuvant Chemotherapy**	**Observation**	**P Value**	**Hazards Ratio [95% CI]**
JBR-10: Overall Survival	69% 5-year	54% 5-year	0.03	0.70, [0.45,0.81]
JBR-10: Cancer-free Survival	61% 5-year	49% 5-year	0.004	0.60, [0.45,0.79]
CALGB 9633 Overall Survival	71% 4-year	59% 4-year	0.028	0.62 [0.41-0.95]
CALGB 9633 Cancer-Free Survival	61% 4-year	50% 4-year	0.035	0.69 [0.48-0.98]

Table 1. Summary of clinical trials demonstrating survival advantage in patients administered adjuvant chemotherapy.

b. Study Rationale. We have developed a molecular-based tumor model to stratify patients for risk of cancer recurrence. At the time of the initial profiling studies, the opportunity for application of the lung cancer model in clinical decision making was not readily apparent. However, this situation has dramatically changed in the past two years due to the findings of several large multi-institutional clinical trials demonstrating a benefit of adjuvant chemotherapy for early stage NSCLC patients. As a result, there is now a clear opportunity to refine prognosis across the Stage IA/IB boundary that defines the use of chemotherapy whereby adjuvant therapy would increase the survival in the high risk group, while limiting low risk patients' exposure to potentially harmful treatments.

c. Study Objectives. Through a randomized prospective study of 1000 patients, we aim to validate the clinical validity of the Lung Metagene Model to provide individualized prediction of risk recurrence in stage 1a NSCLC patients and the clinical utility of the predicted risk recurrence to guide the use of adjuvant chemotherapy.

Primary Objectives

1. To prospectively validate a prognostic indicator of survival in stage I NSCLC patients identified with a low Lung Metagene Score who are observed after resection.

2. To demonstrate a survival advantage for the remaining patients randomized to adjuvant chemotherapy compared to an observation arm (present standard of care).

Secondary objectives

1. To demonstrate a cancer-free survival advantage for the remaining patients randomized to adjuvant chemotherapy compared to an observation arm (present standard of care).

2. To compare the Lung Metagene Score with best clinico-pathologic model for cancer-free survival

3. Characterize the rate of chemotherapy toxicity for the chemotherapy treatment arm

4. To validate the analytical performance of the Lung Metagene Score from archived paraffin-embedded tumor samples.

d. Institutional Review Board Approval. Approval is pending.

e. Informed Consent. This study will utilize a two-step consent process (Figure 8). For the first stage, potential research participants meeting the initial eligibility criteria (Table 2) will be consented for tissue collection and LMS assessment. For the second stage, research participants will be consented to be randomized into a treatment or observation arm. Draft informed consent documents are currently under development and will be submitted for review with the study proposal to the IRB.

f. Patient Eligibility Criteria. All patients who meet the initial inclusion criteria will be consented for the tissue collection, creation of individual metagene score by microarray and consent for enrollment into the treatment trial.

g. Arms/Regimens. See Figure 8 and 9.

h. Specimen Preparation. The CALGB and ACOSOG thoracic surgeons have extensive experience harvesting, preparing, shipping and banking high-quality fresh-frozen tumor samples. Immediately following resection, the specimen is brought to the Pathology Frozen Section Facility. A pathologist identifies and procures as many 5x5x5 mm samples as possible from the leading edge of the tumor, "berry-picking" areas that are grossly homogeneous and viable, without obvious inflammation, necrosis or fibrosis. These are transported (if necessary) on wet ice, and snap frozen immediately in liquid nitrogen or on powdered dry ice, and stored at –80°C until shipment. Tissue preparation and transportation kits have been developed and will be distributed to each participating site with pre-printed shipping labels. Specimens are shipped on dry ice to the Duke University Health System Clinical Diagnostic Laboratory by Federal Express for next AM delivery to arrive on (non-holiday)

Tuesday through Saturday. This facility handles all clinical specimens from multiple hospitals and is a CAP (College of American Pathologist) certified facility that performs high-throughput complex molecular diagnostic testing.

i. Quality Control and RNA extraction. Specimens received will be logged at the CALGB Lung Cancer Tissue Bank and assigned identification codes. Each aliquot will be bisected. Half will be embedded in OCT medium, a frozen section from its cut face prepared and H&E stained. All slides will be reviewed by a pathologist and scored for percent
distribution of categories of viable nucleated cells: tumor, normal lung, fibroblasts, lymphocytes, and other (to be specified). Total RNA will be prepared from a subsample (~25 mg) cut from the unembedded half of each specimen that exceeds established histologic quality thresholds. Each subsample will be subjected to homogenization and phenol-chloroform extraction (Trizol), followed by DNAse treatment. Gel electrophoresis will be used as an initial RNA quality screen and an A260/A280 Spectrophotometer reading will be taken to assess RNA purity (contamination with DNA). Twenty micrograms of RNA from each sample that demonstrates intact and comparable 18s and 28s bands will be shipped to Duke on dry ice by Federal Express for next AM delivery to arrive on (non-holiday) Monday through Friday. Remaining RNA, embedded and un-embedded tissue will be stored in the LCTB at –80 degrees C.

- Verification of high-quality RNA (10% will not meet criteria)
- Verification of pathological stage I. A few patients will have IB-IIIA (estimated 13.5 % by
- previous studies)
- Sites will be compensated a nominal amount for all patients not eligible for randomization.
- Sites will be notified if the patient is eligible for randomization
- After obtaining consent, sites will be notified of the assigned treatment strata

j. Feasibility studies. The requirements for utilization of genomic tests in clinical practice, including as the basis for selecting patients for this trial, are clearly different than those in the pure research setting. This involves questions of methods for sample acquisition, sample processing, assay reproducibility, assay services that are compliant with requirements for clinical testing. Assay reproducibility, the extent to which the same answer or prediction is obtained when a sample is assayed multiple times, is obviously critical to clinical testing. In the research setting, variability in sample assay is incorporated into the development of the predictive models, reducing the certainty in the predictions. But, in the context of applying such a test in the clinic, where the clinical decision will be dictated by the results of a single assay, it is obviously critical to understand and minimize the extent to which assay variables influence a result. To this end, we have initiated studies prior to the initiation of the actual prospective trial to address the extent to which experimental variation is a factor in these assays and are developing a series of standard operating procedures (SOPs) that will guide the operation of the trial.

k. Sample Acquisition. The CALGB and ACOSOG thoracic surgeons have extensive experience harvesting, preparing, shipping and banking high-quality fresh-frozen tumor samples. CALGB 9761 collected 500 fresh-frozen tumors and ACOSOG Z4031 (David Harpole, PI) accrued over 1000/1000 patients in less than two years (50-70 patient per month accrual) with excellent quality fresh-frozen tumors. Both sets have high-quality RNA from extracted from >90% of samples submitted.

Table 2. Study Eligibility Criteria.

A. Pre-and Intraoperative Patient Eligibility

Age ≥18
Performance Status 0-1
Women must be non-pregnant and non-lactating
No concomitant malignancy
Known or suspected NSCLC with a mass 1.8 cm to 3.5 cm on CT scan
Biopsy must be obtained to confirm diagnosis prior to registration (This may include intra-operative diagnosis)
Medical staging: chest, liver & adrenal CT.

Surgical staging: Evidence of hilar or mediastinal node involvement by Chest CT (>1 cm diameter) must be assessed with either mediastinoscopy, endo-esophageal ultrasound, or mediastinal nodal sampling before or at time of thoracotomy.
No prior chemotherapy or radiotherapy for NSCLC
Must be surgical candidate

No patients with known infectious disease, such as HIV

No history of previous or concomitant malignancy, other than curatively treated carcinoma in situ of the cervix, or basal cell or squamous cell carcinoma of the skin, or surgically treated in situ carcinoma of the breast, or other cancer for which the patient has been disease free for 5 years

B. Post-Operative Eligibility

Anatomical resection with lobectomy

Pathologic stage I NSCLC tumor size ≥1.75 cm in diameter

Randomization must occur within 4-8 weeks of resection

Adequate tumor specimen: Histopathology review at BWH CALGB Lung Cancer Bank and RNA at Duke IGSP Microarray Facility

Granulocytes ≥ 1800/ul

Platelets ≥ 100,000/ul

Bilirubin <1.5 mg/dl

SGOT (AST) <2.0 x ULN

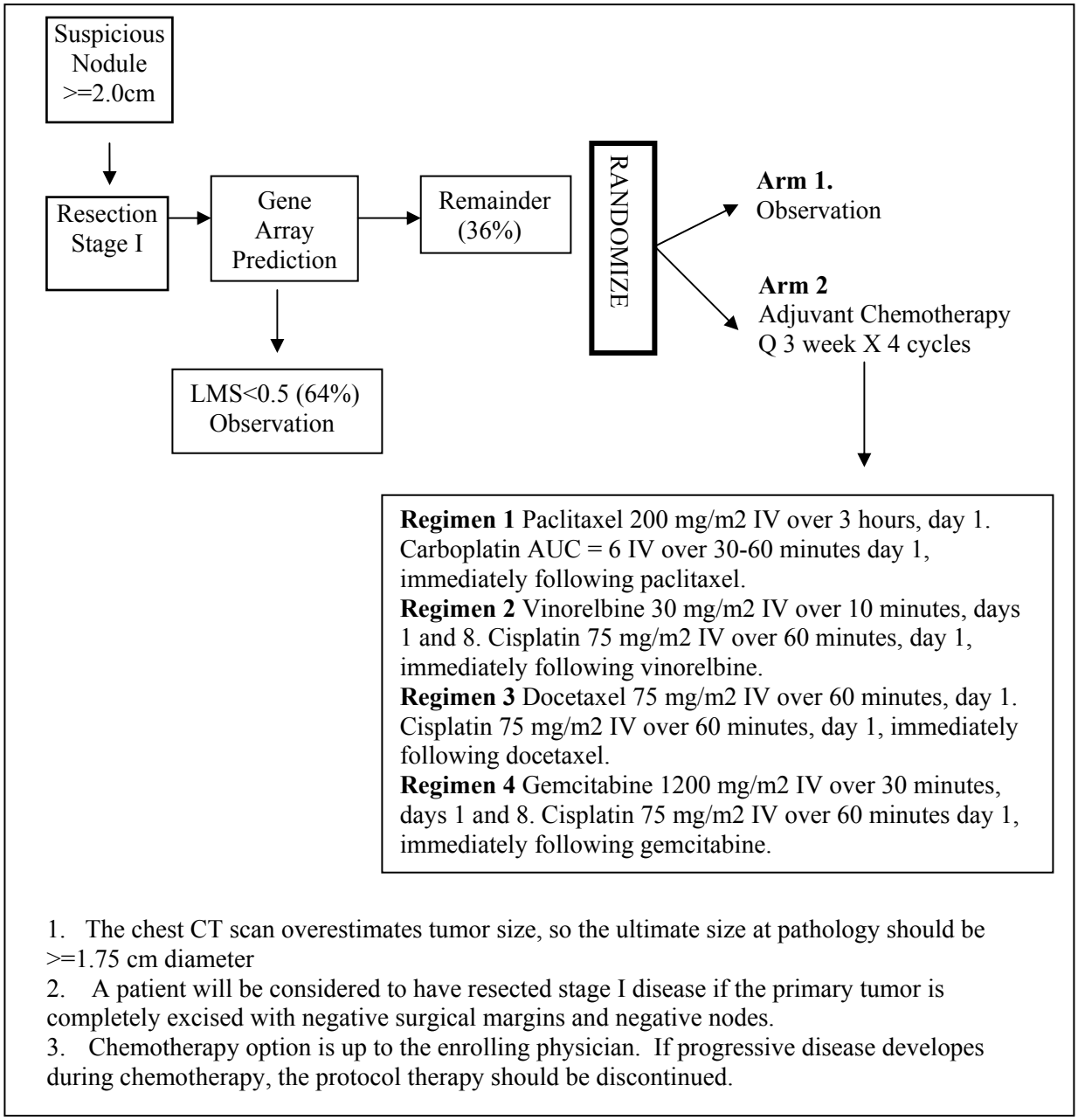


Figure 8. Study Arms/Regimens -- Overall Schema.

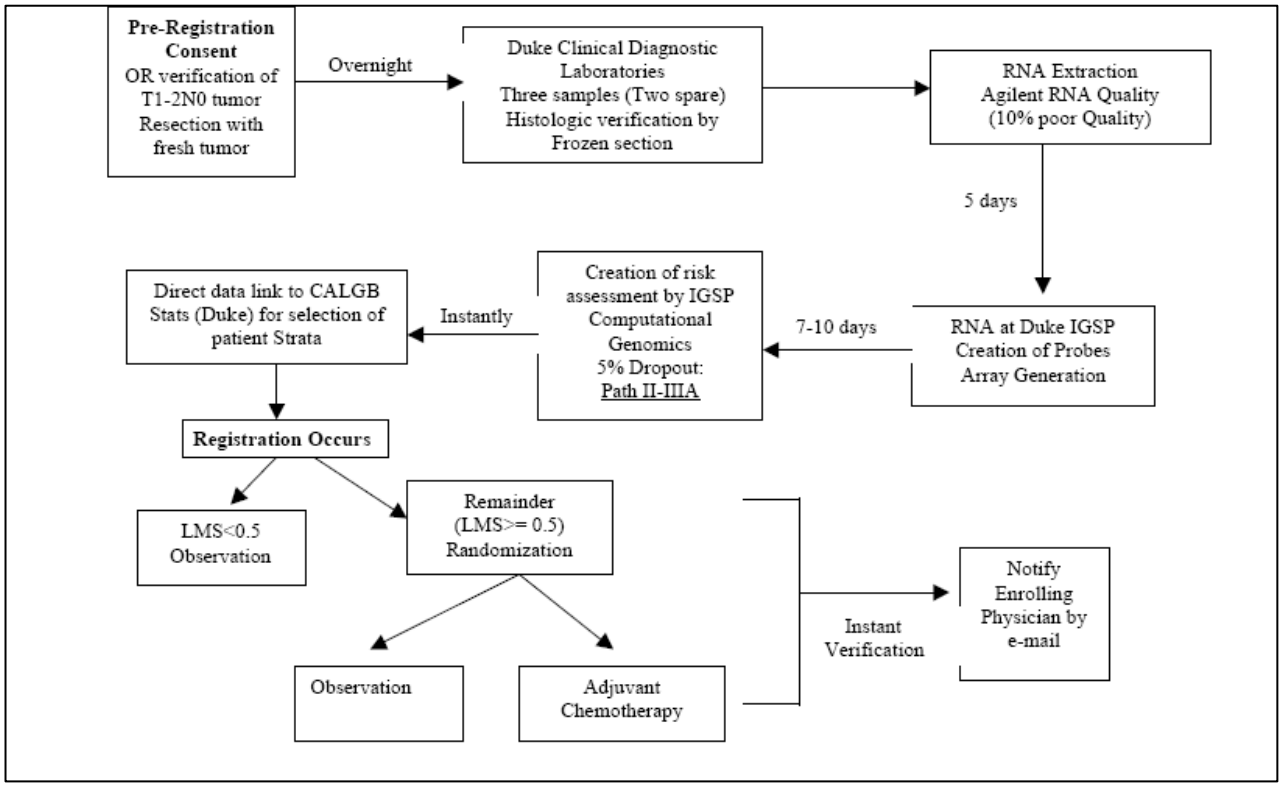


Figure 9. Trial Logistics (18-21 days).

l. Sample Processing. The Clinical Molecular Diagnostics Laboratory at Duke University Medical Center is a CAP-accredited laboratory that performs most of the high complexity DNA and RNA-based clinical molecular diagnostics tests for Duke University Health System patients. This laboratory has in place the infrastructure to perform several key components of the microarray-based lung metagene test, including RNA processing, temporary lung tumor tissue banking, and frozen section diagnosis. We routinely perform RNA extractions, and we process approximately 10,000 samples per year for clinical diagnostic testing.

Due to a recent increase in demand, a small branch of the clinical laboratory has been designed to specifically handle fee-for service RNA and DNA extractions and to aid in coordination of sample acquisition, histological analysis, processing, shipping and banking for clinical trials. The director of this branch is Dr. Michael Datto, who also serves as the Associate Director of the Clinical Molecular Diagnostics Laboratory as a whole. We have developed a robust method for lung tumor sample acquisition and RNA preparation. Using these methods, we have generated RNA that has yielded high quality Affymetrix U133 plus 2.0 microarray data. We have assayed the reproducibility of these methods, and they show a high degree of precision. These methods form the basis for the SOPs that will be used in this upcoming clinical trial. In collaboration with the Department of Pathology Informatics Division, we are also developing an internet-based sample tracking system. This system will be invaluable part for the current study and other up-coming multi-institutional clinical trials. All tissues are processed and banked in secure dedicated freezers with appropriate power and liquid nitrogen back up and with an encryption-protected databank for relevant information.

m. Assay Reproducibility. Logistics for our stage I trial are described above. A feasibility trial from sample acquisition to data analysis has been completed. The results of these studies will establish a benchmark for judging the extent to which variability due to sample acquisition, handling, and assay contributes to the end result. As an initial step in assessing sample collection and assay reproducibility, we collected 10 tumor samples from 5 patients (2 each) with stage IA NSCLC resected during 1 week at Duke. Tumor samples were snap-frozen and sent to the CALGB Lung Cancer Tumor Bank for a feasibility study. All activities through the data analysis phase were assessed by this pilot study (feasibility in an 18-21 day window). The pathologic review at Brigham and Women's Hospital revealed adequate tumor samples for all patients. Three of the duplicate samples contained <50% viable tumor. The pathologist recommendation was to increase the number of samples from two to three for each patient. Gel electrophoresis and Agilent array demonstrated excellent RNA quality and quantity was observed for all samples. Probe generation and Affymetrix array hybridizations were accomplished within one week. Paired samples had nearly identical gene expression profiles and LUNG METAGENE predictions, signifying reproducibility. The entire process was completed in less than 18 days, demonstrating the feasibility of this trial.

Additionally, 4 samples from each of 5 tumors were processed and analyzed using the SOP's developed for this trial at the Duke Molecular Diagnostics Laboratory and revealed high assay precision with all samples from any given tumor giving the same array data and same LMS. The turn around time for the entire process from acquisition to LMS generation was within the 18 to 21 day window. All remaining archived frozen samples will be transferred to the CALGB Lung Cancer Tumor Bank at the Brigham and Women's Hospital for storage and future investigations.

n. Assay Services. Each tumor sample will be run in duplicate using the U133 2.0 plus Genechip and an Affymetrix custom-designed LUNG METAGENE SCORE chip (2100 genes plus controls). We will take advantage of the services of an Affymetrix service provider, Expression Analysis (EA), as the mechanism by which these assays are performed. EA was established as an outgrowth of the Duke Microarray Facility and continues to provide services to Duke investigators. Importantly, EA has completed requirements and internal audits associated with good laboratory practice (GLP) regulations making it the first company capable of providing Affymetrix GeneChip assays in compliance with GLP regulations. The next step is to meet the guidelines for CLIA certification which will allow EA to perform tests for clinical use. It is anticipated that CLIA certification will be achieved in 2006.

o. Chemotherapy Administration Schedule. Chemotherapy will be chosen from one of four regimens. The regimens were selected to match an ongoing intergroup adjuvant chemotherapy trial (ECOG 1505 A Phase III Randomized Trial of Adjuvant Chemotherapy With or Without Bevacizumab for Patients With Completely Resected Stage IB-IIIA Non-Small Cell Lung Cancer (NSCLC,) with the exception that bevacizumab will not be used. ECOG 1505 is examining adjuvant chemotherapy for more advanced stage resected NSCLC.

All doses will be based on the patient's actual weight. The actual weight at screening will be used for calculating BSA. The BSA should only be recalculated if a patient's weight changes by ≥ 10%. At the discretion of the investigator, all patients will receive one of 4 chemotherapy regimens. Each cycle is 3 weeks (21 days). All chemotherapy regimens will be given for a total of 4 cycles. The investigator is required to choose the chemotherapy regimen for the patient (1, 2, 3, or 4) prior to randomization.

Chemotherapy Regimen 1: Carboplatin/Paclitaxel
Paclitaxel 200 mg/m2 IV over 3 hours, day 1.

Carboplatin AUC = 6 IV over 30-60 minutes day 1, immediately following paclitaxel.

Premedication
Prior to receiving paclitaxel, all patients will receive the following premedications:
• Dexamethasone (or equivalent steroid) 20 mg p.o. 12 and 6 hours prior to paclitaxel infusions OR dexamethasone 20 mg IV <1 hour prior to infusion with paclitaxel if oral dexamethasone not taken.
• Diphenhydramine 50 mg IV (or equivalent) < 1 hour prior to paclitaxel infusion.
• Cimetidine 300 mg IV OR ranitidine 50 mg IV OR equivalent H2-blocker given < 1 hour prior to paclitaxel infusion.

Carboplatin Dosing
Carboplatin dosing will be based on the Calvert formula and should be recalculated for each dose:

Carboplatin dosing (mg) = AUC x (GFR +25).

GFR is estimated using the Cockroft-Gault formula for creatinine clearance:

$$\frac{(140 - \text{patient's age}) \times (\text{patient's weight in kilograms})}{72 \times \text{patient's serum creatinine}}$$

(for females, multiply the result by 0.85).

The GFR calculated by the Cockroft-Gault formula above will be used for GFR in the Calvert formula.

Chemotherapy Regimen 2: Cisplatin/Vinorelbine
Vinorelbine 30 mg/m2 IV over 10 minutes, days 1 and 8.
Cisplatin 75 mg/m2 IV over 60 minutes, day 1, immediately following vinorelbine.

Antiemetics
It is strongly recommended that all patients receive adequate anti-emetics with cisplatin-based chemotherapy. The specifics of the regimen are at the discretion of the treating physician, provided adequate control is achieved. One potential regimen consists of 20 mg of oral dexamethasone and a high dose of oral or IV 5HT3 antagonist (such as 2 mg oral or 10 mcg/kg IV granisetron, or 32 mg oral or IV ondansetron) on the day of cisplatin administration. Followed by additional anti-emetics consisting of 4 days of oral dexamethasone (8 mg po bid for 2 days (days 2, 3) then 4 mg po bid for 2 days (days 4, 5) and scheduled metoclopramide or 5HT3 antagonist for days 2-5 for delayed emesis.

Hydration Requirements
Hydration guidelines may be modified at the discretion of the treating physician provided adequate pre and post cisplatin hydration is achieved and renal function remains adequate. One suggested regimen consists of administering cisplatin in 500 cc to 1000 cc of IV fluids following adequate hydration and the establishment of adequate urinary output. It is suggested the pre-cisplatin hydration consist of NS at 500 cc/hr x 1 liter and post-cisplatin hydration consist of ½ NS + 10 meq KCl/liter + 1 gram magnesium sulfate/liter + 25 grams mannitol/liter at 500 cc/hr for at least one hour, followed by additional hydration at the discretion of the investigator.

Chemotherapy Regimen 3: Cisplatin/Docetaxel
Docetaxel 75 mg/m2 IV over 60 minutes, day 1.
Cisplatin 75 mg/m2 IV over 60 minutes, day 1, immediately following docetaxel.

Antiemetics
It is strongly recommended that all patients receive adequate anti-emetics with cisplatin-based chemotherapy. The specifics of the regimen are at the discretion of the treating physician, provided adequate control is achieved. One potential regimen consists of 20 mg of oral dexamethasone and a high dose of oral or IV 5HT3antagonist (such as 2 mg oral or 10 mcg/kg IV granisetron, or 32 mg oral or IV ondansetron) on the day of cisplatin administration. Followed by additional anti-emetics consisting of 4 days of oral dexamethasone (8 mg po bid for 2 days (days 2, 3) then 4 mg po bid for 2 days (days 4, 5) and scheduled metoclopramide or 5HT3 antagonist for days 2-5 for delayed emesis.

Hydration Requirements
Hydration guidelines may be modified at the discretion of the treating physician provided adequate pre and post cisplatin hydration is achieved and renal function remains adequate. One suggested regimen consists of administering cisplatin in 500 cc to 1000 cc of IV fluids following adequate hydration and the establishment of adequate urinary output. It is suggested the pre-cisplatin hydration consist of NS at 500 cc/hr x 1 liter and post cisplatin hydration consist of 1/2 NS + 10 meq KCl/liter + 1 gram magnesium sulfate/liter + 25 grams mannitol/liter at 500 cc/hr for at least one hour, followed by additional hydration at the discretion of the investigator.

Premedications
Dexamethasone (or equivalent steroid) regimen will be given 8 mg po every 12 hours for 5 doses starting 24 hours prior to docetaxel infusion, continuing the day of docetaxel infusion and finishing the day after the docetaxel infusion.

Chemotherapy Regimen 4: Cisplatin/Gemcitabine
Gemcitabine 1200 mg/m2 IV over 30 minutes, days 1 and 8.
Cisplatin 75 mg/m2 IV over 60 minutes day 1, immediately following gemcitabine.

Antiemetics
It is strongly recommended that all patients receive adequate anti-emetics with cisplatin-based chemotherapy. The specifics of the regimen are at the discretion of the treating physician, provided adequate control is achieved. One potential regimen consists of 20 mg of oral dexamethasone and a high dose of oral or IV 5HT3 antagonist (such as 2 mg oral or 10 mcg/kg IV granisetron, or 32 mg oral or IV ondansetron) on the day of cisplatin administration. Followed by additional anti-emetics consisting of 4 days of oral dexamethasone (8 mg po bid for 2 days (days 2, 3) then 4 mg po bid for 2 days (days 4, 5) and scheduled metoclopramide or 5HT3 antagonist for days 2-5 for delayed emesis.

Hydration Requirements
Hydration guidelines may be modified at the discretion of the treating physician provided adequate pre and post cisplatin hydration is achieved and renal function remains adequate. One suggested regimen consists of administering cisplatin in 500 cc to 1000 cc of IV fluids following adequate hydration and the establishment of adequate urinary output. It is suggested the pre-cisplatin hydration consist of NS at 500 cc/hr x 1 liter and post cisplatin hydration consist of 1/2 NS + 10 meq KCl/liter + 1 gram magnesium sulfate/liter + 25 grams mannitol/liter at 500 cc/hr for at least one hour, followed by additional hydration at the discretion of the investigator.

p. Dose Modifications. All toxicities should be graded according to the Common Toxicity Criteria Adverse Events (version 3.0). If more restrictive dose reductions are required by a treating

institution for specific toxicities than those listed in the protocol, the stricter restrictions will be allowed. For all toxicities, if one drug in a chemotherapy regimen is held, the other must be held, as well.

Chemotherapy Regimen 1: Carboplatin and Paclitaxel

Hematologic Toxicity (Carboplatin and Paclitaxel)

Dose reductions are not required for neutropenia, unless febrile neutropenia occurs. If a platelet nadir of < 25,000 is reached, future cycles require dose reduction. Day 1 dosing may only resume for platelet count >100,000 and ANC >1500. Please see table below:

Dose Reductions for Hematologic Toxcity

	Paclitaxel	**Carboplatin**
1st episode *	Dose reduce by 1 level **	Dose reduce by 1 level**
2nd episode *	Dose reduce by 1 level **	Dose reduce by 1 level **
3rd episode	Discontinue protocol therapy	Discontinue protocol therapy
Anemia	No adjustment	No adjustment

* Episodes = Febrile Neutropenia or Platelet Nadir <25,000

Alternatively, if current episode is febrile neutropenia, growth factor support (G-CSF or pegylated G-CSF) may be used with all subsequent cycles instead of dose reduction. If growth factor support has already been instituted, than dose reduction is required for the next episode of febrile neutropenia. Use of G-CSF should be in accordance with the American Society of Clinical Oncology (ASCO) guidelines as published in the Journal of Clinical Oncology. **ANC must be at least 1,500/µl and platelet count must be at least 100,000/µl on day 1 of each cycleDose Reduction Levels

	Original Dose	**Reduced Dose**
Carboplatin	AUC of 6	AUC of 5
	AUC of 5	AUC of 4
Paclitaxel	200 mg/m^2	150 mg/m^2
	150 mg/m^2	100 mg/m^2

Treatment should be delayed for up to 3 weeks until the day 1 ANC is at least 1500/ul and the platelet count is at least 100,000/ul. However, if the counts have not recovered in 3 weeks, the patient's protocol treatment will be discontinued. The patient will still be followed for toxicity and response. Patient and investigators need to be attentive to the possibility of fever and infection so that these complications can be promptly and appropriately managed.

If chemotherapy must be withheld due to hematologic toxicity, CBC and platelet counts should be obtained weekly until the counts reach the lower limits for treatment. The treatment schedule will then proceed in the usual schedule.

No dose reductions will be made for anemia. Patients should be supported per the treating physician's discretion.The use of growth factor support for anemia will be allowed, as will blood transfusions as indicated.

Dose reductions, once initiated, are permanent. If both febrile neutropenia and thrombocytopenia of < 25,000 occur, the dose reduction will be to the lower dose specified.

Gastrointestinal Toxicity (Carboplatin, Paclitaxel)

Nausea and/or vomiting should be controlled with adequate antiemetics. If grade 3 or 4 nausea/vomiting occur in spite of antiemetics, the dose should be reduced by 20% for the next course. If tolerated, increase back to 100% dose as soon as possible.

If, on day 1 of any treatment cycle, the patient has stomatitis, the treatment should be withheld until the stomatitis is cleared. If the stomatitis has not cleared in 3 weeks, the patient's protocol treatment will be discontinued. (Refer to the CTCAE version 3.0 for specific grading criteria.) If acute grade 3 stomatitis occurs, at any time, the dose should be reduced to 75% when the stomatitis is completely cleared. This dose reduction is permanent.

Hepatic Toxicity (Paclitaxel)

Day 1 value should be used in determining the dose.

SGOT (AST)		**Bilirubin**	**Paclitaxel Dose**
≤5 X ULN	And	WNL	200 mg/m^2
> 5 X ULN	OR	> ULN – 1.5 X ULN	150 mg/m^2
		> 1.5 X ULN	0

If paclitaxel is withheld due to hepatic toxicity, carboplatin should also be withheld and administered only when the paclitaxel is resumed. If paclitaxel is withheld, hepatic values must recover within 3 weeks; if not, patient's protocol treatment will be discontinued. There are no dose reductions of carboplatin for hepatic toxicity.

Cardiovascular Toxicity (Paclitaxel)

Cardiac rhythm disturbances have occurred infrequently in patients in clinical trials; however, most patients were asymptomatic and cardiac monitoring is not required.Transient asymptomatic bradycardia has been noted in as many as 29% of patients. More significant, AV block has rarely been noted. Cardiac events should be managed as follows:

Asymptomatic bradycardia - no treatment required.

Symptomatic arrhythmia during infusion- stop paclitaxel infusion; manage arrhythmia according to standard practice. **Protocol treatment will be discontinued.**

Chest pain and/or symptomatic hypotension (< 90/60/mm Hg or required fluid replacement) - stop paclitaxel infusion. Perform an EKG. Give intravenous diphenhydramine and dexamethasone. if hypersensitivity is considered. Also, consider epinephrine or bronchodilators if chest pain is not thought to be cardiac. Protocol treatment will be discontinued.

Neurologic Toxicity (Paclitaxel)

Paclitaxel doses should be modified for neuropathy-sensory as follows. There are no dose reductions in carboplatin for neurologic toxicity.

Grade of toxicity	**Dose**
0	200 mg/m^2
1	200 mg/m^2
2	Hold treatment until patient recovers to grade 1; then resume treatment at 160 mg/m^2 (20% reduction)
≥ 3	Hold treatment until patient recovers to grade 1 toxicity, then resume treatment at 140 mg/m^2 (a 30% reduction).

Dose modifications made for neurotoxicity are permanent reductions. If recovery to grade 1 does not occur within 3 weeks, discontinue protocol therapy. NOTE: Carboplatin will not be given without Paclitaxel.

Allergic Reaction/Hypersensitivity (Paclitaxel)

CAUTION: Patients who experienced a mild to moderate hypersensitivity reaction have been successfully rechallenged, but careful attention to prophylaxis and bedside monitoring of vital signs is recommended.

Mild symptoms: Complete paclitaxel infusion. Supervise at bedside. No treatment required.

Moderate symptoms: Stop paclitaxel infusion. Give intravenous diphenhydramine 25 - 50 mg and intravenous dexamethasone 10 mg. Resume paclitaxel infusion after recovery of symptoms at a low rate, 20 mL/hour for 15 minutes, then 40 mL/hour for 15 minutes, then, if no further symptoms, at full dose rate until infusion is complete. If symptoms recur, stop paclitaxel infusion. **Protocol treatment will be discontinued.**

Severe life-threatening symptoms: Stop paclitaxel infusion. Give intravenous diphenhydramine and dexamethasone as above. Add epinephrine or bronchodilators if indicated. **Protocol treatment will be discontinued.**

Other Toxicity

For any grade 3 or 4 toxicity not mentioned above which is judged to be clinically significant, chemotherapy treatment should be withheld until the patient recovers completely or to grade 1. The treatment should then be resumed at 50% dose (permanent dose reduction). If recovery to grade 1 does not occur within 3 weeks, the patient's protocol treatment will be discontinued. For grade 1 and 2 toxicities, no dose reduction should be made.

Multiple Toxicity

If multiple toxicities occur and conflicting dose modification guidelines exist, the more stringent dose modification criteria should be chosen.

Chemotherapy Regimen 2: Cisplatin/ Vinorelbine

Hematologic Toxicity (Vinorelbine)

• Intra-cycle adjustments (e.g. day 8):

Absolute Neutrophil Count (ANC) (x 10^6/L)		**Platelets (X10^6/L)**	**Vinorelbine Dose**
> 1000	AND	> 75,000	100%
500-1000	OR	50,000-74,999	75%
≤ 500	OR	< 50,000	0%

Omitted day 8 dose of vinorelbine will not be made up.

• Day 1 cycle dose adjustments:

Dose reductions are not required for neutropenia, unless febrile neutropenia occurs. If a platelet nadir of < 25,000 is reached, future cycles require dose reduction. Day 1 dosing may only resume for platelet count >100,000 and ANC >1500. Please see table below:

Dose Reductions for Hematologic Toxicity

	Cisplatin	Vinorelbine Days 1, 8
1st episode *	No adjustment	Dose reduce by 1 level **
2nd episode *	No adjustment	Dose reduce by 1 level **
3rd episode	Discontinue protocol therapy	Discontinue protocol therapy
Anemia	No adjustment	No adjustment

* Episodes = Febrile Neutropenia or Platelet Nadir <25,000

Alternatively, if current episode is febrile neutropenia, growth factor support (G-CSF or pegylated G-CSF) may be used with all subsequent cycles instead of dose reduction. If growth factor support has already been instituted, than dose reduction is required for the next episode of febrile neutropenia. Use of G-CSF should be in accordance with the American Society of Clinical Oncology (ASCO) guidelines as published in the Journal of Clinical Oncology. **ANC must be at least 1,500/ µl and platelet count must be at least 100,000/µl on day 1 of each cycle.

Dose Reduction Levels

	Original dose	Reduced dose
Vinorelbine Days 1,8	30 mg/m2	22.5 mg/m2
	22.5 mg/m2	15 mg/m2

Treatment should be delayed for up to 3 weeks until the day 1 ANC is at least 1500/µl and the platelet count is at least 100,000/ul.. However, if the counts have not recovered in 3 weeks, the patient's protocol treatment will be discontinued. The patient will still be followed for toxicity and response. Patient and investigators need to be attentive to the possibility of fever and infection so that these complications can be promptly and appropriately managed.

If chemotherapy must be withheld due to hematologic toxicity, CBC and platelet counts should be obtained weekly until the counts reach the lower limits for treatment. The treatment schedule will then proceed in the usual schedule.

No dose reductions will be made for anemia. Patients should be supported per the treating physician's discretion.The use of growth factor support for anemia will be allowed, as will blood transfusions as indicated.

Dose reductions, once initiated, are permanent for all future cycles. If both febrile neutropenia and thrombocytopenia of < 25,000 occur, the dose reduction will be to the lower dose specified.

Gastrointestinal Toxicities (Cisplatin/vinorelbine)

Nausea and/or vomiting should be controlled with antiemetics. If grade 3 nausea/vomiting occurs in spite of maximum antiemetics (steroid pre-medication for 4 days after administration, 5HT3 antagonist, and aprepitant or similar agent if available), the dose of cisplatin should be reduced by 25% for the next course. If tolerated, increase back to 100% as soon as possible.

Significant constipation has been associated with vinca alkaloid administration. Patients experiencing constipation should be treated with laxatives and other measures at the discretion of the investigator. Enemas are not advised for patients who are, or may be, neutropenic.

If, on day 1 of any treatment cycle, the patient has stomatitis, the treatment should be withheld until the stomatitis is cleared. If the stomatitis has not cleared in 3 weeks, the patient's protocol treatment will be discontinued. (Refer to the CTC, version 3.0 for specific grading criteria.). If

acute grade 3 stomatitis occurs at any time, the dose should be given at 75% dose when the stomatitis is completely cleared. This is a permanent dose reduction.

Renal Toxicity (Cisplatin)

Creatinine	Percent dose of cisplatin to give	Percent dose of vinorelbine to give
≤1.5 x ULN	100%	100%
> 1.5 but ≤ 2.0 x ULN	50%	100%
> 2.0 x ULN	0%	0%

If serum creatinine is elevated such that treatment is not administered as scheduled (i.e. > 2.0 X ULN), repeat the abnormal tests at weekly intervals. If the serum creatinine returns to < 2.0 x ULN within 3 weeks, cisplatin may be reinstituted at 50% of the full dose during the next cycle (permanent dose reduction). A more aggressive hydration regimen must be instituted as well with all subsequent doses of cisplatin. If the cisplatin is held, the vinorelbine will be held as well so that both drugs are kept on the same schedule. If creatinine is still > 2.0 x ULN after 3 weeks, either discuss with study chair, or discontinue protocol therapy.

Neurologic Toxicity (Cisplatin, Vinorelbine)

Vinorelbine and cisplatin doses should be modified as follows for neurologic toxicity. The day 1 value should be used in determining dose. Dose reductions for neurotoxicity are permanent.

Grade of Toxicity	Vinorelbine and Cisplatin Doses
0	100%
1	100%
2	Hold treatment until patient recovers to grade 1; then resume treatment at 75% dose
≥ Grade 3	Hold treatment until patient recovers to grade1; then resume treatment at 50% dose

Auditory Toxicity (Cisplatin)

Cisplatin is well known to cause high-frequency hearing loss. Continued use of the drug does not always result in hearing loss, although it may do so. If grade 2 or worse hearing loss is noted, the patient should be presented with a discussion of the relative risks of hearing loss versus the potential benefit of continuing cisplatin therapy, and a decision made on the continuation of cisplatin. Severe hearing loss (grades 3 and 4) is an indication to discontinue the drug.

Hepatic Toxicity (Vinorelbine)

Day 1 value should be used in determining dose.

SGOT/AST		Alkaline phosphatase		Bilirubin	Vinorelbine dose
≤ 1.5 x ULN	AND	< 1.5 x ULN	AND	WNL	100%
> 1.5 - 5 x ULN	AND	> ULN-1.5 x ULN	OR	> ULN-1.5 x ULN	75%
> 5 x ULN	AND	> 5 x ULN	OR	> 1.5 x ULN	Hold *

* Repeat LFTs weekly. If recovered, reduce dose by 25%. If not recovered within 3 weeks, discontinue protocol therapy. If vinorelbine is withheld due to hepatic toxicity, cisplatin should also be withheld and administered when vinorelbine is resumed. There are no dose reductions of cisplatin for hepatic toxicity.

Any elevation in bilirubin **ALONE** qualifies for dose reduction. However, an elevation in **BOTH** the SGOT/AST and alkaline phosphatase is required to qualify for a dose reduction.

Other Toxicities
For any clinically significant grade 3 or 4 toxicity, not mentioned above, the treatment should be withheld until the patient recovers completely or to grade 1. The treatment should then be resumed at 50% dose (permanent dose reduction). For grade 1 and 2 toxicities, no dose reduction should be made.

Chemotherapy Regimen 3: Cisplatin/Docetaxel

Hematologic Toxicity (Docetaxel)
Day 1 cycle dose adjustments (hematologic toxicity):
Dose reductions are not required for neutropenia, unless febrile neutropenia occurs. If a platelet nadir of < 25,000 is reached, future cycles require dose reduction. Day 1 dosing may only resume for platelet count >100,000 and ANC >1500. Please see table below:

Dose Reductions for Hematologic Toxicity

	Cisplatin	**Docetaxel**
1st episode*	No adjustment	Dose reduce by 1 level**
2nd episode*	No adjustment	Dose reduce by 1 level**
3rd episode*	Discontinue protocol therapy	Discontinue protocol therapy
Anemia	No adjustment	No adjustment

*Episodes = Febrile Neutropenia or Platelet Nadir <25,000
Alternatively, if current episode is febrile neutropenia, growth factor support (G-CSF or pegylated G-CSF) may be used with all subsequent cycles instead of dose reduction. If growth factor support has already been instituted, than dose reduction is required for the next episode of febrile neutropenia. Use of G-CSF should be in accordance with the American Society of Clinical Oncology (ASCO) guidelines as published in the Journal of Clinical Oncology. **ANC must be at least 1,500/µl and platelet count must be at least 100,000/µl on day 1 of each cycle.

Dose Reduction Levels

	Original Dose	**Reduced Dose**
Docetaxel	75 mg/m^2	56 mg/m^2
	56 mg/m^2	37.5 mg/m2

Treatment should be delayed for up to 3 weeks until the day 1 ANC is at least 1500/µl and the platelet count is at least 100,000/ul.. However, if the counts have not recovered in 3 weeks, the patient's protocol treatment will be discontinued. The patient will still be followed for toxicity and response. Patient and investigators need to be attentive to the possibility of fever and infection so that these complications can be promptly and appropriately managed.

If chemotherapy must be withheld due to hematologic toxicity, CBC and platelet counts should be obtained weekly until the counts reach the lower limits for treatment. The treatment schedule will then proceed in the usual schedule.

No dose reductions will be made for anemia. Patients should be supported per the treating physician's discretion. The use of growth factor support for anemia will be allowed, as will blood transfusions as indicated.

Dose reductions, once initiated, are permanent for all future cycles. If both febrile neutropenia and thrombocytopenia of <25,000 occur, the dose reduction will be to the lower dose specified.

Gastrointestinal Toxicities (Cisplatin and Docetaxel)

Nausea/Vomiting

Nausea and/or vomiting should be controlled with antiemetics. If grade 3 nausea/vomiting occurs in spite of maximum antiemetics (steroid pre-medication for 4 days after administration, 5HT3 antagonist, and aprepitant or similar agent if available), the dose of cisplatin should be reduced by 25% for the next course. If tolerated, increase back to 100% as soon as possible.

Diarrhea

In the case of severe diarrhea, octreotide is recommended. If the patient has significant diarrhea (>3 loose stools/24 hours), the patient should be treated in the subsequent cycles with 2 tablets of loperamide or diphenoxylate in addition to 1 or 2 tablets after each loose stool. In case of diarrhea >grade 2 despite the prophylactic treatment, patient must go off treatment. Appropriate symptomatic treatment with loperamide or diphenoxylate hydrochloride with atropine sulfate should be given. If loperamide is used, a maximum of 16 mg/day should be used and loperamide should be discontinued if results are not seen in 48 hours.

If, on day 1 of any treatment cycle, the patient has stomatitis, the treatment should be withheld until the stomatitis is cleared. If the stomatitis has not cleared in 3 weeks, the patient's protocol treatment will be discontinued. (Refer to the CTC, version 3.0 for specific grading criteria.) If acute grade 3 stomatitis occurs at any time, the dose should be given at 75% dose when the stomatitis is completely cleared. This is a permanent dose reduction.

Renal Toxicity (Cisplatin)

Creatinine	Cisplatin Dose	Docetaxel Dose
≤1.5 x ULN	100%	100%
> 1.5 but ≤ 2.0 x ULN	50%	100%
> 2.0 x ULN	0%	0%

If serum creatinine is elevated such that treatment is not administered as scheduled (i.e. > 2.0 X ULN), repeat the abnormal tests at weekly intervals. If the serum creatinine returns to < 2.0 x ULN within 3 weeks, cisplatin may be reinstituted at 50% of the full dose during the next cycle (permanent dose reduction). A more aggressive hydration regimen must be instituted as well with all subsequent doses of cisplatin. If the cisplatin is held, the docetaxel will be held as well so that both drugs are kept on the same schedule. If creatinine is still > 2.0 x ULN after 3 weeks, either discuss with study chair, or discontinue protocol therapy.

Neurologic Toxicity (Docetaxel, Cisplatin)

Cisplatin and docetaxel doses should be modified as follows for neurologic toxicity. The day 1 value should be used in determining dose. Dose reductions for neurotoxicity are permanent.

Grade of Toxicity	Docetaxel and Cisplatin Doses
0	100%

1	100%
2	Hold treatment until patient recovers to grade 1, then resume treatment at 75% dose
≥ Grade 3	Hold treatment until patient recovers to grade 1; then resume treatment at 50% dose

Auditory Toxicity (Cisplatin)
Cisplatin is well known to cause high-frequency hearing loss. Continued use of the drug does not always result in hearing loss, although it may do so. If grade 2 or worse hearing loss is noted, the patient should be presented with a discussion of the relative risks of hearing loss versus the potential benefit of continuing cisplatin therapy, and a decision made on the continuation of cisplatin. Severe hearing loss (grades 3 and 4) is an indication to discontinue the drug.

Hepatic Toxicity (Docetaxel)

Day 1 value should be used in determining dose.

SGOT/AST		**Alkaline phosphatase**		**Bilirubin**	**Docetaxel dose**
≤ 1.5 x ULN	AND	< 1.5 x ULN	AND	WNL	100%
> 1.5 - 5 x ULN	AND	1.5 – 5 x ULN	OR	> ULN-1.5 x ULN	75%
> 5 x ULN	AND	> 5 x ULN	OR	> 1.5 x ULN	Hold*

* Repeat LFTs weekly. If recovered, reduce dose by 25%. If not recovered within 3 weeks, discontinue protocol therapy. If docetaxel is withheld due to hepatic toxicity, cisplatin should also be withheld and administered when docetaxel is resumed. There are no dose reductions of cisplatin for hepatic toxicity.

Any elevation in bilirubin **ALONE** qualifies for dose reduction. However, an elevation in **BOTH** the SGOT/AST and alkaline phosphatase is required to qualify for a dose reduction.

Hypersensitivity Reactions (Docetaxel)
CAUTION: Patients who experienced a mild to moderate hypersensitivity reaction have been successfully rechallenged, but careful attention to prophylaxis and bedside monitoring of vital signs is recommended.

Mild symptoms: Complete docetaxel infusion. Supervise at bedside. No treatment required.

Moderate symptoms: Stop docetaxel infusion. Give intravenous diphenhydramine 25 - 50 mg and intravenous dexamethasone 10 mg. Resume docetaxel infusion after recovery of symptoms at a low rate, 20 mL/hour for 15 minutes, then 40 mL/hour for 15 minutes, then, if no further symptoms, at full dose rate until infusion is complete. If symptoms recur, stop docetaxel infusion. **Protocol treatment will be discontinued.**

Severe life-threatening symptoms: Stop docetaxel infusion. Give intravenous diphenhydramine and dexamethasone as above. Add epinephrine or bronchodilators if indicated. **Protocol treatment will be discontinued**.

Fluid Retention
Fluid retention should be graded as follows.

Edema*	**Severity Grading**	**Effusion***
Asymptomatic	Mild 1	Asymptomatic

Symptomatic	Moderate 2	Symptomatic – Intervention may be required
Symptomatic - resulting in drug Discontinuation	Severe 3	Symptomatic – Intervention required

* Report the highest grade of edema or effusion. No dose reduction is planned for fluid retention. Patients developing new onset or symptomatic edema or other signs of increasing fluid retention should be treated with oral diuretics. Regimens found to be effective in the management of fluid retention due to docetaxel are listed below. Diuretic therapy may be initiated in the order listed at the discretion of the investigator:

• Spironolactone 50 mg PO, once to three times daily.
• Furosemide 40 mg PO daily if not responsive to spironolactone.

Potassium supplementation may be given as needed.

• If, after a trial of > 2 weeks, this is ineffective, treat with furosemide 20 mg PO daily plus metolazone 2.5 mg PO daily with potassium supplementation as needed.
Further therapy following fluid retention should be customized depending upon the clinical situation.

Other Toxicities
For any clinically significant grade 3 or 4 toxicity not mentioned above, the treatment should be withheld until the patient recovers completely or to grade 1. The treatment should then be resumed at 50% dose (permanent dose reduction). For grade 1 and 2 toxicities, no dose reduction should be made.

Chemotherapy Regimen 4: Cisplatin/Gemcitabine

Hematologic Toxicity (Gemcitabine)
• Intra-cycle adjustments (e.g. day 8)

Absolute Neutrophil Count (x 10^6/L)		**Platelets ($X10^6$/L)**	**% Full dose of Gemcitabine**
> 1000	AND	> 75,000	100%
500-999	OR	50,000-74,999	75%
≤500	OR	50,000	0%

Omitted day 8 doses of gemcitabine will not be made up.

• Day 1 cycle dose adjustments (hematologic toxicity):

Dose reductions are not required for neutropenia, unless febrile neutropenia occurs. If a platelet nadir of < 25,000 is reached, future cycles require dose reduction. Day 1 dosing may only resume for platelet count >100,000 and ANC >1500. Please see table below:

Dose Reductions for Hematologic Toxicity

	Cisplatin	**Gemcitabine**
1st episode *	No adjustment	Dose reduce by 1 level**
2nd episode*	No adjustment	Dose reduce by 1 level**
3rd episode	Discontinue protocol therapy	Discontinue protocol therapy
Anemia	No adjustment	No adjustment

*Episodes = Febrile Neutropenia or Platelet Nadir <25,000
Alternatively, if current episode is febrile neutropenia, growth factor support (G-CSF or pegylated G-CSF) may be used with all subsequent cycles instead of dose reduction. If growth factor support has already been instituted, than dose reduction is required for the next episode of febrile neutropenia. Use of G-CSF should be in accordance with the American Society of Clinical Oncology (ASCO) guidelines as published in the Journal of Clinical Oncology. **ANC must be at least 1,500/µl and platelet count must be at least 100,000/µl on day 1 of each cycle.

Dose Reduction Levels

	Original Dose	Reduced Dose
Gemcitabine	1200 mg/m^2	900 mg/m^2
	900 mg/m^2	600 mg/m2

Treatment should be delayed for up to 3 weeks until the day 1 ANC is at least 1500/µl and the platelet count is at least 100,000 /µl. However, if the counts have not recovered in 3 weeks, the patient's protocol treatment will be discontinued. The patient will still be followed for toxicity and response. Patient and investigators need to be attentive to the possibility of fever and infection so that these complications can be promptly and appropriately managed.

If chemotherapy must be withheld due to hematologic toxicity, CBC and platelet counts should be obtained weekly until the counts reach the lower limits for treatment. The treatment schedule will then proceed in the usual schedule.

No dose reductions will be made for anemia. Patients should be supported per the treating physician's discretion. The use of growth factor support for anemia will be allowed,as will blood transfusions as indicated.

Dose reductions, once initiated, are permanent for all future cycles. If both febrile neutropenia and thrombocytopenia of < 25,000 occur, the dose reduction will be to the lower dose specified.

Gastrointestinal Toxicities (Cisplatin)
Nausea and/or vomiting should be controlled with antiemetics. If grade 3 nausea/vomiting occurs in spite of maximum antiemetics (steroid pre-medication for 4 days after administration, 5HT3 antagonist, and aprepitant or similar agent if available), the dose of cisplatin should be reduced by 25% for the next course. If tolerated, increase back to 100% as soon as possible.
If, on day 1 of any treatment cycle, the patient has stomatitis, the treatment should be withheld until the stomatitis is cleared. If the stomatitis has not cleared in 3 weeks, the patient's protocol treatment will be discontinued. (Refer to the CTC, version 3.0 for specific grading criteria.) If acute grade 3 stomatitis occurs at any time, the dose should be given at 75% dose when the stomatitis is completely cleared. This is a permanent dose reduction.

Renal Toxicity (Cisplatin)

Creatinine	Cisplatin Dose	Gemctiabine Dose
≤1.5 x ULN	100%	100%
> 1.5 but ≤ 2.0 x ULN	50%	100%
> 2.0 x ULN	0%	0%

If serum creatinine is elevated such that treatment is not administered as scheduled (i.e. > 2.0 X ULN), repeat the abnormal tests at weekly intervals. If the serum creatinine returns to < 2.0 x ULN within 3 weeks, cisplatin may be reinstituted at 50% of the full dose during the next cycle (permanent dose reduction). A more aggressive hydration regimen must be instituted as well with

all subsequent doses of cisplatin. If the cisplatin is held, the gemcitabine will be held as well so that both drugs are kept on the same schedule. If creatinine is still > 2.0 x ULN after 3 weeks, either discuss with study chair, or discontinue protocol therapy.

Neurologic Toxicity (Gemcitabine, Cisplatin)
Gemcitabine and cisplatin doses should be modified as follows for neurologic toxicity. Day 1 value should be used in determining dose. Dose reductions for neurotoxicity are permanent.

Grade of Toxicity	Gemcitabine and Cisplatin Doses
0	100%
1	100%
2	Hold treatment until patient recovers to grade 1, then resume treatment at 75% dose
≥ Grade 3	Hold treatment until patient recovers to grade 1; then resume treatment at 50% dose

Auditory Toxicity (Cisplatin)
Cisplatin is well known to cause high-frequency hearing loss. Continued use of the drug does not always result in hearing loss, although it may do so. If grade 2 or worse hearing loss is noted, the patient should be presented with a discussion of the relative risks of hearing loss versus the potential benefit of continuing cisplatin therapy, and a decision made on the continuation of cisplatin. Severe hearing loss (grades 3 and 4) is an indication to discontinue the drug.

Other Toxicities
For any clinically significant grade 3 or 4 toxicity not mentioned above, the treatment should be withheld until the patient recovers completely or to grade 1. The treatment should then be resumed at 50% dose (permanent dose reduction). For grade 1 and 2 toxicities, no dose reduction should be made.

p. Statistical Considerations.

Overview of Study Design. Patients with suspected Stage I NSCLC and have undergone a complete surgical resection of their disease will be eligible for pre-registration to the study. The risk of cancer recurrence will be predicted by the LMP for each patient. Those patients who have correct pathological stage I with a tumor size of 1.75 cm in diameter or greater with or without visceral pleural invasion and valid LMS will be assigned to one of the three study arms. In particular, patients with LMS < 0.5 will be assigned to observation (arm A); the rest of patients (LMS ≥ 0.5) will be randomized with equal probability to receive either adjuvant chemotherapy (arm B) or observation (arm C). Randomization will be implemented with a permuted-block scheme stratified by stage (T1N0 vs. T2N0).

The primary objectives are (P1) to validate the superiority of arm A (LMS<0.5 Observation) to arm C (LMS≥ 0.5 Observation) on overall survival and (P2) to determine the efficacy of adjuvant chemotherapy in patients with LMS≥0.5, i.e. a survival benefit for patients on arm B (LMS≥ 0.5 Chemo) compared to those on arm C (LMS≥ 0.5 Observation). The endpoint for both objectives will be overall survival (OS), defined as the time from formal registration to death of all causes. Formal registration of a patient occurs right after the LMS model prediction on the patient is finished, the tumor is pathological stage I and the CALGB Statistical Center is notified by email. Secondary objectives of this study are (S1) to compare progression free survival (PFS) of

patients on three study arms, (S2) to compare prediction accuracy of the risk of cancer-related death at 3 and 5 years based on the clinical prediction model and the LMS model, (S3) to characterize the adverse events related to the adjuvant chemotherapy.

To address the primary objectives, the study is designed to have adequate power to compare overall survivals of arm B and arm C (P1) and those of arm A and arm C (P2). In order to control the Type I error to the level of 0.05 for the two planned comparisons, we will use Hochberg procedure (Hochberg 1988). Specifically, at first, both comparisons (arm A vs. arm C, arm B vs. arm C) will be tested at a two-sided significance level of 0.05. If both p-values are less than 0.05, both null hypotheses are rejected. If one of the p-values is greater than 0.05, the other will be tested at a two-sided significance level of 0.025. The adjusted p values will be reported at the final analysis.

Approximately 1170 patients suspected with stage I non-small cell lung cancer will be initially registered to the study. With an allowance of 15% ineligible rate due to incorrect pathological stage and tumor size and non-usable genomic data, we expect that about 994 patients to be formally registered and assigned to one of the three study arms. Based on the result of a random sample of stage I patients from ACOSOG Z003 and CALGB 9761, we estimate that 64% patients with LMS<0.5 and 36% with LMS≥ 0.5. Therefore, about 636 patients with LMS<0.5 are to be assigned to arm A and 358 patients with LMS≥ 0.5 are to be randomized to arm B and arm C. At a monthly rate of 24 patients, the study accrual is expected to accrue the total of 1170 patients over 4 years. An additional follow-up of 3 years is needed before the final analysis takes place at 7 years after the first enrollment. The final sample size would be smaller if early stopping occurred for futility or superiority of survival benefits of arm B relative to arm C.

Sample Size Considerations. From an analysis of a random sample of stage I patients from ACOSOG Z003 and CALGB 9761, the 5-year overall survival in T1N0 and T2N0 patients is 60%. Of these, the 5-year overall survivals for patients with LMS≥ 0.5 and LMS<0.5 are 85% and 25%, respectively.

The following assumptions are made while determining the size of the study: (1) 15% patients will be formally registered the study due to either incorrect pathological stage or un-usable genomic data; (2) 64% formally registered patients have LMS<0.5 and the rest of 36% patients have LMS≥ 0.5; (3) patients with LMS≥0.5 will be randomized to arm B (Chemo) and arm C (Observation) with equal probability, and patients with LMS<0.5 will be assigned to arm A (Observation); (4) the 5-year overall survival rate of arm A (LMS<0.5 Observation) is 85%, that of arm C (LMS≥ 0.5 Observation) is 25%, and that of arm B (LMS≥ 0.5 Chemo) is 39.7%. Under constant hazards, the hazard ratio of arm C/arm B is 1.50 and that of arm C/arm A is 8.53; (5) an accrual length of 4 years at an accrual rate of 24 patients per month; (6) additional follow-up of 3 years after the last enrollment; and (7) a two-sided significance level of 0.05 for both planned comparisons. Under these assumptions, we expect a total of 1170 patients initially registered to the study, 994 patients formally registered, 179 patients randomized to arm B and 179 patients randomized to arm C and 636 patients in arm A. At the time of final analysis, or 7 years after the first enrollment, a total of 238 deaths are expected among arm C (LMS≥ 0.5 Observation) and arm B (LMS≥ 0.5 Chemo) under alternative hypotheses. Under fixed sample size design, the power in detecting the expected survival benefit for arm B relative to arm C is about 88% using a stratified log-rank test with 132 deaths on arm B and 106 deaths on arm C. Taking interim analyses into account (see next section), the power will slightly decrease to about 87%. On the other hand, the power in detecting the expected survival benefit for arm A relative to arm C is 100% using a log-rank test with 95 deaths on arm A and 132 deaths on arm C.

Interim analysis**.** The first interim analysis for survival efficacy of arm B over arm C will occur 2 years after the first enrollment, or when 36 deaths have been observed on arm B and arm C combined. After that, interim analyses will occur once a year until the final analysis at 7 years. A total of 5 interim analyses and one final analysis are planned at year 2, 3, 4, 5, 6 and 7. Early stopping at any of the interim analyses can occur either for superiority (arm B is superior to the arm C) or for futility (arm B is equivalent or inferior to arm C) on overall survival. Using S+SeqTrial [ref2], we will construct two-sided boundaries in the spirit of O'Brien Fleming (O'Brien 1979). We will truncate alpha level at 0.005 for the futility boundary to echo the spirit in Freidlin and Korn (Freidlin 2002). Without adjusting for multiple testing on the two planned comparisons, the final analysis at 7 years will conclude a superiority of arm B over arm C if the p-value of a two-sided stratified log rank test is less than 0.05. The following table displays the operating characteristics, including power, average study size, stopping probabilities under true hazard ratios of 0.75, 1.0, 1.3, and 1.5.

Hazard Ratio (λ_C/λ_B)	Expected Number of Deaths among arm B and arm C	Prob. to Reject H0 (Power)	Early Stop Prob. for Superiority	Early Stop Prob. for Futility
0.75	83	0.0000	0.0000	0.9941
1.0	160	0.0250	0.0163	0.6913
1.3	202	0.5094	0.3926	0.1033
1.5	176	0.8661	0.7730	0.0170
1.75	142	0.9880	0.9676	0.0021

The CALGB DSMB will review the data available from the trial at each of its semiannual meetings. This will include toxicity, disease recurrence and survival information. The DSMB will determine which of its biannual meetings most appropriately fits the protocol-dictated one-year formal interim analysis. This determination will be based on a recommendation of the study statistician. In determining whether the trial should be continued, the DSMB will consider the results at each interim analysis, as described above. The DSMB will use its discretion in weighing the combined impact of treatment-related morbidity, disease recurrence and overall survival.

Assumption Monitoring. After the first 100 patients have been formally registered to the study, the proportion of patients assigned to each treatment arm will be re-estimated. If there are significantly more than 64% patients with a LMS <0.5, the sample size of the study will be re-estimated to ensure at least 358 patients with LMS≥0.5 to be accrued. As a result, the final size of initially registered patients may be modified in an amendment after study activation.

Accrual (accrual Rate: 24 patients / month). ACOSOG Z0030 accrued 30-40 patients per month in a randomized trial of mediastinal lymph node assessment. CALGB 9633 accrued 7-8 patients per month (over 2/3 of which came from CALGB institutions). Our proposed trial will be initially supported by CALGB and ACOSOG (including NCI-C centers) because these are the only groups with a tract record in collecting fresh tumor. SWOG, ECOG, NCCTG, NCI-C will be approached about joining once their banking techniques are proven reliable. Given the high interest in genomic-targeted randomized adjuvant trials, we expect that the total of 1170 patients with suspected stage IA and IB NSCLC can be accrued over a period of 4 years at a rate of 24 patients per month (15 minimum/30+ maximum per month). All patients will be followed after the last enrollment for at least 3 years.

Analytic Methods. Overall survival is defined as the time between formal registration and death of all causes. Progression free survival is defined as the time between formal registration and disease relapse or death of all causes, whichever comes first. The primary analysis for the overall survival comparison of arm A and arm C or arm B over arm C will be performed using the intent-to-treat principal, which will include all randomized patients. The primary analysis at 7 years will conclude a survival superiority of arm B over arm C or arm A over arm C if the p-values of a two-sided log rank test for both comparisons are less than 0.05 or either p-value is less than 0.025 according to Hochberg procedure for the multiplicity adjustment. The Hochberg adjusted p values will be provided for the primary analysis. The function of overall survival and failure free survival will be characterized by the Kaplan-Meier method (Kaplan 1958).

The comparisons of treatment arms on overall survival will be done using the log ranked or its stratified version (Kalbfleisch 2002). Secondary multivariate survival analysis for the effect of chemotherapy will be performed using a Cox's proportional hazard model (Cox 1972) with the significant prognostic factors as initial model covariates, such as age, gender, smoking status, tumor size, and histological type. A step-down procedure that consists of dropping the least significant covariates, one at a time, will be used to obtain a more parsimonious model. The probabilities of death due to different causes (cancer or other causes) will be estimated and modeled for three treatment arms using the methodology developed by Gray (Gray 1988, Fine 1999). These secondary analyses will be done for both intent-to-treat population and as-treated population. Similarly analyses will be conducted for progression free survival.

The prediction accuracy (sensitivity and specificity) of the LMS model for the risk of cancer recurrence (LMS cut off of 0.5) will be validated using the long term survival data of arm A and arm C, where a positive outcome is defined as a death occurring within 5 years of registration. The extent of agreement of the risk of cancer recurrence based on the clinical model and the genomic model will be evaluated using Kappa statistics, and their performance relative to the binary survival outcome (die within 5 years or not) will be compared using a generalized estimating equation model (Diggle 2002) with logit link with empirical variance estimator to account for correlation of multiple measures.

Contingency tables will be used to summarize the frequency of toxicity by type and severity for patient receiving chemotherapy.

Feasibility. Stage I is the largest and fastest growing subset of patients with early stage NSCLC, with an estimated 30,000+ cases per year resected in North America. No data exists on the utility of adjuvant therapy. The idea for this trial has been vetted nationally to thoracic oncologists and patient advocates and has received broad support as it is believed it will help answer an important question. However, a standard phase III design would require more than one thousand patients randomized without the LMS to remove those patients at low risk for recurrence and thus not likely to benefit from adjuvant chemotherapy. It is doubtful that a rrandomized trial which requires more than 1000 patients to be randomized is feasible, while a genomic-based trial has significant appeal to patients and investigators.

VII. REFERENCES

Alam N, Shepherd FA, Winton T, et al. Compliance with post-operative adjuvant chemotherapy in non-small cell lung cancer: an analysis of National Cancer Institute of Canada and intergroup trial JBR.10 and a review of the literature. Lung Cancer 47:385-394, 2005.

Arriagada R, Bergman B, Dunant A, Le Chevalier T and Pignon JP. Vansteenkiste J. International Adjuvant Lung Cancer Trial Collaborative Group. Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. New Engl J Med 350(4):351-60, 2004.

Aaronson NK, Bakker W, Stewart A L, van Dam F, van Zandwijk N, Yarnold J R, et al. Multidimensional approach to the measurement of quality of life in lung cancer clinical trials. In N. K. Aaronson & J. Beckman (Eds.), The quality of life of cancer patients (pp. 63-82). New York: Raven Press, 1987.

Bergman B, Aaronson N K, Ahmedzai S, Kaasa S and Sullivan M. The EORTC-QLQ-LC13: A modular supplement to the EORTC core quality of life questionnaire (QLQ-C30) for use in lung cancer clinical trials. EORTC study group on quality of life. Eur J Cancer, 30A(5), 635-642, 1994.

Beer DG, Kardia SL, Huang CC, Giordano TJ, Levin AM, Misek DE, Lin L, Chen G, Gharib TG, Thomas DG, Lizyness ML, Kuick R, Hayasaka S, Taylor JM, Iannettoni MD, Orringer MB and Hanash S. Gene-expression profiles predict survival of patients with lung adenocarcinoma. Nat Med. 8(8):816-24, 2002.

Belani, C. Carboplatin plus paclitaxel in the treatment of advanced non-small cell lung cancer and carboplatin plus paclitaxel plus radiation in stage III NSCLC. in The Fox Chase Cancer Center Consensus Workshop. St John, U.S. Virgin Islands: Sem Oncol, 1995.

Belani CP, et al. Phase I study of paclitaxel and carboplatin (CBDCA) in advanced and metastatic non-small cell lung cancer (NSCLC). Proceedings of ASCO, 14: 174, 1995.

Beziak A, Winton T, Ding K, et al. Quality of life in a trial of adjuvant chemotherapy for early stage completely resected non-small cell lung cancer (NCIC CTG BR.10). Lung Cancer 41:Suppl 2:S20-S20, 2003.

Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, Ladd C, Beheshti J, Bueno R, Gillette M, Loda M, Weber G, Mark EJ, Lander ES, Wong W, Johnson BE, Golub TR, Sugarbaker DJ and Meyerson M. Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. PNAS 98(24):13790-5, 2001.

Bishop J. Taxoids in lung cancer. Lung Cancer, 1994. 11(supplement 2): p. 250-251.
Bore P. Pharmacokinetics of a new anti-cancer drug, Navelbine in patients. Cancer Chemother Pharmacol 23:24-251, 1989.

Bunn P. The University of Colorado experience with paclitaxel and carboplatin in non-small cell lung cancer. in The Fox Chase Cancer Consensus Workshop. St John, U.S. Virgin Islands: Seminars in Oncology, 1995.

Burroughs Wellcome Co., Navelbine (venorelbine tartrate) Invesigators brochure 1993.

Calvert AH and Newell DR. Update on optimal carboplatin dosing using AUC. 1994, Princeton, New Jersey: Bristol-Myers Squibb.

Calvert AH, et al. Carboplatin dosage: prospective evaluation of a simple formula based on renal function. Journal of Clinical Oncology 7:1748-1756, 1989.

Chang A, et al. Phase II study of taxol in patients with stage IV non-small cell lung cancer (NSCLC); the Eastern Cooperative Oncology Group Results (ECOG). Proc Am Soc Clin Onc. 11:293, 1992.

Chang A, Kim K, and Glick J. Phase II study of taxol, merbarone, and piroxantrone in stage IV non-small cell lung cancer: the Eastern Cooperative Oncology Group results. JNCI. 85:388-394, 1993.

Cockcroft DW and Gault MH. Prediction of creatinine clearace from serum creatinine. Nephron, 16(1):31-41, 1976.

Cox LS, Africano NL, Tercyak KP and Taylor KL. Nicotine dependence treatment for patients with cancer. Cancer, 98 : 632-44, 2003.

D'Amico TA, Massey M, Herndon JE II, Moore MB, Harpole DH, Jr: A Biologic risk model for Stage I lung cancer: Immunohistochemical analysis of 408 patients using 10 molecular markers. J Thorac Cardiovasc Surg.117:736-743, 1999.

Donehower R and Rowinsky E. An overview of experience with TAXOL (paclitaxel) in the USA. CA Treat Rev. 19 (Suppl C):63-78. 1993.

Donehower R et al. Phase I trial of taxol in patients with advanced cancer. Cancer Treat Rep. 71(12):1171-1177, 1987.

Dorr RT and Alberts DT. Use of estimated creatinine clearance for dose adjustments with renally eliminated anticancer agents. Princeton, New Jersey: Bristol-Myers Squibb, 1993.
Egorin MJ, et al., Pharmacokinetics a and dosage reduction of cis-diammine-(1,1-cyclobutanedicarboxylato) platinum in patients with impaired renal function. Cancer Res. 44:5432-5438, 1984.

Ettinger D, et al., Phase II study of Taxol in patients with extensive-stage small cell lung cancer (SCLC): An Eastern Cooperative Oncology Group study. Proc Am Soc Clin Oncol. 12:A1094, 1993.

Fellous A, Ohayon R, Vacassin T, Binet S, Lataste H, Krikorian A, Couvinier JP, Meininger V. Biochemical effects of navelbine on tubulin and associated proteins. Sem Oncol. 16(2 suppl 4):9-14, 1989.

Fine J and Gray R. A proportional hazards model for the subdistribution of a competing risk. J Amer Statis Assoc. 94:496-509, 1999.

Fossella, F., et al., A phase II study of taxol (NSC 125973) in patients with non-small cell lung cancer (NSCLC). Proc Am Soc Clin Onc. 1992. 11:294.

Fossa, S.D. Quality of life assessment in unselected oncologic out-patients: A pilot study. Int J

Oncol. 4:1393-1397, 1994.

Gibbons FX, McGovern PG, Lando HA. Relapse and risk perception among members of a smoking cessation clinic. Health Psychol. 10(1):42-45, 1991. UI: 2026129

Freidlin B, Korn EL, George SL. Data monitoring committees and interim monitoring guidelines. Control Clin Trials. 20(5):395-407, 1999.

Garber ME. Troyanskaya OG. Schluens K. Petersen S. Thaesler Z. Pacyna-Gengelbach M. van de Rijn M. Rosen GD. Perou CM. Whyte RI. Altman RB. Brown PO. Botstein D. Petersen I. Diversity of gene expression in adenocarcinoma of the lung. Proc Nat Acad Sci USA. 98(24):13784-9, 2001.

Gray R. A class k-sample tests for comparing the cumulative incidence of a competing risk. Ann Statis. 16:1141-54, 1988.

Grem JL, et al., Phase I study of taxol administered as a short IV infusion daily for 5 days. Cancer Treat Rep. 71:53-59, 1987.

Hainsworth J and Greco F. Paclitaxel administered by 1 hour infusion. Preliminary results of a phase I/II trial comparing two schedules. Cancer. 74(4):1377-82, 1994.

Hainsworth J, et al., Taxol administered by one-hour infusion: preliminary results of a Phase I/II study comparing two dose schedules Proc Annu Meet Am Soc Clin Oncol. 13:A413, 1994.

Harpole DH Jr, Herndon JE, Wolfe WG, Iglehart JD, Marks JR. A prognostic model of recurrence and death in stage I non-small cell lung cancer utilizing presentation, histopathology, and oncoprotein expression. Cancer Res. 55:51-56, 1995.

Harpole DH Jr, Marks JR, Richards W, Sugarbaker DJ. Localized adenocarcinoma of the lung: oncogene expression of erbB-2 and p53 in 150 patients. Clin Cancer Res. 1:659-664, 1995.

Harpole DH Jr, Richards WG, Herdon JE, Sugarbaker DJ. Angiogenesis and molecular biologic sub-staging in patients with stage I non-small cell lung cancer. Ann Thorac Surg. 61:1470-1476, 1996.

Harpole DH, Herndon JE, Young WG, Wolfe WG, Sabiston DC. Stage I non-small cell lung cancer: a multivariate analysis of treatment and patterns of recurrence. Cancer. 76:787-796, 1995.

Hjermstad, M.J., Fossa, D.S., Bjordal, K., Kaasa, S. Test/retest study of the European organization for Research and Treatment of Cancer core quality-of-life questionnaire. J Clin Oncol. 13(5):1249-1254, 1995.

Hogan, JW, Laird, NM.. Mixture models for the joint distribution of repeated measures and event times. Statistics in Medicine 16: 239-257, 1997.

Horowitz M, Wilner N and Alvarez W. Impact of events scale: A measure of subjective distress. Psychosom Med. 41(3):209-218, 1979.

Hochberg Y. A sharper Bonferroni procedure for multiple tests of significance. Biometrika. 75:800–802, 1988.

Huang E, Cheng SH, Dressman H, et al. Gene expression predictors of breast cancer outcomes. Lancet. 361:1590-6, 2003.

Jeliffe RW. Creatinine clearance: bedside estimate. Ann Internal Med. 79:604-605, 1973.

Jodrell DI, et al., Relationships between carboplatin exposure and tumor response and toxcicity in patients with ovarian cancer. J Clin Oncol. 10:520-528, 1992.

Johnson, J. A. et al. Valuation of EuroQOL (EQ-5D) health states in an adult US sample. Pharmacoeconomics. 13:421-433, 1998.

Kato H. Ichinose Y. Ohta M. Hata E. Tsubota N. Tada H. Watanabe Y. Wada H. Tsuboi M. Hamajima N. Ohta M. Japan Lung Cancer Research Group on Postsurgical Adjuvant Chemotherapy. A randomized trial of adjuvant chemotherapy with uracil-tegafur for adenocarcinoma of the lung. N Engl J Med. 350(17):1713-21, 2004.

Kearns CM, et al., Reduced platelet toxicity with combination carboplatin & paclitaxel; pharmacodynamic modulation of carboplatin associated thrombocytopenia. Proc Amer Soc Clin Oncol. 14:170, 1995.

Kelly K and Bunn P. Phase I trials of taxol-containing chemotherapy regimens for the treatment of advanced non-small cell and small cell lung cancer. Lung Cancer. 11(Supplement 1):122, 1994.

Kikuchi T, Daigo Y, Katagiri T, Tsunoda T, Okada K, Kakiuchi S, Zembutsu H, Furukawa Y, Kawamura M, Kobayashi K, Imai K, Nakamura Y. Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. Oncogene. 10;22(14):2192-205, 2003.

Kind P. The EuroQol instrument: An index of health-related quality of life (pp. 191-201). In B. Spilker (ed.), Quality of life and pharmacoeconomics in clinical trials, 2nd edition. Philadelphia: Lippincott-Raven Publishers, 1996.

Kornblith AB, Holland JC. A model for quality-of-life research from the Cancer and Leukemia Group B: The telephone interview, conceptual approach to measurement, and theoretical framework. JNCI Mono. 20:55-62, 1996.

Krikorian A, Rahmani R, Bromet M, Bore P and Cano JP. Pharmacokinetics of Navelbine. Sem Oncol. 16(suppl 4):21-25, 1989.

Kumar N. Taxol induced polymerization of purified tubulin. J Biol Chem. 256: p. 10435-10441, 1981.

Lan K, DeMets D. Discrete sequential boundaries for clinical trials. Biometrika. 70:659-653, 1983.
Langer, C.J., Leighon, J. Comis, #004#R., et al., Paclitaxel and carboplatin in advanced non-small cell lung cancer: A Phase II Toxicity, response and survival Analysis. J Clin Oncol. 13:1860-1870, 1995.

Legha S, Tenney D and Krakoff I. Phase I study of taxol using a 5-day intermittent schedule. J Clin Oncol. 4:762-766, 1986.

Lilenbaum RC, Herndon JE 2nd, List MA, Desch C, Watson DM, Miller AA, Graziano SL, Perry MC. Saville W. Chahinian P. Weeks JC. Holland JC. Green MR. Single-agent versus combination chemotherapy in advanced non-small-cell lung cancer: the cancer and leukemia group B (study 9730) J Clin Oncol. 23(1):190-6, 2005.

Little RJA and Rubin DB. Statistical Analysis with Missing Data. Wiley, New York, 1987.
Liu ET. Mechanism-derived gene expression signatures and predictive biomarkers in clinical oncology. PNAS 102:3531-3532, 2005.

Long HJ. Paclitaxel (Taxol): a novel anticancer chemotherapeutic drug. Mayo Clinic Proc. 69:341-345, 1994.

Muggia FM. Overview of carboplatin: replacing, complementing, and extending the therapeutic horizons of cisplatin. Sem Oncol. 16:7-13, 1989.

Murphy W, Fossella F, Winn R, et al., Phase II study of taxol in patients with untreated advanced non-small cell lung cancer. JNCI. 85:384-388, 1993.

Naruke T, Goya T, Tsuchiya R, and Surmasu K. Prognosis and survival in resected lung carcinoma based on the international staging system. J Thorac Cardiovasc Surg, 96:440-447, 1988.

O'Brien PC, Fleming TR. A multiple testing procedure for clinical trials. Biometrics. 35(3):549-556, 1979.

Ozols RF. Optimal dosing of carboplatin. Sem Oncol. 16(2 Suppl 5):14-18, 1989. UI:2655094
Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics. CA-Cancer J Clin. 47(1):5-27, 1997.

Pittman J, Huang E, Dressman H, et al. Integrated modeling of clinical and gene expression information for personalized prediction of disease outcomes. Proc Nat'l Acad Sci. 101:8431-6, 2004.

Potti A, Mukherjee S, Petersen R, Dressman HK, Bild A, Koontz J, Kratzke R, Watson MA, Kelley M, Ginsburg GS, West M, Harpole DH Jr, Nevins JR. A genomic strategy to refine prognosis in early-stage non-small-cell lung cancer. N Engl J Med 355(6): 570-80, 2006.

Robins JM, Rotnitzky A, Zhao L-P and Lipsitz S. Estimation of regression coefficients when some regressors are not always observed. J Amer Stat Assoc. 89:846-866, 1994.

Rosenbaum PR and Rubin DB. Reducing bias in observations studies using subclassification on the propensity score. J Amer Stat Assoc. 79:516-524, 1984.

Rowinsky EK, et al., Paclitaxel on a 3-hour schedule and carboplatin in non small cell lung cancer: Use of maximally tolerated and clinicaly relevant single agent doses in combination is feasible. Proc Amer Soc Clin Oncol. 14:354, 1995.

Rowinsky EK, Cazenave LA and Donehower RC. Taxol: a novel investigational antimicrotubule agent. JNCI. 82:1247-1259, 1990.

Schiff PB and Horwitz SB. Taxol assembles tubulin in the absence of exogeneous guanosine 5"-triphosphate or microtubule associated proteins. Biochem. 20(11):3242-3252, 1981.

Schiff PB, Faut J and Horwitz SB. Promotion of microtubule assembly in vitro by taxol. Nature. 277:665-667, 1979.

Schnoll RA, Rothman RL, Newman H, Lerman C, Miller S, Movsas B, Sherman E, Ridge JA. Characteristics of cancer patients entering a smoking cessation program and correlates of quit motivation: implications for the development of tobacco control programs for cancer patients. Psycho-Oncology. 13:346-358, 2004.

Sorensen BT, et al., Dose-toxicity relationship of carboplatin in combination with cyclophosphamide in ovarian cancer patients. Cancer Chemother Pharmacol. 28(5):397-401, 1991.

Strauss GM, Herndon J, Maddaus MA, et al. Randomized clinical trial of adjuvant chemotherapy with paclitaxel and carboplatin following resection in Stage IB non-small cell lung cancer: Report of Cancer and Leukemia Group B (CALGB) Protocol 9633. J Clin Oncol. 22:Suppl 14S:7019, 2004.

Strauss GM, Kwiatkowski DJ, Harpole DH, Lynch TJ, Skarin AT and Sugarbaker DJ. Molecular and pathologic markers in stage I non-small cell carcinoma of the lung. J Clin Oncol. 13(5):1265-1279, 1995.

Thewes B, Meiser B and Hickie IB. Psychometric properties of the Impact of Event Sclae amongst women at increased risk for hereditary breast cancer. Psycho-Onc. 10(6):459-468, 2001.

Vafai D, et al. Phase I/II trial of combination carboplatin and taxol in non-small cell lung cancer (NSCLC). Proc Amer Soc Clin Oncol. 14:352, 1995.

Van Echo DA, Egorin MJ and Aisner J. The pharmacology of carboplatin. Sem Oncol. 16(2 Suppl 15):1-6, 1989.

Vickberg, S. M. The concerns about recurrence scale (CARS): A systematic measure of women's fears about the possibility of breast cancer recurrence. Ann Behav Med. 25(1):16-24, 2003.

Weiss R, et al. Hypersensitivity reactions from taxol. J Clin Oncol. 8(7):1263-1268, 1990.
Weinstein, N. D. Unrealistic optimism about future life events. J Pers Social Psych. 39:806-820, 1980.

West M, Blanchette C, Dressman H, Huang ., Ishida S, Spang R., Zuzan H, Olson JA, Jr., Marks JR and Nevins JR. Predicting the clinical status of human breast cancer by using gene expression profiles. Proc Natl Acad Sci USA. 98(20):11462-11467, 2001.

Wiernik P, et al. Phase I clinical and pharmacokinetic study of taxol. Cancer Res. 47(9):2486-2493, 1987.

Wigle DA, Jurisica I, Radulovich N, Pintilie M, Rossant J, Liu N, Lu C, Woodgett J, Seiden I, Johnston M, Keshavjee S, Darling G, Winton T, Breitkreutz BJ, Jorgenson P, Tyers M, Shepherd FA and Tsao MS. Molecular profiling of non-small cell lung cancer and correlation with disease-free survival. Cancer Res. 62(11):3005-8, 2002.

Winton T, Livingston R, Johnson D, et al. Vinorelbine plus Cisplatin vs. observation in resected non-small cell lung cancer. NEJM. 352(25):2589-97, 2005.

VIII. APPENDICES

APPENDIX 1. LMP Agilent 2100 Bioanalyzer RNA Integrity Check

APPENDIX 2. LMP Cleanup and Quantification of Biotin-Labeled cRNA

APPENDIX 3. LMP Cleanup of Double-Stranded cDNA

APPENDIX 4. IGSP First Strand cDNA Synthesis Protocol

APPENDIX 5. LMP Fragmenting the cRNA for Target Preparation

APPENDIX 6. LMP NanoDrop: Determining RNA Concentration

APPENDIX 7. LMP Qiagen RNeasy Plus RNA Isolation

APPENDIX 8. IGSP Second Strand cDNA Synthesis Protocol

APPENDIX 9. LMP Synthesis of Biotin-Labeled cRNA

APPENDIX 10. LMP Tissue Procurement Protocol (for RNA)

APPENDIX 1. LMP Agilent 2100 Bioanalyzer RNA Integrity Check

BACKGROUND:

The Agilent 2100 Bioanalyzer is a microfluidics-based platform for the analysis of DNA, RNA, proteins and cells. For any RNA based molecular diagnostics assay, high quality RNA is an essential starting component. Therefore, RNA isolation has to be followed by a stringent RNA quality control. The Agilent 2100 bioanalyzer in combination with RNA 6000 Nano LabChip kit is a simple and efficient means of producing a high-resolution sizing analysis of small RNA samples. This allows for the visualization of ribosomal RNAs. The relative amounts of these rRNAs can be used a measure of RNA degradation.

PRINCIPLE:
The RNA 6000 Nano LabChip kit can be considered as an extremely small capillary electrophoresis instrument. Rather than migrating through capillaries, samples are run through polymer filled channels etched into a glass substrate. Results are comparable to standard gel based and capillary electrophoretic methods of RNA visualization

SPECIMEN: Isolated RNA

LOCATION: This protocol must be performed in the RNA Extraction Room.

EQUIPMENT AND SUPPLIES:

Equipment:
2100 Agilent Bioanalyzer (Agilent Technologies, Cat# G2940CA, 1-877-424-4536)
Microcentrifuge (Beckman Coulter 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
MiniVortexer (VWR cat#58816-121, 1-800-932-5000)

Supplies:
RNaseZAP® (Ambion, Inc. Cat# 9780, 1 800 888-8804)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550)
1.5 mL microcentrifuge tubes (RNase free) (VWR cat#14231-062, 1-800-932-5000)
Heating block (VWR cat#13259-030, 1-800-932-5000)
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)

Reagents:
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water
RNase Away (VWR, Cat# 17810-491, 1-800-932-5000)

Preparation:

Setting up the Assay Equipment and Bioanalyzer

Before beginning the chip preparation protocol, ensure that the chip priming station and the bioanalyzer are set up and ready to use.
You have to
• replace the syringe at the chip priming station with each new RNA kit
• adjust the base plate of the chip priming station
• adjust the syringe clip at the chip priming station
• adjust the bioanalyzer's chip selector
• set up the vortex mixer
• finally, make sure that you start the software before you load the chip.

Setting up the Chip Priming Station

1 Replace the syringe with each new reagent kit:
a Unscrew the old syringe from the lid of the chip priming station.
b Release the old syringe from the clip. Discard the old syringe.
c Remove the plastic cap of the new syringe and insert it into the clip.
d Slide it into the hole of the luer lock adapter and screw it tightly to the chip priming station.
2 Adjust the base plate:
a Open the chip priming station by pulling the latch.
b Using a screwdriver, open the screw at the underside of the base plate.
c Lift the base plate and insert it again in position. Retighten the screw.
3 Adjust the syringe clip:
a Release the lever of the clip and slide it up to the top

Setting up the Bioanalyzer

Adjust the chip selector:
1 Open the lid of the bioanalyzer and make sure that the electrode cartridge is inserted in the instrument. If not, open the latch, remove the pressure cartridge and insert the electrode cartridge.
2 Remove any remaining chip and adjust the chip selector to position (1).

Vortex Mixer

IKA - Model MS2-S8/MS2-S9 (supplied with the Agilent 2100 Bioanalyzer system)
To set up the vortex mixer, adjust the speed knob to 2000 rpm.

Starting the 2100 expert software

1. Go to your desktop and double-click the following icon. 
The screen of the software appears in the *Instrument* context. The icon in the upper part of the screen represents the current instrument/PC communication status:
Lid closed, no chip or chip empty, Lid open, Dimmed icon: no communication, Lid closed, chip inserted, RNA or demo assay selected

2 If more than one instrument is connected to your PC, select the instrument you want to use in the tree view.

Performance Parameters:
Handle and store all reagents according to the instructions on the label of the individual box.
• Avoid sources of dust or other contaminants. Foreign matter in reagents and samples or in the wells of the chip will interfere with assay results.
• Keep all reagent and reagent mixes refrigerated at 4 °C when not in use.
• Allow all reagents and samples to equilibrate to room temperature for 30 minutes before use.
• Protect dye and dye mixtures from light. Remove light covers only when pipetting. The dye decomposes when exposed to light and this reduces the signal intensity.
• Always insert the pipette tip to the bottom of the well when dispensing the liquid. Placing the pipette at the edge of the well may lead to poor results.
• Always wear gloves when handling RNA and use RNase-free tips, microfuge tubes and water.
• It is recommended to heat denature all RNA samples and RNA ladder before use
• Do not touch the Agilent 2100 bioanalyzer during analysis and never place it on a vibrating surface.
• Always vortex the dye concentrate for 10 seconds before preparing the gel-dye mix.
• Use a new syringe and electrode cleaners with each new kit.
Use loaded chips within 5 minutes. Reagents might evaporate, leading to poor results.
• To prevent contamination (e.g. RNase) problems, it is strongly recommended to use a dedicated electrode cartridge for RNA assays.
• Perform the following RNase decontamination procedure for the electrodes on a daily basis before running any assays.

SAFTEY:

Handling DMSO

Kit components contain DMSO. Because the dye binds to nucleic acids, it should be treated as a potential mutagen and used with appropriate care.

Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples.
Handle the DMSO stock solutions with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample.

PROCEDURE - STEPWISE:

For proper handling of the ladder, following steps are necessary:
1 After reagent kit arrival, pipette the ladder in RNase-free vial. The ladder can be ordered separately (reorder number 5067-1529).
2 Heat denature it for 2 min at 70 °C.
3 Immediately cool down the vial on ice.
4 Prepare aliquots in RNase-free vials with the required amount for a typical daily use.

5 Store aliquots at -70°C.
6 Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process).

Prepare the assay, Load the chip, and Run the assay

Decontaminating the Electrodes
To avoid decomposition of your RNA sample, follow this electrode decontamination procedure on a daily basis before running any RNA Nano assays.
1 Slowly fill one of the wells of an electrode cleaner with 350 µl RNaseZAP.
2 Open the lid and place electrode cleaner in the Agilent 2100 bioanalyzer.
3 Close the lid and leave it closed for about 1 minute.
4 Open the lid and remove the electrode cleaner. Label the electrode cleaner and keep it for future use. You can reuse the electrode cleaner for all 25 chips in the kit.
5 Slowly fill one of the wells of another electrode cleaner with 350 µl RNase-free water.**TE**
6 Place electrode cleaner in the Agilent 2100 bioanalyzer.
7 Close the lid and leave it closed for about 10 seconds.
8 Open the lid and remove the electrode cleaner. Label it and keep it for further use.
9 Wait another 10 seconds for the water on the electrodes to evaporate before closing the lid.

Preparing the Gel

1 Allow all reagents to equilibrate to room temperature for 30 minutes before use.
2 Place 550 µl of Agilent RNA 6000 Nano gel matrix (red !) into the top receptacle of a spin filter.
3 Place the spin filter in a microcentrifuge and spin for 10 minutes at 1500 g ± 20 %
4 Aliquot 65 µl filtered gel into 0.5 ml RNase-free microfuge tubes that are included in the kit. Store the aliquots at 4 °C and use them within one month of preparation.

Preparing the Gel-Dye Mix
1 Allow all reagents to equilibrate to room temperature for 30 minutes before use. Protect the dye concentrate from light while bringing it to room temperature.
2 Vortex RNA 6000 Nano dye concentrate (blue !) for 10 seconds and spin down.
3 Add 1 µl of RNA 6000 Nano dye concentrate (blue !) to a 65 µl aliquot of filtered gel.
4 Cap the tube, vortex thoroughly and visually inspect proper mixing of gel and dye. Store the dye concentrate at 4 °C in the dark again.
5 Spin tube for 10 minutes at room temperature at 13000 g (for Eppendorf microcentrifuge, this corresponds to 14000 rpm). Use prepared gel-dye mix within one day.
Gel-dye mix filtered gel
A larger volume of gel-dye mix can be prepared in multiples of the 65+1 ratio, if more than one chip will be used within one day. Always re-spin the gel-dye mix at 13000 g for 10 minutes before each use.

Loading the Gel-Dye Mix
Before loading the gel-dye mix, make sure the base of the plate of the chip priming station is in

position ‘C’ and the adjustable clip set to top position.

1 Allow the gel-dye mix to equilibrate to room temperature for 30 minutes before use and protect the gel-dye mix from light during this time.
2 Take a new RNA Nano chip out of its sealed bag .
3 Place the chip on the chip priming station.
4 Pipette 9.0 µl of the gel-dye mix at the bottom of the well marked and dispense the gel-dye mix.
5 Set the timer to 30 seconds, make sure that the plunger is positioned at 1 ml and then close the chip priming station. The lock of the latch will click when the Priming Station is closed correctly.
6 Press the plunger of the syringe down until it is held by the clip.
7 Wait for exactly 30 seconds and then release the plunger with the clip release mechanism.
8 Visually inspect that the plunger moves back at least to the 0.3 ml mark.
9 Wait for 5 seconds, then slowly pull back the plunger to the 1 ml position.
10 Open the chip priming station.
11 Pipette 9.0 µl of the gel-dye mix in each of the wells marked.

Loading the RNA 6000 Nano Marker
1 Pipette 5 µl of the RNA 6000 Nano marker (green !) into the well marked with the ladder symbol and each of the 12 sample wells.

- **N** Please discard the remaining vial with gel-dye mix.

NOTE Do not leave any wells empty or the chip will not run properly. Unused wells must be filled with 5 µl of the RNA 6000 Nano marker (green !) plus 1 µl of the buffer in which the samples are diluted.

Loading the Ladder and Samples
1 Before use, thaw ladder aliquots and keep them on ice (avoid extensive warming upon thawing process)
2 To minimize secondary structure, heat denature (70 °C, 2 minutes) the samples before loading on the chip.
3 Pipette 1 µl of the RNA ladder into the well marked with the ladder symbol .
4 Pipette 1 µl of each sample into each of the 12 sample wells.
5 Set the timer to 60 seconds.
6 Place the chip horizontally in the adapter of the IKA vortex mixer and make sure not to damage the buldge that fixes the chip during vortexing.
If there is liquid spill at the top of the chip, carefully remove it with a tissue.
7 Vortex for 60 seconds at 2000 rpm.
8 Refer to the next topic on how to insert the chip in the Agilent 2100 bioanalyzer. Make sure that the run is started within 5 minutes.

Inserting a Chip in the Agilent 2100 Bioanalyzer
1 Open the lid of the Agilent 2100 bioanalyzer.
2 Check that the electrode cartridge is inserted properly and the chip selector is in position (1).
3 Place the chip carefully into the receptacle. The chip fits only one way.
4 Carefully close the lid. The electrodes in the cartridge fit into the wells of the chip.
5 The 2100 expert software screen shows that you have inserted a chip and closed the lid by displaying the chip icon at the top left of the *Instrument* context.
NOTE
Sensitive electrodes and liquid spills
Forced closing of the lid may damage the electrodes and dropping the lid may cause liquid spills resulting in bad results.
Do not use force to close the lid and do not drop the lid onto the inserted chip.

Starting the Chip Run

1 In the *Instrument* context, select the appropriate assay from the *Assay* menu.
2 Accept the current *File Prefix* or modify it. Data will be saved automatically to a file with a name using the prefix you have just entered. At this time you can also customize the file storage location and the number of samples that will be analyzed.
3 Click the *Start* button in the upper right of the window to start the chip run. The incoming raw signals are displayed in the *Instrument* context.
4 To enter sample information like sample names and comments, select the *Data File* link that is highlighted in blue or go to the *Assay* context and select the *Chip Summary* tab. Complete the sample name table.
5 To review the raw signal trace, return to the *Instrument* context.
6 After the chip run is finished, remove the chip from the receptacle of the bioanalyzer and dispose it according to good laboratory practices.
CAUTION
Contamination of electrodes
Leaving the chip for a period longer than 1 hour (e.g. over night) in the bioanalyzer may cause contamination of the electrodes. Immediately remove the chip after a run.

Cleaning up after a RNA 6000 Nano Chip Run
1 Slowly fill one of the wells of the electrode cleaner with 350 µl RNase-free water.
2 Open the lid and place the electrode cleaner in the Agilent 2100 bioanalyzer.
3 Close the lid and leave it closed for about 10 seconds.
4 Open the lid and remove the electrode cleaner.
5 Wait another 10 seconds to allow the water on the electrodes to evaporate before closing the lid.
NOTE Use a new electrode cleaner with each new kit.
CAUTION

Prepared by	Date Adopted	Supersedes Procedure #
Erika Foster	8/1/06	

Review Date	Revision Date	Signature

APPENDIX 2. LMP Cleanup and Quantification of Biotin-Labeled cRNA

BACKGROUND:

PRINCIPLE:

SPECIMEN: double-stranded cDNA products after first and second strand cDNA synthesis

LOCATION:

EQUIPMENT AND SUPPLIES:

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water
80% (v/v) Ethanol in water
RNase free water (VWR, Cat#EM-9610, 1-800-932-5000)

Preparation:
RNA isolation should be performed before using this kit.
Use only nuclease-free water, buffers, and pipette tips.

Performance Parameters:
BEFORE STARTING, please note:
■ *cDNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 24 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
■ *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*
■ *If cDNA synthesis was performed in a reaction tube smaller than 1.5 mL, transfer the reaction mixture into a 1.5 or 2 mL microfuge tube (not supplied) prior to addition of cDNA Binding Buffer.*

Storage Requirements:
The cDNA Cleanup Columns and IVT cRNA Cleanup Columns should be stored at 2–8ºC upon arrival. The whole kit can be stored at 2–8ºC, but in this case the buffers should be redissolved before use. Make sure that all buffers are at room temperature when used.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette.

PROCEDURE - STEPWISE:
BEFORE STARTING *please note:*
■ *It is essential to remove unincorporated NTPs, so that the concentration and purity of cRNA can be accurately determined by 260 nm absorbance.*
■ *DO NOT extract biotin-labeled RNA with phenol-chloroform. The biotin will cause some of the RNA to partition into the organic phase. This will result in low yields.*
■ *Save an aliquot of the unpurified IVT product for analysis by gel electrophoresis.*
■ *IVT cRNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 20 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
■ *IVT cRNA Binding Buffer may form a precipitate upon storage. If necessary, redissolve by warming in a water bath at 30°C, and then place the buffer at room temperature.*
■ *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*
1. Add 60 µL of **RNase-free Water** to the IVT reaction and mix by vortexing for 3 seconds.
2. Add 350 µL **IVT cRNA Binding Buffer** to the sample and mix by vortexing for 3 seconds.
3. Add 250 µL ethanol (96-100%) to the lysate, and mix well by pipetting. Do not centrifuge.
4. Apply sample (700 µL) to the IVT **cRNA Cleanup Spin Column** sitting in a **2 mL Collection Tube**. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through and Collection Tube.
5. Transfer the spin column into a new **2 mL Collection Tube** (supplied). Pipet 500 µL **IVT cRNA Wash Buffer** onto the spin column. Centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm) to wash. Discard flow-through
IVT cRNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to the IVT cRNA Wash Buffer before use
6. Pipet 500 µL 80% (v/v) ethanol onto the spin column and centrifuge for 15 seconds at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.
7. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.
- Place columns into the centrifuge using every second bucket.
- Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps.

Label the collection tubes with the sample name. During centrifugation some column caps may break, resulting in loss of sample information.
- Centrifugation with open caps allows complete drying of the membrane.

8. Transfer spin column into a new **1.5 mL Collection Tube** (supplied), and pipet 11 µL of **RNase-free Water** directly onto the spin column membrane.

- Ensure that the water is dispensed directly onto the membrane.
- Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.

9. Pipet 10 µL of **RNase-free Water** directly onto the spin column membrane.

- Ensure that the water is dispensed directly onto the membrane. Centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute.
- For subsequent photometric quantification of the purified cRNA, we recommend dilution of the eluate between 1:100 fold and 1:200 fold.

10. Store cRNA at -20°C, or -70°C if not quantitated immediately. Alternatively, proceed to *Step 2: Quantification of the cRNA*.

Step 2: Quantification of the cRNA

Use spectrophotometric analysis to determine the cRNA yield. Apply the convention that 1 absorbance unit at 260 nm equals 40 µg/mL RNA.

■ Check the absorbance at 260 nm and 280 nm to determine sample concentration and purity.

■ Maintain the A260/A280 ratio close to 2.0 for pure RNA (ratios between 1.9 and 2.1 are acceptable).

- For quantification of cRNA when using total RNA as starting material, an adjusted cRNA yield must be calculated to reflect carryover of unlabeled total RNA. Using an estimate of 100% carryover, use the formula below to determine adjusted cRNA yield:

  Adjusted cRNA yield = RNAm - (total RNAi) (y) cRNA measured after IVT (µg) total RNAi = starting amount of total RNA (µg) y = fraction of cDNA reaction used in IVT

- **Example:** Starting with 10 µg total RNA, 50% of the cDNA reaction is added to the IVT, giving a yield of 50 µg cRNA. Therefore, adjusted cRNA yield = 50 µg cRNA - (10 µg total RNA) (0.5 cDNA reaction) = 45.0 µg. Use adjusted yield in *Fragmenting the cRNA for Target Preparation*

Step 3: Checking Unfragmented Samples by Gel Electrophoresis

Gel electrophoresis of the IVT product is done to estimate the yield and size distribution of labeled transcripts. cRNA products will be examined on an Agilent 2100 Bioanalyzer.

Prepared by	Date Adopted	Supersedes Procedure #
Erika Foster	8/1/06	

Review Date	Revision Date	Signature

APPENDIX 3. LMP Cleanup of Double-Stranded cDNA

BACKGROUND:

PRINCIPLE:

SPECIMEN: cDNA products after first and second strand cDNA synthesis

LOCATION:

EQUIPMENT AND SUPPLIES:

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water

Preparation:
RNA isolation should be performed before using this kit.

Performance Parameters:

BEFORE STARTING, please note:
■ *cDNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 24 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
■ *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*
■ *If cDNA synthesis was performed in a reaction tube smaller than 1.5 mL, transfer the reaction mixture into a 1.5 or 2 mL microfuge tube (not supplied) prior to addition of cDNA Binding Buffer.*

Storage Requirements:
The cDNA Cleanup Columns and IVT cRNA Cleanup Columns should be stored at 2–8ºC upon arrival. The whole kit can be stored at 2–8ºC, but in this case the buffers should be redissolved before use. Make sure that all buffers are at room temperature when used.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette.

PROCEDURE - STEPWISE:

1. Add 600 µL of **cDNA Binding Buffer** to the double-stranded cDNA synthesis preparation. Mix by vortexing for 3 seconds.

2. Check that the color of the mixture is yellow (similar to cDNA Binding Buffer without the cDNA synthesis reaction).
If the color of the mixture is orange or violet, add 10 µL of 3M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

3. Apply 500 µL of the sample to the **cDNA Cleanup Spin Column** sitting in a **2 mL Collection Tube** (supplied), and centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

4. Reload the spin column with the remaining mixture and centrifuge as above. Discard flow-through and Collection Tube.

5. Transfer spin column into a new **2 mL Collection Tube** (supplied). Pipet 750 µL of the **cDNA Wash Buffer** onto the spin column. Centrifuge for 1 minute at ≥ 8,000 x g (≥ 10,000 rpm). Discard flow-through.

cDNA Wash Buffer is supplied as a concentrate. Ensure that ethanol is added to the cDNA Wash Buffer before use

6. Open the cap of the spin column and centrifuge for 5 minutes at maximum speed (≤ 25,000 x g). Discard flow-through and Collection Tube.
Label the collection tubes with the sample name. During centrifugation some column caps may break, resulting in loss of sample information.

- Place columns into the centrifuge using every second bucket. Position caps over the adjoining bucket so that they are oriented in the opposite direction to the rotation (i.e., if the microcentrifuge rotates in a clockwise direction, orient the caps in a counterclockwise direction). This avoids damage of the caps.
- Centrifugation with open caps allows complete drying of the membrane.

7. Transfer spin column into a 1.5 mL Collection Tube, and pipet 14 µL of **cDNA Elution Buffer** directly onto the spin column membrane. Incubate for 1 minute at room temperature and centrifuge 1 minute at maximum speed (≤ 25,000 x g) to elute. Ensure that the cDNA Elution Buffer is dispensed directly onto the membrane. The average volume of eluate is 12 µL from 14 µL Elution Buffer.

8. After cleanup, please proceed to *Synthesis of Biotin-Labeled cRNA*

Prepared by	Date Adopted	Supersedes Procedure #
Erika Foster	8/1/06	

Review Date	Revision Date	Signature

APPENDIX 4. IGSP First Strand cDNA Synthesis Protocol

BACKGROUND:

PRINCIPLE:

SPECIMEN: 10 μg of total RNA

LOCATION: This protocol must be performed in the RNA Extraction Room.

EQUIPMENT AND SUPPLIES:

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000μL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000μL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000μL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200μL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)

Reagents:
One-cycle cDNA Synthesis Kit (Affymetrix Cat#900431, 1-888-362-2447)
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)

Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water

Preparation:
RNA isolation should be performed before using this kit

Performance Parameters:
Performance of the kit has been shown to be unaffected for up to twenty freeze-thaw cycles

Storage Requirements:
This kit should be kept at -20°C in a non-frost-free freezer

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette.

PROCEDURE - STEPWISE:

One-Cycle cDNA Synthesis Kit is used for this step.

1. Briefly spin down all tubes in the Kit before using the reagents.
2. Perform all of the incubations in thermal cyclers. The following program can be used as a reference to perform the first-strand cDNA synthesis reaction in a thermal cycler; the 4°C holds are for reagent addition steps:
70°C 10 minutes
4°C hold
42°C 2 minutes
42°C 1 hour
4°C hold

1. Mix RNA sample, diluted poly-A RNA controls, and T7-Oligo(dT) Primer.

a. Place total RNA (10 µg) in a 0.2 mL PCR tube.
b. Add 2 µL of the appropriately diluted poly-A RNA controls (See *Step 1: Preparation of Poly-A RNA Controls for One-Cycle cDNA Synthesis (Spike-in Controls)* on page 2.1.13 of Affymetrix Eukaryotic Sample and Array Processing Manual).
c. Add 2 µL of 50 µM T7-Oligo(dT) Primer.
d. Add RNase-free Water to a final volume of 11 or 12 µL (see Table 2.1.3 and Table 2.1.4).
e. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.
f. Incubate the reaction for 10 minutes at 70°C.
g. Cool the sample at 4°C for at least 2 minutes.
h. Centrifuge the tube briefly (~5 seconds) to collect the sample at the bottom of the tube.

2. In a separate tube, assemble the First-Strand Master Mix.

a. Prepare sufficient First-Strand Master Mix for all of the RNA samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.5, is for a single reaction.
b. Mix well by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the master mix at the bottom of the tube.

3. Transfer 7 µL of First-Strand Master Mix to each RNA/T7-Oligo(dT) Primer mix for a final volume of 18 µL. Mix thoroughly by flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube, and immediately place the tubes at 42°C.

4. Incubate for 2 minutes at 42°C.

5. Add 2uL of **SuperScript II** to each RNA sample for a final volume of 20 µL.

6. Incubate for 1 hour at 42°C; then cool the sample for at least 2 minutes at 4°C.
Cooling the samples at 4°C is required before proceeding to the next step. Adding the Second-Strand Master Mix directly to solutions that are at 42°C will compromise enzyme activity.

After incubation at 4°C, centrifuge the tube briefly (~5 seconds) to collect the reaction at the bottom of the tube and immediately proceed to *Second-Strand cDNA Synthesis*.

Table 2.1.3
RNA/T7-Oligo(dT) Primer Mix Preparation for 1 to 8 µg of total RNA, or 0.2 to 1 µg of mRNA
Component Volume
Sample RNA variable
Diluted poly-A RNA controls 2 µL
T7-Oligo(dT) Primer, 50 µM 2 µL
RNase-free Water variable
Total Volume 12 µL

Table 2.1.4
RNA/T7-Oligo(dT) Primer Mix Preparation for 8.1 to 15 µg of total RNA, or > 1 µg of mRNA
Component Volume
Sample RNA variable
Diluted poly-A RNA controls 2 µL
T7-Oligo(dT) Primer, 50 µM 2 µL
RNase-free Water variable
Total Volume 11 µL

Table 2.1.5
Preparation of First-Strand Master Mix
Component Volume
5X 1st Strand Reaction Mix 4 µL
DTT, 0.1M 2 µL
dNTP, 10 mM 1 µL
Total Volume 7 µL

Prepared by	**Date Adopted**	**Supersedes Procedure #**
Erika Foster	8/1/06	

Review Date	**Revision Date**	**Signature**

APPENDIX 5. LMP Fragmenting the cRNA for Target Preparation

BACKGROUND:

PRINCIPLE:

SPECIMEN:

LOCATION:

EQUIPMENT AND SUPPLIES:

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
96-100% (v/v) Ethanol in water
80% (v/v) Ethanol in water
RNase free water (VWR, Cat#EM-9610, 1-800-932-5000)

Preparation:
RNA isolation should be performed before using this kit.
Use only nuclease-free water, buffers, and pipette tips.

Performance Parameters:
Fragmentation of cRNA target before hybridization onto GeneChip probe arrays has been shown to be critical in obtaining optimal assay sensitivity. Affymetrix recommends that the cRNA used in the fragmentation procedure be sufficiently concentrated to maintain a small volume during the procedure. This will minimize the amount of magnesium in the final hybridization cocktail. Fragment an appropriate amount of cRNA for hybridization cocktail preparation and gel analysis.

Storage Requirements:
The cDNA Cleanup Columns and IVT cRNA Cleanup Columns should be stored at 2–8ºC upon arrival. The whole kit can be stored at 2–8ºC, but in this case the buffers should be redissolved before use. Make sure that all buffers are at room temperature when used.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette.

PROCEDURE - STEPWISE:

1. The Fragmentation Buffer has been optimized to break down full-length cRNA to 35 to 200 base fragments by metal-induced hydrolysis.

- The following table shows suggested fragmentation reaction mix for cRNA samples at a final concentration of 0.5 µg/µL. Use **adjusted** cRNA concentration, as described in *Step 2: Quantification of the cRNA*. The total volume of the reaction may be scaled up or down dependent on the amount of cRNA to be fragmented.

Table 2.1.18	
Sample Fragmentation Reaction by Array Format*	
Component 49/64 Format 100 Format	
CRNA 20 µg (1 to 21 µL) 15 µg (1 to 21 µL)	
5X Fragmentation Buffer 8 µL 6 µL	
RNase-free Water (variable) to 40 µL final volume to 30 µL final volume	
Total Volume 40 µL 30 µL	

*Please refer to specific probe array package insert for information on array format.

2. Incubate at 94°C for 35 minutes. Put on ice following the incubation.
3. Save an aliquot for analysis on the Bioanalyzer.

- The standard fragmentation procedure should produce a distribution of RNA fragment sizes from approximately 35 to 200 bases.

4. Store undiluted, fragmented sample cRNA at -20°C (or -70°C for longer-term storage) until ready to perform the hybridization, as described in the *Eukaryotic Target Hybridization*.

Prepared by	Date Adopted	Supersedes Procedure #
Erika Foster	8/1/06	

Review Date	Revision Date	Signature

APPENDIX 6. LMP NanoDrop: Determining RNA Concentration

BACKGROUND:

The NanoDrop® ND-1000 is a full-spectrum (220-750) spectrophotometer that measures small volume samples with high accuracy and reproducibility. It has the capability to measure highly concentrated samples without dilution. The NanoDrop® ND-1000 is ideally suited for measuring concentration and quality measurements without dilution (DNA, RNA, oligos, PCR product, and hybridization probe).

PRINCIPLE:

A 1 µl sample is pipetted onto the end of a fiber optic cable (the receiving fiber). A second fiber optic cable (the source fiber) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fiber optic ends. The gap is controlled to both 1mm and 0.2mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array is used to analyze the light after passing through the sample. The instrument is controlled by special software run from a PC, and the data is logged in an archive file on the PC.

SPECIMEN:

Although sample size is not critical, it is essential that the liquid column be formed so that the gap between the upper and lower measurement pedestals is bridged with sample. Aqueous solutions of nucleic acids require a volume of 1 µl. A precision pipettor (0-2 µl) (VWR cat#40000-264) with precision tips (Genesee Sci Cat#24-401) to assure that sufficient sample (1-2 µl) is used. If you are unsure about your sample characteristics or pipettor accuracy, a 2µl sample is recommended.

LOCATION: This protocol must be performed in the RNA Extraction Room.

EQUIPMENT AND SUPPLIES:

Equipment:
NanoDrop® ND-1000 (NanoDrop Technologies, Inc., phone# +1-302-479-7707)

Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
Tissue wipes (VWR cat#82003-820, 1-800-932-5000)
Sterile, RNAse-free pipette tips (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)

Reagents:
5.25% solution of sodium hypochlorite (VWR cat#37001-060)
(bleach-freshly prepared) can be used to ensure that no biologically active material is present on the measurement pedestals.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette. Following RNA purification, the concentration and A_{260}/A_{280} ratio is determined. The A_{260}/A_{280} ratio should be in the range of 1.6-1.9.

PROCEDURE - STEPWISE:

1. Ideally, the concentration of an RNA sample should be determined immediately after purification. However, if the RNA has been stored frozen, thaw it on ice
2. Quick-spin the RNA samples, 20 sec 4000 RPM, microcentrifuge
3. With the sampling arm open, pipette 1 uL of the sample onto the lower measurement pedestal
4. Close the sampling arm and initiate a spectral measurement using the operating software on the PC (see Operating Software below). The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement is made
5. When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. This will prevent carryover. Wipe the pedestals with a dilute bleach solution if biologically active materials are being measured.

Operating Software
- **Module Startup**: When the software starts, a message should appear "Ensure sample pedestals are clean and then load a water sample. After loading water sample, click OK to initialize instrument"
- **Measure (F1)** Each time a software module is opened (initiated), the Measure' button is inactive. A blank must first be measured
- **Blank (F3)** Before making a sample measurement, a blank must be measured and stored. After this measurement, a straight line will appear on the screen

Blanking Cycle
1. Load a blank sample (the buffer, solvent, or carrier liquid used with your samples) onto the lower measurement pedestal and lower the sampling arm into the 'down' position
2. Click on the 'Blank' F3 button
3. Wipe the blanking buffer from both pedestals using a laboratory wipe
4. Analyze an aliquot of the blanking solution as though it were a sample. Do this by using the 'Measure' button (F1). The result should be a spectrum with a relatively flat baseline. Wipe the blank from both measurement pedestal surfaces and repeat the process until the spectrum is flat

- With the sampling arm in the down position, start the Nanodrop software by selecting the following path:

Start → Programs → Nanodrop → ND-1000 3.3.0

- Choose the application module 'Nucleic Acid'
- Primary data storage of archive files is stored at c:\nanodropdata. Also, choose the 'auto reporting' option in the User Preferences window, this will allow data to automatically be saved to the report for all samples.
- Use the **ng/µL** screen feature to check RNA concentrations and quality – follow the stepwise prompts.

Start Report/Recording (F6)

- To log measurement results in a report table and print them:
  - Select 'Start Report' button
  - When the maximum number of entries for that specific report has been reached, there are 2 options:
    - Print Report & Clear
    - Save Report & Clear

Prepared by	Date Adopted	Supersedes Procedure #
Erika Foster	8/1/06	

Review Date	Revision Date	Signature

APPENDIX 7. LMP Qiagen RNeasy Plus RNA Isolation

BACKGROUND:

Qiagen RNeasy plus kits provide a fast and simple method for the preparation of total RNA from human fresh/frozen tissue. All contaminants and enzyme inhibitors are removed, making the RNA suitable for use in all downstream applications such as: RT-PCR; Northern, dot, and slot blotting; RNase/S1 nuclease protection; differential display; Poly A^+RNA selection; Primer extension; cDNA synthesis; and microarray analysis.

PRINCIPLE:

Fresh frozen tissue is mechanically disrupted under highly denaturing conditions, which immediately inactivate RNAses to allow the isolation of intact RNA. After mechanical disruption, the tissue is homogenized by a brief spin through a QIAshredder spin column. Ethanol is added to adjust the binding conditions and the sample is applied to a QIAamp spin column. Total RNA is bound to the silica-gel membrane during a brief spin in a microcentrifuge. Contaminants are washed away and total RNA is eluted in 30µL of RNAse-free water for direct use in any downstream application.

SPECIMEN: 50-100 mg of fresh frozen tissue. Tissue must be frozen with 45 minutes of surgical resection. Delays in freezing will result in inferior RNA quality (see LMP Tissue Procurement protocol)

LOCATION: This protocol must be performed in the RNA Extraction Room.

EQUIPMENT AND SUPPLIES:

Equipment:

Mini-Beadbeater (Catalog#3110BX (Instructions for the Mini-Beadbeater 3110BX (Biospec Products, Inc. 800-617-3363))
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:

Microvials, 2mL with caps (Cat# 10831 BioSpec Products Inc.)
Zirconia/silica beads, 2.5mm (Cat# 11079125z BioSpec Products Inc.)
QiaShredder (Cat# 79654 Qiagen, 800-426-8157)
Sterile 15mL polypropylene tube
Sterile, RNAse-free pipette tips (20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550)
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)

Reagents:

RNeasy Plus Mini Kit (Cat#74134 Qiagen, 800-426-8157)
2-Mercaptoethanol (B-ME) (Cat# 21985-023 Gibco)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)

70% Ethanol in water

Preparation:

Prior to initial use of kit:
RPE Buffer: add 4 volumes of 100% ethanol to obtain a working solution.

For Daily Use:
RLT buffer: add 1µL of β-mercaptoethanol to 100µL of RLT buffer (adjust to needed volume using 1:100 ratio).

Performance Parameters:
Buffer RLT is stable for 1 month after addition of β-ME (at room temperature).

Storage Requirements:
Tissue lysates (in Buffer RLT) can be stored at -70^{o}C. To process frozen lysates, thaw and incubate for 10 minutes at 37^{o}C to ensure all salts have dissolved.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette. Following RNA purification, the concentration and A_{260}/A_{280} ratio is determined (using nanodrop spectrophotometer). The A_{260}/A_{280} ratio should be in the range of 1.6-1.9.

PROCEDURE - STEPWISE:

I. Disruption of frozen tissue

- Fill the 2 mL screw-cap vial at least ½ full (½ - ¾ is okay) with beads and the rest of the volume with buffer and biomaterial (lung tissue core).
    - For an approximately 50mg tissue sample, need to use 1.2 mL Buffer RLTPlus* (Qiagen kit)
    - SO: add 600uL beads, tissue sample, and 1.2 mL Buffer RLTPlus** to the top.

* Ensure that β-ME is added to Buffer RLTplus before use (β-mercaptoethanol must be added to Buffer RLT). Add 10 uL of β-ME per 1 mL of Buffer RLT. The solution is stable for 1 month after the addition of β-ME.

** The vial should be filled to the top to exclude as much air as possible when the vial cap is screwed on. Dry beads can entrap air, you may have to invert the vial several times to wet the beads, then top-up the vial with more buffer RLTplus

- Insert the vial securely in the arm assembly of the Mini-Beadbeater
- Close the black safety cover
- Operate the BeadBeater for a total of three minutes by selecting a speed (the rabbit) of 4800 rpm (48 on display)
- Set a time (the clock) of 180 seconds (18 on display)

- At the end of the 3-minute run, the temperature of the homogenate will be about 25 degrees C
- Most of the homogenate can be recovered by decanting after the beads settle to the bottom of the vial
- Place homogenized sample on ice and proceed to next tissue sample.
- When all tissue samples are homogenized, proceed to RNeasy Mini Protocol

II. RNeasy Plus Mini Protocol for Isolation of Total RNA from Animal Tissue

Important points before starting

- β-mercaptoethanol (β-ME) must be added to Buffer RLT Plus before use. See *note above.
- Buffer RPE is supplied as a concentrate. Before using the first time, add 4 volumes of ethanol as indicated on the bottle. Date the bottle.
- Perform all steps of the procedure at room temperature.
- During the procedure, work quickly.
- All centrifugation steps are performed at 20-25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.
- ***(**flow-through contains buffer RLT or Buffer RW1 and is therefore not compatible with bleach.)

1. Homogenize the sample using QiaShredders.

- Each sample gets split between 2 QiaShredders and are subsequently processed separately.
- Pipette 600uL lysate directly onto QiaShredder column placed in a 2mL collection tube (max volume 700uL).
- Centrifuge 2min, maximum speed

2. Transfer the homogenized lysate to a gDNA Eliminator spin column placed in a 2 mL collection tube

- Centrifuge for 30s at ≥8000 x g (≥ 10,000 rpm)
- Discard the column, and save the flow-through
- Make sure that no liquid remains on the column membrane after centrifugation.

3. Add 1 volume (600uL) of 70% ethanol to the flow-through, and mix well by pipetting. Do NOT centrifuge.

- 70% ETOH (3.5mL ETOH + 1.5mL RNase free ddH2O).

4. Transfer up to 700uL of the sample to an RNeasy mini column placed in a 2 ml collection tube (supplied).

- Close the lid gently, and centrifuge for 15 s at ≥8000 x g (11,000 rpm). Discard the flow through*.
- Reuse the collection tube in step 5. if the volume exceeds 700uL, load aliquots successively onto the RNeasy column and centrifuge as above. Discard the flow-through after each centrifugation step.

5. Add 700uL Buffer RW1 to the RNeasy column. Close the lid gently, and centrifuge for 15s at ≥8000 x g (11,000 rpm) to wash the column. Discard the flow-through. Reuse the collection tube in step 6.

6. Pipet 500uL Buffer RPE onto the RNeasy column. Close the lid gently, and centrifuge for 15s at ≥8000 x g (11,000 rpm) to wash the column.

- Discard the flow-through. Reuse the collection tube in step 7.

7. Add another 500uL buffer RPE to the RNeasy column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (11,000 rpm).

8. Place the RNeasy column in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1min. This centrifugation ensures that no ethanol is carried over during elution.

9. To elute, transfer the RNeasy column to a new 1.5 mL collection tube. Pipet 30 of RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and set on bench, at room temperature, for 10min.

10. Centrifuge for 1 min at ≥8000 x g (11,000 rpm) to elute.

Prepared by	Date Adopted	Supersedes Procedure #
Erika Foster	8/1/06	

Review Date	Revision Date	Signature

APPENDIX 8. IGSP Second Strand cDNA Synthesis Protocol

BACKGROUND:

PRINCIPLE:

SPECIMEN:

LOCATION:

EQUIPMENT AND SUPPLIES:

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)

Reagents:
One-cycle cDNA Synthesis Kit (Affymetrix Cat#900431, 1-888-362-2447)
Sample Cleanup Module (Affymetrix Cat#900371, 1-888-362-2447)
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)
70% Ethanol in water

Preparation:

Performance Parameters:
Performance of the kit has been shown to be unaffected for up to twenty freeze-thaw cycles

Storage Requirements:
This kit should be kept at -20°C in a non-frost-free freezer

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette.

PROCEDURE - STEPWISE:

Second-Strand cDNA Synthesis

The following program can be used as a reference to perform the second-strand cDNA synthesis reaction in a thermal cycler.
16°C 2 hours
4°C hold
16°C 5 minutes
4°C hold

One-Cycle cDNA Synthesis Kit is used for this step.

1. In a separate tube, assemble Second-Strand Master Mix.
It is recommended to prepare Second-Strand Master Mix immediately before use.

a. Prepare sufficient **Second-Strand Master Mix** for all of the samples. When there are more than 2 samples, it is prudent to include additional material to compensate for potential pipetting inaccuracy or solution lost during the process. The following recipe, in Table 2.1.6, is for a single reaction.
b. Mix well by gently flicking the tube a few times. Centrifuge briefly (~5 seconds) to collect the solution at the bottom of the tube.

2. Add 130 µL of **Second-Strand Master Mix** to each first-strand synthesis sample from *Step 2: First-Strand*
cDNA Synthesis for a total volume of 150 µL. Gently flick the tube a few times to mix, and then centrifuge briefly (~5 seconds) to collect the reaction at the bottom of the tube.

3. Incubate for 2 hours at 16°C.

4. Add 2 µL of **T4 DNA Polymerase** to each sample and incubate for 5 minutes at 16°C.

5. After incubation with **T4 DNA Polymerase** add 10 µL of **EDTA, 0.5M.** Can proceed to Cleanup procedure. Do not leave the reactions at 4°C for long periods of time.

Table 2.1.6. Preparation of Second-Strand Master Mix
RNase-free Water 91 µL
5X 2nd Strand Reaction Mix 30 µL
dNTP, 10 mM 3 µL
E. coli DNA ligase 1 µL
E. coli DNA Polymerase I 4 µL
RNase H 1 Ml
Total Volume 130 µL

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APPENDIX 9. LMP Synthesis of Biotin-Labeled cRNA

BACKGROUND:

The GeneChip® HT IVT Labeling Kit is optimized specifically for producing amplified and biotinylated targets to hybridize to eukaryotic GeneChip® brand arrays for expression analysis when using the GeneChip® Array Station. Each kit contains sufficient reagents for completing 96 in vitro transcription reactions when following the detailed procedures described in the GeneChip® Expression Analysis Technical Manual (For Cartridge Arrays Using the GeneChip Array Station).

PRINCIPLE:

The template DNA for this IVT reaction is typically double-stranded cDNA containing the T7 promoter sequence. In GeneChip target labeling experiments, the T7 promoter sequence is incorporated into the cDNA template by using a T7-Oligo(dT) Promoter Primer in the initial reverse transcription reaction. Using this HT IVT Labeling Kit with MEGAscript® reagents (IVT Labeling Enzyme Mix and T7 RNA Polymerase), biotinylated cRNA targets are generated in the presence of an optimized mixture of the four natural ribonucleotides and one biotin-conjugated nucleotide analog (IVT Labeling NTP Mix). The synthetic analog is efficiently incorporated into the cRNA target during the in vitro transcription labeling reaction mediated by T7 RNA polymerase as a pseudouridine reagent. The biotinylated cRNA targets are then purified, fragmented,
and hybridized to GeneChip expression arrays.

SPECIMEN: double-stranded cDNA products after first and second strand cDNA synthesis

LOCATION: This protocol must be performed in the RNA Extraction Room.

EQUIPMENT AND SUPPLIES:

Equipment:
MyCycler™Thermal Cycler (BioRad, Cat#170-9703 1-800-424-6723)
Microcentrifuge (Beckman Coulter Microfuge 18w/F241.5P, part# 367160, 1-800-742-2345)
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)

Supplies:
20/200/1000µL pipettes (VWR cat# 83009-726, 83009-732, 83009-736, 1-800-932-5000)
Sterile, RNAse-free pipette tips(20/200/1000µL) (Genesee Scientific, filter tips cat#24-404, 24-412, 24-430. Refills 20-200µL cat#24-150RL, 1000 24-165RL) 1-800-789-5550
Disposable gloves (Genesee Scientific, cat#37-102M, 1-800-789-5550)
0.2mL PCR tubes (2170-010, VWR cat# , 1-800-932-5000)
1.7mL microcentrifuge tubes (Genesee Scientific, cat#24-282, 1-800-789-5550)

Reagents:
GeneChip IVT Labeling Kit (Affymetrix Cat#900449, 1-888-362-2447)
Eukaryotic Poly-a RNA Control Kit (Affymetrix Cat#900433, 1-888-362-2447)
One-Cycle cDNA Synthesis Kit (Affymetrix Cat#900431, 1-888-362-2447)
Hybridization Controls (Affymetrix Cat#900454 and 900457, 1-888-362-2447)
Ethanol (100%) (VWR, Cat# DK2004L, 1-800-932-5000)

96-100% (v/v) Ethanol in water
RNase free water (VWR, Cat#EM-9610, 1-800-932-5000)

Preparation:
RNA isolation should be performed before using this kit.
This kit is only used for the IVT labeling step for generating biotin-labeled cRNA. For the IVT amplification step using unlabeled ribonucleotides in the First Cycle of the Two-Cycle cDNA Synthesis Procedure, a separate kit is recommended (MEGAscript® T7 Kit, Ambion, Inc.). Use only nuclease-free water, buffers, and pipette tips.

Performance Parameters:
The Target Hybridizations and Array Washing protocols have been optimized specifically for this IVT Labeling Protocol. Closely follow the recommendations described below for maximum array performance.
BEFORE STARTING, please note:
■ *cDNA Wash Buffer is supplied as a concentrate. Before using for the first time, add 24 mL of ethanol (96-100%), as indicated on the bottle, to obtain a working solution, and checkmark the box on the left-hand side of the bottle label to avoid confusion.*
■ *All steps of the protocol should be performed at room temperature. During the procedure, work without interruption.*
■ *If cDNA synthesis was performed in a reaction tube smaller than 1.5 mL, transfer thereaction mixture into a 1.5 or 2 mL microfuge tube (not supplied) prior to addition of cDNA Binding Buffer.*

Storage Requirements:
Store all reagents in a -20°C freezer that is not self-defrosting. Prior to use, centrifuge all reagents briefly to ensure that the solution is collected at the bottom of the tube.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. No aliquot is returned to the original container. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample. Transfer of sample should be done with a sterile transfer pipette.

PROCEDURE - STEPWISE:

1. Use the following table to determine the amount of cDNA used for each IVT reaction following the cDNA cleanup step.

Table 2.1.16
IVT Reaction Set Up
Starting Material Volume of cDNA to use in IVT
Total RNA
10 to 100 ng all (~12 µL)
1.0 to 8.0 µg all (~12 µL)
8.1 to 15 µg 6 µL
mRNA
0.2 to 0.5 µg all (~12 µL)
0.6 to 1.0 µg 9 µL

1 to 2.0 µg 6 µL

2. Transfer the needed amount of template cDNA to RNase-free microfuge tubes and add the following reaction components in the order indicated in the table below. If more than one IVT reaction is to be performed, a master mix can be prepared by multiplying the reagent volumes by the number of reactions. Do not assemble the reaction on ice, since spermidine in the **10X IVT Labeling Buffer** can lead to precipitation of the template cDNA.

Table 2.1.17
IVT Reaction
Reagent Volume
Template cDNA* variable (see Table 2.1.16)
RNase-free Water variable
(to give a final reaction volume of 40 µL)
10X IVT Labeling Buffer 4 µL
IVT Labeling NTP Mix 12 µL
IVT Labeling Enzyme Mix 4 µL
Total Volume 40 µL

*0.5 to 1 µg of the 3'-Labeling Control can be used in place of the template cDNA sample in this reaction as a positive control for the IVT components in the kit.

3. Carefully mix the reagents and collect the mixture at the bottom of the tube by brief (~5 seconds) microcentrifugation.
4. Incubate at 37°C for 16 hours. To prevent condensation that may result from water bath-style incubators, incubations are best performed in oven incubators for even temperature distribution, or in a thermal cycler.

Overnight IVT reaction time has been shown to maximize the labeled cRNA yield with high-quality array results. Alternatively, if a shorter incubation time (4 hours) is desired, 1 µL (200 units) of cloned T7 RNA polymerase (can be purchased directly from Ambion, P/N 2085) can be added to each reaction and has been shown to produce adequate labeled cRNA yield within 4 hours. The two different incubation protocols generate comparable array results, and users are encouraged to choose the procedure that best fits their experimental schedule and process flow.

5. Store labeled cRNA at -20°C, or -70°C if not purifying immediately. Alternatively, proceed to *Cleanup and Quantification of Biotin-Labeled cRNA*.

Prepared by	**Date Adopted**	**Supersedes Procedure #**
Erika Foster	8/1/06	

Review Date	**Revision Date**	**Signature**

APPENDIX 10. LMP Tissue Procurement Protocol (for RNA)

BACKGROUND:

The ability to obtain high quality RNA from fresh tissue is highly dependent on the tissue procurement protocol. Delays in tissue processing usually lead to RNA degradation. Inadequate freezing and shipping conditions can also be a major source of pre-analytic variance in downstream applications. In addition, estimating the amount of tissue needed to generate a sufficient quantity of RNA can be difficult. The following protocol addresses these issues.

PRINCIPLE:

Lung tumor tissue is harvested in the frozen section or gross dissection area of the surgical pathology suite. The tissue is first examined by a certified anatomic pathologist or surrogate (resident, pathology assistant). Relevant margins are inked, removed and examined by frozen section analysis, if necessary. It is imperative that harvesting tissue for use in the LMP does not impede accurate initial assessment of critical feature of the tumor resection, most notably margin status. A defined amount of tumor tissue is then harvested using a stainless steel dermal punch biopsy.

SPECIMEN: Lung resection specimen

LOCATION: Surgical Pathology Suite

EQUIPMENT AND SUPPLIES:

Equipment:

Scale (Ohaus Adventurer Scale 0.1 mg – 100gm AR1140 or comprable substitute)
Small liquid nitrogen storage container

Supplies:

5 mM dermal punch biopsy (Miltex, Ref 33-35)
Cryovials (Nalgene VWR cat # 66008-706)
Styrofoam shipping container
Scalpel blade
10 cm stainless steel probe

Reagents:

Liquid nitrogen or alternative freezing media
Dry ice

Preparation:

Performance Parameters:

Storage Requirements:
Tissue samples should be kept frozen on dry ice or at -80 C until shipped.

QUALITY CONTROL:

Lab guidelines for safe handling of all samples must be followed. All tubes used for specimen processing must be labeled with the unique MD# before transfer of aliquot or sample.

PROCEDURE - STEPWISE:

1. After the specimen has been processed for margin status, and the frozen section assessment of the specimen is complete, cut an approximately 1.0 x 1.0 x 0.5 cm block of firm grossly homogeneous lung tumor using a clean scalpel blade.
2. Using the dermal punch biopsy cut three core biopsies of equal size – each biopsy should be 0.5 cm long.
    a. Although three is the ideal number of biopsies, LMP testing can be performed on a single 0.5 cm long, 0.5 cm diameter core of tissue. Additional pieces are requested so that they can be processed in the event of sample exclusion secondary to failure to meet histologic criteria, poor RNA quality or insufficient RNA quantity. Clinical judgment is required to determine if three core biopsies of tissue can be spared from a particular resection specimen.
    b. The core biopsy may be difficult to extract from the dermal punch device. If so it can be pushed out from the back end using the 10 cm probe.
3. Designate the core biopsies as "A", "B", and "C"
4. Weigh each core biopsy, and record the weight on the sample submission form
5. For each core biopsy shave approximately 0.1 to 0.2 cm off of one end using a clean scapel blade.
    a. This piece will ultimately be used for frozen section evaluation.
6. Place both pieces for each core into an individual labeled cryovial.
7. Submerge the three cryovials into liquid nitrogen for 1 minute.
8. Transfer the cryovials from liquid nitrogen into the styrofoam shipping container, and cover with dry ice.
9. Store at -80C until shipped.

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