STANDARD OPERATING PROCEDURE (SOP) FOR NINE MARKER MICROSATELLITE INSTABILITY (MSI) ANALYSIS BY POLYMERASE CHAIN REACTION

I. SCOPE AND PURPOSE

Microsatellites are short, tandem repeat (STR) DNA sequences with repeating units from 1-6 base pairs in length. Microsatellites are distributed throughout the human genome, and individual repeat loci often vary in length from one individual to another. Microsatellite instability (MSI) is the change in length of a microsatellite allele due to either insertion or deletion of repeating units and a failure of the DNA mismatch repair (MMR) system to fix these replication errors. This genomic instability arises in a variety of human neoplasms where tumor cells have a decreased ability to faithfully replicate DNA. MSI is particularly associated with colorectal cancer where 15-20% of sporadic tumors show MSI, in contrast to the more common chromosomal instability (CIN) phenotype (seen in 65-70% of sporadic colorectal cancer tumors), with MSI status being an independent prognostic indicator. MSI analysis is also clinically useful in identifying patients at increased risk of hereditary nonpolyposis colorectal cancer (HNPCC)/Lynch Syndrome, where a germline mutation of a MMR gene causes a familial predisposition to colorectal cancer. MSI analysis alone is not sufficient to make a diagnosis of a germline MMR mutation given the high rate of sporadic MSI positive colorectal tumors, but a positive result is an indication for follow-up genetic testing and counseling.

DNA from tumor tissue and corresponding adjacent normal tissue or normal blood is subjected to multiplex PCR using fluorescently-labeled primers for co-amplification of nine markers, including 4 mononucleotide and 3 dinucleotide repeat markers for MSI determination and two pentanucleotide markers (Penta D and Penta E) for establishing sample identity. The resulting PCR fragments are separated using capillary electrophoresis. Allelic profiles of normal versus tumor tissue are compared by two independent reviewers, and MSI is scored as the presence of novel microsatellite lengths in tumor DNA compared to normal DNA.

II. PROCEDURE

A. Safety Procedure

1. Use universal safety precautions when handling when handling all body fluids, tissues, and cell cultures; wear personal protective equipment (PPE).

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B. Quality Control

1. STR analysis is subject to contamination by very small amounts of non-template human DNA. Extreme care should be taken to avoid cross-contamination when preparing sample DNA, handling primer pairs, setting up amplification reactions and analyzing amplification products.
2. Worksheets are used for cocktail component calculations. Lot numbers, concentrations and expiration dates of reagents used are recorded where applicable. Unusual observations in set up of assays are also noted on these worksheets. All control samples are tested at the same time, in an identical manner and by the same technologist as the CCG BCR samples included with each assay group.
   Aerosol barrier pipet tips are used for the set-up of all PCR reactions to prevent cross contamination.
3. PCR assays require separate areas for set-up and amplification. The DNA extraction is performed in the Pre-PCR room. PCR reactions are set-up in a separate room from the thermal cycler and post-PCR analysis area. No amplified products are allowed in the PCR set-up area.
4. Dedicated equipment (pipettes), filtered tips and other supplies are used in each area. Powder-free gloves are always used and goggles are used as needed.
5. No aliquot of original specimen, DNA or any other reagent should ever be returned to the original container after sampling.
6. All new lots of reagents are tested in parallel with the one in current use before being put into use; results are recorded in QC log. All kit components must be quality control tested and used together thereafter. All reagents supplied in a kit must be used only with other reagents in the same kit lot number; reagents with identical lot numbers cannot be used interchangeably between kit lot numbers.
7. The threshold values (MSI-H, MSI-L, MSS) of a known case will be verified every six months if a new lot of reagents has not been tested within that time period.
8. For all assays, specimens should be ordered and set up in the following sequence: participant samples, positive controls, negative controls. This is done to minimize the chance for cross contamination of patient samples while providing the greatest chance for detecting contamination in the negative control.
9. Analytical Controls: Two controls are used for the MSI Analysis System:
   a. Negative Control: Nuclease Free Water. This blank control should contain no peaks in the markers being analyzed.
   b. Positive Control: DNA from the control cell line HCT 116 (positive for MSI) is set up with each run to ensure reproducibility of the MSI Analysis System and to ensure that capillary electrophoresis and analysis software are functioning correctly.
10. Amplification from any loci in control or patient samples must have amplification height greater than 100 intensity units. If the intensity if less than 100 units, the sample will be repeated.

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11. The control results are verified for acceptability before the data are released.
    a. Reactions that do not have acceptable results from analytical controls must be repeated.
    b. Annotations will be added to the setup worksheet for assays with unacceptable analytical controls and a link to the repeat test. For example, if MSI-92 demonstrated amplification from the negative control, the assay would be repeated as MSI-92b. The MSI-92 assay worksheet would be annotated to convey the assay failed for amplification of negative control and repeated as MSI-92b.
12. Some common situations that may cause analytically inaccurate results are PCR reagent contamination, capillary malfunction, and/or decreased fluorescent intensity.
13. Primers are light sensitive. All reagents, reactions, and products containing labeled primers should be protected from light when possible.

C. Specimen information

DNA isolated from matched normal and tumor samples.

E. Required equipment, supplies, and reagents

1. Equipment

Capsule Centrifuge
PCR Hood
Pipettes – adjustable
Thermal Cycler
Vortex Mixer
ABI PRISM 3730XL Genetic Analyzer
Heat Block (95°C)

2. Supplies

Aerosol Barrier Pipet Tips
Personal Protective Equipment (PPE)
MicroAmp Optical 96-well reaction plate (AB, N801)
Plate Septa-96 well (AB, 4315933)
ABI 3730XL 48-Capillary Array (AB, 4331247)
Ice
Microcentrifuge tubes, 1.5 mL

3. Reagents

Nuclease-Free Water (Fisher, BP2484-50)
HCT 116 Genomic DNA (10 ng/µL) (ATCC, CCL-247)
LIZ Size Standard (Applied Biosystems, Cat# 4322682)

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Matrix Standards Dye Set G5 (Applied Biosystems, Cat# 4345833)
Amplitaq Gold DNA Polymerase (Applied Biosystems Cat# N808-0249)
Hi-Di Formamide (Applied Biosystems Cat# 4311320)
ABI 3730XL Pop-7 Polymer (Applied Biosystems Cat# 4332241)
dNTPs (Roche, Catalog# 11-969-064-001)
50 mM $MgCl_2$ (Invitrogen Life Technologies, catalog# 10966-034)
10X PCR Buffer (Invitrogen Life Technologies, catalog# 10966-034)
10X Genetic Analyzer Buffer (Applied Biosystems, catalog# 402824)
GeneScan 500 LIZ internal lane standard

F. Reagent preparation (including storage conditions)

1. Oligonucleotide Primers for Human Loci amplified by the MSI Analysis System:

Marker Name	GenBank Number	Major Repeat Sequence	Size Range (bp)	Primer Dye
BAT-40	M38180	$(A)_x$	85-140	PET
BAT-26	U41210	(A)26	90-135	NED
BAT-25	L04143	(A)25	99-130	6-FAM
TGFBRII	Unigen HS: 82028		60-80	VIC
D5S346	181171	CA repeat	85-135	VIC
D17S250	177030	CA repeat	130-185	6-FAM
D2S123	187953	CA repeat	175-250	NED
Penta D	AC000014	$(AAAAG)_{2-17}$	376-449	6-FAM
Penta E	AC027004	AAAGA	379-474	VIC

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Marker Name	Forward Primer Sequence	Reverse Primer Sequence
BAT-40	PET-ATT AAC TTC CTA CAC CAC AAC	GTAGAGCAAGACCACCTTG
BAT-26	NED-TGACTACTTTTGACTTCAGCC	AACCATTCAACATTTTTAACCC
BAT-25	6FAM - TCGCCTCCAAGAATGTAAGT	TCTGCATTTTAACTATGGCTC
TGFBRII	VIC-CTTTATTCTGGAAGATGCTGC	GAAGAAAGTCTCACCAGGC
D5S346	VIC-ACTCACTCTAGTGATAAATCGGG	AGC AGA TAA GAC AGT ATT ACT AGT T
D17S250	6FAM-GGAAGAATCAAATAGACAAT	GCTGGCCATATATATATTTAAACC
D2S123	NED-AAACAGGATGCCTGCCTTTA	GGACTTTCCACCTATGGGAC
Penta D	GAAGGTCGAAGCTGAAGTG	ATTAGAATTCTTTAATCTGGACACAAG
Penta E	ATTACCAACATGAAAGGGTACCAATA	TGGGTTATTAATTGAGAAAACTCCTTACAATTT

III. PROCEDURE-STEPWISE

A. Polymerase Chain Reaction Set-Up:

1. Obtain the appropriate worksheet for the NCH 9 Marker MSI Assay.
2. Clean work area.
3. Prepare enough Primer master mix (PMM) for the plates to be run plus an additional 15%. Place primer master mix on ice.

Primer (Forward, Reverse)	Stock Concentration	Final Concentration	Volume (1ml total)
BAT25	100 µM	0.4 µM	4 µL F + 4 µL R
D17S250	100 µM	2.0 µM	20 µL F + 20 µL R
TGFBR2	100 µM	0.2 µM	2 µL F + 2 µL R
D5S346	100 µM	0.5 µM	5 µL F+ 5 µL R
BAT26	100 µM	0.36 µM	3.6 µL F+ 3.6 µL R
Penta E	100 µM	1.0 µM	10 µL F + 10 µL R
Penta D	100 µM	1.0 µM	10 µL F + 10 µL R
BAT40	100 µM	0.75 µM	7.5 µL F + 7.5 µL R
D2S123	100 µM	0.4 µM	4 µL F + 4 µL R
			867.8 µL water

4. Label one 1.5 mL microfuge tube with the name of the MSI master mix (MMM) reaction to be set up, i.e. MSI xx.
5. Using the calculated volumes from the worksheet, add each component of the master mix to the MMM 1.5 mL microfuge tube. Mix gently and spin down briefly to collect liquid at the bottom of the tube.

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Component	Volume Per Sample
Nuclease-Free water	2.80 µL
10X PCR Buffer II	1.20 µL
25 mM $MgCl_2$	1.20 µL
2.5 mM dNTPs	1.40 µL
Primer Master Mix (PMM)	3.00 µL
Amplitaq Gold DNA polymerase	0.40 µL
Total reaction volume	**10.0 µL**

6. Add 10 µL of the MMM cocktail to each well of an appropriately labeled 96 well PCR Plate.
7. Prepare a dilution plate for the template DNA so that the final concentration is 10 ng/µL.
8. Add 2 µL of each diluted sample DNA (10 ng/µL) to the appropriate well containing the master mix cocktail, pipet up and down several times to mix.
9. Add 2 µL of the HCT 116 (10 ng/µL) control DNA to its appropriate tube.
10. Add 2 µL of nuclease free water to the negative/blank control tube.
11. Place the tubes in the MJ thermal cycler and run method "MSI":

95°C for 7 minutes

94°C for 60 seconds
58°C for 30 seconds — 2 cycles
72°C for 45 seconds

93°C for 45 seconds
54°C for 30 seconds — 41 cycles
72°C for 40 seconds

72°C for 5 minutes
4°C hold indefinitely

12. When the program is complete, remove the tubes from the thermal cycler. The PCR products can be stored at 4°C until ready for electrophoresis.
13. Remove an aliquot of Hi-Di formamide and vial of LIZ size standard from the –20°C freezer and thaw at room temperature. Combine 9 µL of formamide with 0.5 µL of LIZ size standard for each sample and control to be run in a 1.5 mL microfuge tube. Vortex briefly and spin down to collect liquid.
14. In a 96-well plate, pipet 9.5ul of formamide/LIZ mixture into separate wells of a 96 well plate.

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15. Prepare a dilution plate (1:5) of the PCR product by adding 1 µL PCR product to 4 µL Nuclease-Free water. Mix by pipetting, and then add 0.5 µL of the dilute PCR product to the appropriate wells of the plate prepared in the previous step (Step 14).
16. Place a 96-well plate septa on the plate, making sure it is seated properly on the wells. Centrifuge plate briefly.
17. Place the plate on the 95°C heat block to denature for 3 minutes. Immediately chill on ice for 3 minutes.
18. Prepare the plate assembly by placing the reaction plate into the plate base.
19. Snap the plate retainer onto the reaction plate and base. Verify that the holes of the plate retainer and the plate septum are aligned.

B. Create a Plate Record:

1. In the Data Collection director, click on Plate Manager. Click on "New" to define a new run. A dialog box for plate description will open.
2. Complete the dialog box by entering a name for the plate "date of run (MMDDYY) and MSI run number." A description of the plate is not necessary.
3. In the "Application" pull –down menu, select GeneMapper-DGMXVHL1.
4. In the [Plate Type] pull-down menu, select "96-well."
5. In the [Plate Sealing] pull-down menu, select "Septa."
6. Enter initials for the owner and operator.
7. Click OK and the Plate Editor opens.
8. In the Sample Name column of each row, enter the sample ID and in the Sample Type column, select the appropriate sample type from the pull down list.
9. In the Size Standard column, select "-250 LIZ" from the pull-down menu.
10. In the Panel column, select "None."
11. In the Analysis Method column, select "G5insstd."
12. In the Results Group column, select "Molecular Genetics_BCR" from the pull-down menu.
13. In the Instrument Protocol 1 column, select "G5" from the pull-down menu.
14. Click "OK" to save.

C. Running the Sample Plate:

1. In the Data Collection software directory, click on "Run Scheduler." This page displays all sample plates that have been created and run on the ABI 3730XL.
2. Select "Search All" to find the Plate Record that was just created. The plate can be found by typing in the name of the plate and clicking on Search, or by selecting Find All. All plates will be in order of date. Choose the plate you want to run, click "Add" and "Done".

3. In the top left corner, a small green arrow will become highlighted to indicate that the instrument is ready to begin the run. Click on the green arrow. A dialog box will open and ask if the run is ready. Click on "yes" and the run will begin.

E. Data Analysis

When the electrophoresis run is complete, the data must be analyzed with the GeneMapper ID software program.

ABI 3130XL Data Analysis:

1. Launch the GeneMapper program by clicking on the icon in the desktop. Log onto the program, using the supplied password.
2. Once the software is launched, it will open to an empty Project page. To add the sample data files collected, go to the "File" menu and select "Add samples to project". This opens a new window.
3. Locate the run folder then click on "Add to List" at the bottom of the window. This will move the run including all sample files to the right side of the screen for analysis in the Project window. Click "Add".
4. Next, the analysis parameters must be defined for the software to perform the correct analysis on the data files. The analysis parameters should be the same as what was defined in the Plate Manager when setting up the ABI run with the following changes: set the Analysis Method column to "Microsatellite Default," and the Panel column to "New Panel" under MSI Folder.
5. Click on the green "Analyze" button at the top of the page. The "Save Project" box will appear. Enter the project name as "MSI" followed by the date and initials. Click OK, and the samples will be analyzed using the parameters specified above. After analysis is complete, the "Status" column (first column on the left side of the project window) should change from showing a green arrow to being empty.
6. On the right side of the project table are several columns with letters as the header. These are PQV (Process Quality Values) values that flag problem samples. As the samples are analyzed, they are subjected to specific criteria defined within the PQV. If the sample passes a specific PQV, a green square will be visible in that column. If the sample data is questionable and should be reviewed by the technologist, a yellow triangle will be visible in that column. If a red octagon is visible in that column, that sample has failed. Consult the supervisor for direction on investigation of the specific PQV problem and solution.
7. All assay controls must be examined prior to interpretation of sample results. If the controls do not yield the correct results, the assay is not valid and the samples should not be interpreted. Consult the supervisor or director for further instruction.
   a. The following describes the analysis of each of the controls and the decisions necessary based upon the results of the capillary electrophoresis. All must be true before continuing to patient analysis:

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i. **Blank Control (Acceptable Negative):** Shows no peaks greater than 100 Fluorescent Units (FU) in the blue, green, red or black channels.
ii. **Blank Control (Unacceptable Positive):** Shows peaks greater than 100 Fluorescent Units (FU) in the blue, green, red or black channels. This result indicates possible contamination of all PCR amplification reactions. Prepare fresh master mix and repeat amplification of all samples and controls or consult with supervisor or director if unsure on how to proceed.
iii. **HCT 116 Positive Control (Acceptable Positive):** Allele peaks should be present for all loci and sizes should be plus or minus 1bp from values seen on previous runs.
iv. **HCT 116 Positive Control (Unacceptable Negative):** Allele peak(s) are not present (i.e. low amplification). This indicates there may be a dilution error, reagent problem or thermal cycling issue. If allele peaks are present but allele(s) are not within 1 bp of posted size, there may be a problem with the capillary electrophoresis or LIZ size standard.

8. For each patient case, select both the normal and tumor file and go to the "Analysis" menu. Select "Display Plots" or click the display plots icon. This will open a new window with the analyzed data displayed graphically. Ensure that Tumor and Normal pairs appear on the same page with Tumor always residing above Normal. Go to the "Plot Settings" menu in the upper left corner of the Samples Plot window and select "MSI" from the pull down menu. Markers in the blue channel will be displayed and can be analyzed first.
9. Adjust the scale of the X-Axis of each plot to exclude any low molecular weight background signal if present.
10. Adjust the scale of the Y-Axis of each plot to allow easy viewing of the associated peaks by positioning the cursor over the Y axis scale and right clicking the mouse. Select "Zoom To..." and enter the appropriate axis scale values.
11. Ensure Page Layout is set to “Landscape” before printing the chromatograms for all blue markers.
12. Repeat Steps 8-10 for remaining color channels.
13. Repeat Steps 7-11 for all patient cases. For the HCT116 control and blank you can select peaks and print with all colors (blue, green, and yellow) selected/displayed.
14. Attach the printed electropherograms to the worksheet. Proceed to the next section of the procedure.

F. Data Interpretation

Patient sample interpretations should be completed as follows.

1. Compare allelic profiles of the Penta D and Penta E markers for a given sample. Normal and tumor tissue for a patient should display the same profile. If they do not, a sample mix up may have occurred. Stop with interpretation of the sample and

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consult with supervisor or director for further action. Similarly, if both Penta D and E markers fail to amplify, stop with interpretation of the sample and consult with supervisor or director for further action. Cases with high MSI may also show MSI at Penta D and E. An alternative identity check (e.g. SNPs) may be necessary.

2. Compare allelic profiles for the 7 MSI markers (all but Penta D + E) between the normal and tumor tissue of a sample. Alleles present in the tumor sample that are not present in the corresponding normal tissue indicate MSI.
3. Consider LOH when comparing allelic profiles. LOH is defined by the presence of an Allelic Index (AI) that is <0.7 or >1.6. The calculation for AI is as follows.

$$AI = \frac{\text{(Ht Normal Allele 1 / Ht Normal Allele 2)}}{\text{(Ht Tumor Allele 1 / Ht Tumor Allele 2)}}$$

4. Use the following numeric code when recording the allelic profile for each MSI marker. This is considered Level 2 data. (Shift = new allele)
    a. 0= Not Interpretable
    b. 1= Shift, Uninformative for LOH
    c. 2= Shift, with LOH
    d. 3= Shift without LOH
    e. 4= No Shift, Uninformative for LOH
    f. 5= No Shift, with LOH
    g. 6= No Shift, without LOH.
5. Determine the overall MSI status of the case as follows. This is considered Level 3 data. MSI status is determined by the number of altered or additional alleles found in the tumor. A single marker found to be "not interpretable" can be tolerated in MSI cases if the marker does not influence the overall call for the case.

    a. MSI-H (high) = ≥40% (≥3/7 markers altered)
    b. MSI-L (low) = 0<40% (1-2 of 7 markers altered)
    c. MSS (stable) = 0% (0/7 markers altered)

6. For samples with low or no amplification, repeat assay with more DNA. Consult with supervisor or director if required action is unclear.
7. Complete the MSI Interpretation Worksheet, initial and date.
8. The MGL Technical Director must review all results.
9. Example of MSI positive and negative mononucleotide markers are in Figure 1 below:

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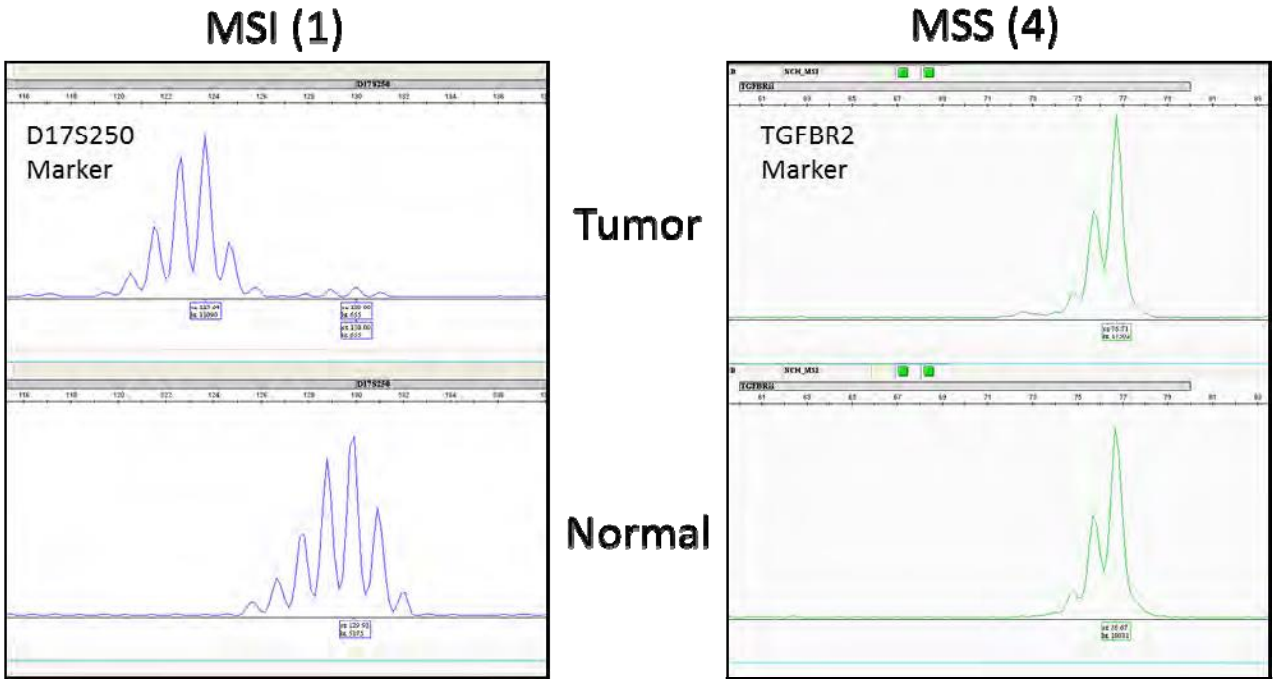

Figure 1. Representative MSI data from case TCGA-AX-A2HD (FSA files 2012-02-28018/018). Two markers are shown from an endometrial case tested from TCGA for MSI. The D17S250 marker on the left is an example of a MSI shift that is uninformative for LOH. The TGFBR2 marker on the right is an example of a marker that does not shift and is uninformative for LOH.

G. Reporting of Results

1. Upon completion of MSI testing for a designated Tumor Study, the Level 2 and 3 results will be summarized in a table.
2. This table will also provide a link to the raw FSA files that gave rise to the Level 2 and 3 data for each case. The FSA files are considered Level 1 data.
3. Prior to finalization of the data set, the table will be spot checked for data entry errors. Cases will be selected at random and the level 2 data confirmed against the original scored FSA printout. All Level 3 calls will be confirmed to have the appropriate number of altered or additional alleles.
4. Level 1 files for each case will be transferred to a folder designated to contain the finalized raw data set. This folder will include the Level 2 and 3 Data file and a description file that defines the assay, scoring criteria, and interpretation key.
5. All finalized Level 1, 2, and 3 data along with the description file will be transferred to BCR informatics for upload to the Data Coordinating Center (DCC).

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IV. REFERENCES

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V. COMPREHENSIVE REVISION HISTORY

A. Changes made in Version 2, Effective Date 1/16/2015
1. Updated formatting
2. Removed any TCGA reference
3. Updated disclaimer
4. Updated reagent catalog numbers

B. Version 1, Effective Date 10/2/2012 - New

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Signatures

Approved By: **Signature on file** **Date:** **Date on file**

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