**MGMT Methylation Detection by MSP**

1. **PRINCIPLE:**
	1. O₆-Methylguanine DNA Methyltransferase (MGMT) is a DNA repair enzyme responsible for removing methyl adducts from the O₆ position of alkylated guanine (O₆-methylguanine), thereby restoring normal nucleotide structure. Failure of this mechanism of DNA repair leads to preferential binding of O₆-methylguanine to thymine during DNA synthesis. This could lead to the replacement of guanine with adenine in subsequent cell cycles. In the event of MGMT failure, the O₆-methylguanine-thymine mismatch leads to the initiation of apoptosis by the cell’s mismatch repair (MMR) machinery at the G2/M checkpoint. It is this process that is assumed to be exploited when temozolomide (TMZ) is used to treat aggressive glioblastomas. TMZ is a strong alkylating agent whose damage to guanine is thought to trigger the MMR response, thereby forcing tumor cells into apoptosis. Normal MGMT functioning appears to combat TMZ therapy, correcting its damage and thus preventing the desired cellular toxicity. However, malignant gliomas may have their *MGMT* gene inactivated due to methylation of their promoter region. Research suggests that glioblastoma patients whose *MGMT* gene promoter is methylated exhibit an increased responsiveness to alkylating therapy. Methylation of *MGMT* has been shown to correlate more closely with TMZ response than MGMT protein expression itself. Given this correlation, the National Comprehensive Cancer Network (NCCN) suggests *MGMT* methylation status may be used as a tool for determining the efficacy of TMZ agents.
	2. DNA methylation pattern may be evaluated using a number of techniques, including Methylation Specific PCR (MSP), Bisulfite Sequencing, Pyrosequencing, and Methylation Specific Restriction Site Fragment Length Polymorphism Analysis. Since the clinically significant sites of methylation have been well-characterized, we have employed a MSP assay to detect *MGMT* promoter methylation status in formalin-fixed, paraffin embedded (FFPE) brain tumor tissue. By this method, genomic DNA is subjected to a bisulfite conversion treatment, during which any unmethylated cytosine is converted to uracil, which ultimately becomes thymine during PCR replication (see Figure 1 below).

Figure 1- Bisulfite Conversion Reaction Schematic: A. Unmethylated cytosine bases are successfully converted to uracil following deamination. B. The 5-carbon methyl group of 5-methylcytosine prevents sulphonation and, thus, deamination of the base.

**B**

**A**

1. **SAMPLE:**
	1. Purified genomic DNA from formalin-fixed, paraffin-embedded (FFPE) tissue
2. **REAGENTS AND STORAGE**:
	1. Oligonucleotide Primers: All stored in -20°C freezer located in PCR room
		1. MTL Fwd- 1 Primer, 100uM in water
			1. 5’-ttt cga cgt tcg tag gtt ttc gc-3’
		2. MTL Rev- 1 Primer, 100uM in water
			1. 5’- gca ctc ttc cga aaa cga aac g-3’
		3. UMTL Fwd- 1 Primer, 100uM in water
			1. 5’- ttg tgt ttt gat gtt tgt agg ttt ttg t-3’
		4. UMTL Rev- 1 Primer, 100uM in water
			1. 5’- aac tcc aca ctc ttc caa aaa caa aac a-3
		5. Combine equal volumes of MTL Fwd primer with MTL Rev primer to create a 50uM mix
		6. Combine equal volumes of UMTL Fwd primer with UMTL Rev primer to create a 50uM mix.
	2. EZ DNA Methylation-Lightning Kit (Cat # D5030; Zymo Research). Store at room temperature in the DNA prep area. Keep contents in dark until ready to use.
	3. AmpliTaqGOLD® DNA polymerase (5 units/µl). Store in -20°C freezer located in PCR room
	4. PCR buffer (10x). Store in -20°C freezer located in PCR room
	5. ThermoFisher deoxynucleotide triphosphates (dATP, dCTP, dGTP, and dTTP), 25mM
		1. Dilute 1:10 to get 2.5mM and then aliquot to create a dNTP mix. Store at -20°C
	6. 25 mM MgCl2 in water.
		1. Heat to 55°C for 1 hour, then store in 4°C refrigerator located in PCR room
3. **CONTROLS:**
	1. Bisulfite DNA Control: Zymo cat#D5015
	2. Sensitivity Positive control: 0.5% SW480 DNA from cell line
	3. Negative controls:
		1. FFPE Tonsil DNA
		2. Non Methylated Control Zymo cat#D5014-1
	4. No Target Control: dH2O
4. **PROCEDURE:**
	1. Bisulfite conversion
		1. **Create your worksheet: MGMT Bisulfite Conversion – Test Worksheet Builder**
			1. Log into Soft Molecular.
			2. Open MGMT Bisulfite Conversion - Test Worksheet Builder by using the tile on the dashboard.
			3. Select **Find**.
			4. If applicable, select **New** in the Pending Worksheets tab.
			5. Click the Barcode# field. Scan product label in the Barcode# field and select **Enter** on the keyboard to add the samples to the worksheet.
			6. If QC is being performed on a new Zymo EZ DNA Methylation-Lightening Kit, select the plate view tab on the left side of the screen.
				1. On the plate view, move the tonsil and patient samples to the right and add the MGMT\_METHYLATED control into the empty A3 slot.
			7. On the Worksheet View tab, verify the control lot numbers by clicking on the **Sample ID** field.
				1. If the control needs to be changed, click on the dropdown arrow and select the correct control in the window that appears.
			8. Verify the reagent lot numbers by clicking on the vertical **Settings** tab on the left side of the screen.
				1. If the reagent lot needs to be changed, click on the dropdown arrow in the **Stock #** column and select the correct lot in the window that appears.
			9. Enter concentration value for controls under **Control Concentration** column.
			10. Mark the **Completed** checkbox and select **Save**.
			11. Select the **printer icon**, verify the correct printer is highlighted and click **Print**.
			12. Close the Print Preview window.
			13. Select **Back** in the MGMT Bisulfite Conversion - Test Worksheet Builder window.
			14. Exit Soft Molecular application.
		2. Thaw patient DNA samples, as well as the positive and negative controls.
		3. Perform the bisulfite conversion using the Zymo Bisulfite Conversion EZ Lightning Kit. If necessary, prepare the wash buffer by adding the specified amount of 100% ethanol to the M-Wash Buffer.
		4. Label the appropriate number of clean 0.2ml PCR tubes as indicated on the worksheet. Each patient will be run in duplicate and will require 2 tubes. Controls are run in singlicate.
		5. 500 ng of each sample must be processed in a total volume of 20ul per Zymo column. Since patient samples are processed in duplicate, a total of 40ul or 25ng/ul DNA solution must be created per sample. Methylated and unmethylated controls are processed in singlicate, so only 20ul dilutions must be created. Control samples are in the extraction freezer.
			1. The Soft Molecular MGMTBC worksheet will calculate the DNA dilutions for patients according to the following formulas:
				1. V1= (25ng/ul\*40ul)/Stock [DNA] or 1000/stock DNA conc in ng
				2. 40ul (total volume) - V1 (Volume of DNA) = Volume of water to add to V1
			2. The Soft Molecular MGMTBC worksheet will calculate the DNA dilutions for controls according to the following formula:
				1. V1=(25ng/ul\*20ul)/Stock [DNA] or 500/stock DNA
			3. Put DNA directly into PCR tube and dilute according to worksheet.
		6. Vortex dilution tubes and transfer 20ul of each patient DNA dilution to its duplicate tube so that each tube contains 20ul of DNA at the appropriate concentration.
		7. Add 130ul of Lightning Conversion Reagent to the 20ul of DNA in the 0.2ml PCR tubes.
		8. Mix and centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.
		9. Place the PCR tubes in a thermal cycler in Pre-PCR room and start program BSEZ:
			1. 98°C for 8 minutes
			2. 54°C for 60 minutes
			3. 4°C for up to 20 hours
		10. **Process your worksheets**: **MGMT Bisulfite Conversion**
			1. Log into Soft Molecular.
			2. Open MGMT Bisulfite Conversion - Test Worksheet Processing by using the tile on the dashboard.
			3. Scan the MGMTBC worksheet into Worksheet# field and select **Find**.
			4. Use the dropdown under **Thermocycler:** to select the correct instrument.
			5. Complete the MGMT Bisulfite Conversion activity by marking the **Completed** checkbox and selecting **Save**.
			6. Click the **Build Next Worksheet** button.
			7. Verify that the Select all tests, MGMTOD To build, and Transfer Controls checkboxes are marked. Select **OK**.
			8. The system will ask the user if they want to open the new worksheet. Select **No**.
			9. Select **Back** in the MGMT Bisulfite Conversion - Test Worksheet Processing window.
			10. Exit Soft Molecular application.
		11. Add 600ul of M-Binding Buffer to the column placed into a provided collection tube.
		12. Flash spin specimen PCR tubes. Load the samples into the prepared column containing M-Binding buffer. Close cap and mix by inverting six times.
		13. Centrifuge at full speed (>10,000xg) for 1 minute. Discard the flow-through into the designated waste container.
		14. Add 100ul of M-Wash Buffer to the column. Centrifuge at full speed for 1 minute.
		15. Add 200ul of L-Desulphonation Buffer to each column and let stand at room temperature for 15-20 minutes. Centrifuge at full speed for 1 minute.
		16. Add 200ul of M-Wash Buffer to column. Centrifuge at full speed for 1 minute. Discard flow-through in designated waste container. Repeat wash twice for a total of three times.
		17. Discard flow-through in designated waste container. Place column back into collection tube and spin 1 minute.
		18. Place each column into a 1.5ml microcentrifuge tube. Add 20ul of M-Elution Buffer directly to the column matrix. Let sit 2 minutes for incubation.
		19. Centrifuge 1 minute at full speed to elute DNA.
		20. Determine the concentration and purity of the bisulfite-converted products using the Nanodrop spectrophotometer (See Quantification of DNA and RNA by Optical Density Procedure).
		21. **Processing your worksheets after Washing and Nanodrop**
			1. Log into Soft Molecular.
				1. Open MGMT Wash and OD – Test Worksheet Processing by using the tile on the dashboard.
				2. Select **Find**.
			2. Nanodrop
				1. Since the absorbance of bisulfite-converted DNA more closely resembles that of RNA, use the RNA function on the Nanodrop.
				2. Input Soft Molecular **Sample ID** in the Nanodrop Sample ID field.

Note: Do not scan the product label. Refer to the MGMT OD Test Worksheet Processing window for the Sample ID.

* + - * 1. Select the **Measure** icon or **F1** on the keyboard.
				2. When all samples have been measured, select the **Reports** button in the menu on the left side of the screen.
				3. If all Nanodrop readings should be exported to an Excel worksheet:

Select the **Export** icon on the right side of the screen.

Save the document to the correct location.

* + - * 1. If only select Nanodrop readings should be exported:

Use the **Ctrl** button on the keyboard to select the Nanodrop readings to be exported.

Select the **Export** icon on the right side of the screen.

Save the document in the correct location.

* + - * 1. Navigate to and open the exported file.
				2. Select **Save**.
				3. A window will appear. Select **No**.
				4. Change the file type from XML Spreadsheet 2003 (\*.xml) to **Excel Workbook** (\*.xlsx).

**Note:** When the file is saved, two documents with the same name will appear in the folder. Only the documents with Type: Microsoft Excel Worksheet will appear when selecting a file to import into Soft Molecular.

* + - * 1. Select **Save**.
			1. Return to Soft Molecular.
				1. Click '**...**' button to the right of the **Import** button.
				2. Select the **Excel** radial button and choose file path for file and click **Import**.
				3. Dilutions will be automatically calculated in the MGMT Wash and OD worksheet. Final concentration should be 6 ng/ul.
				4. Verify the reagent lot numbers by clicking on the vertical **Settings** tab on the left side of the screen.

If the reagent lot needs to be changed, click on the dropdown arrow in the **Stock #** column and select the correct lot in the window that appears.

* + - * 1. Click **Print Worksheet** button.
				2. Select the **printer icon**, verify the correct printer is highlighted and click **Print** button.
				3. Close the Print Preview window.
				4. Complete the MGMT Wash and OD activity by marking the **Completed** checkbox and selecting **Save**.
				5. A window will appear, if the Attached Tests process was previously completed, select **OK**.
				6. If there are multiple (non-FISH) tests on the same order (Such as AMP or BRAF), inform the Director/Pathologist/Manager and proceed with steps a-k.

Highlight the correct patient sample.

Select the **Tools** tab.

Click the **Bridge to Order Entry** button.

Select **Yes** in the window that appears.

Click on the Specimen tab.

Select the **+** sign to expand the child level aliquots.

Locate the **Att Tests** column.

Detach the AMP/BRAF/other assay from the MGMT aliquots (e.g., -03-01 and -03-02) and then attach the other test to the original aliquot (e.g., -03-00).

Select **Back** to return to MGMT OD Worksheet Processing.

**NOTE: Remember to close Order Entry tab before proceeding with testing.**

Repeat steps a-i for all applicable samples.

* + - * 1. Click the **Build Next Worksheet** button.
				2. Verify **Select all tests** is selected. Mark MGMTPCR **To build** checkbox. Select **OK**.

Transfer Controls will automatically mark when the To Build checkbox is marked.

* + - * 1. The system will ask the user if they want to open the new worksheet. Click **No**.
				2. Select **Back** in the MGMT Wash and OD – Test Worksheet Processing window.
				3. Open MGMT PCR – Test Worksheet Builder by using the tile on the dashboard.
				4. Select **Find**.
				5. Select the **Plate View** tab.
				6. Adjust the controls and patient samples to fill in the empty A3 and B3 wells.
				7. Verify the reagent lot numbers by clicking on the vertical **Settings** tab on the left side of the screen.

If the reagent lot needs to be changed, click on the dropdown arrow in the **Stock #** column and select the correct lot in the window that appears.

* + - * 1. Select the **Worksheet View** tab.
				2. Click on **Print Worksheet** button. Select **OK**.
				3. Select the printer icon, verify the correct printer is highlighted and click **Print** button.
				4. Close the Print Preview window.
				5. Click on the **Settings** button to open the Select Printer window. Verify the correct printer is selected in the dropdown field. Select **Print**.

**Note:** Select **View** in the Select Printer window to preview the Section Settings.

* + - * 1. If applicable, close Print Preview.
				2. Select **Back** in the MGMT PCR – Test Worksheet Builder window.
				3. Exit Soft Molecular application.
		1. The bisulfite-converted DNA is ready for immediate analysis or can be stored:
			1. At or below -20°C for up to 4 months.
			2. At or below -70°C for long-term storage.
	1. PCR Setup:
		1. Completely thaw all necessary reagents except AmpliTaq® Gold DNA polymerase.
		2. Create sample dilutions as calculated previously on the MGMTOD worksheet.
		3. Briefly vortex reagents except for Taq and flash spin for 30 sec.
		4. Assemble PCR reactions in an Air Clean PCR setup hood using clean gloves and barrier pipette tips, as appropriate.
		5. Label the top of PCR tubes with the individual reaction number and test, as indicated in the PCR worksheet.
		6. Prepare master mixes M (methylated primers) and U (unmethylated primers) according to the Methylated and Unmethylated Section Settings in the appropriate tubes. Check that the lot number of each reagent matches the Section Settings worksheet.
		7. Mix by carefully pipetting up and down using a barrier pipette tip.
		8. Aliquot 20ul of either Master Mix M or U to the specified tubes using barrier pipette tip.
		9. Add 5ul of DNA or water to each reaction tube, as specified in the MGMT PCR worksheet (except for the Bisulfite Control sample, for which you must add 1ul of sample and 4ul of water, as indicated in the MGMT PCR worksheet).
		10. Spin tubes for 30 sec. at 13,000 rpm.
		11. Initiate File MGMT1 on the thermal cycler:
			1. 95°C for 10 min.
			2. 40 amplification cycles, as follows:
				1. 95°C for 30 sec. (denaturation)
				2. 63° C for 30 sec. (annealing)
				3. 72°C for 30 sec. (extension)
			3. Final extension reaction for 10 min. at 72°C to complete all products
			4. Hold reaction at soak temperature of 4°C
		12. **Process your worksheet: MGMT PCR**
			1. Log into Soft Molecular.
			2. Open MGMT PCR - Test Worksheet Processing by using the tile on the dashboard.
			3. Click **Find** to see the pending MGMT PCR Worksheets.
			4. Double click on the worksheet to open.
			5. Use the dropdown under **Thermocycler:** to select the correct instrument.
			6. Complete the MGMT PCR activity by marking the **Completed** checkbox and selecting **Save**.
			7. Click the **Build Next Worksheet** button.
			8. Verify that the Select all tests, MGMTGEL2 To build checkbox, and Transfer Controls checkboxes are marked. Select **OK**.
			9. The system will ask the user if they want to open the new worksheet. Select **No**.
			10. Select **Back** in the MGMT PCR - Test Worksheet Processing window.
			11. Open MGMT GEL - Test Worksheet Builder by using the icon on the dashboard.
			12. Click **Find** to see the pending MGMT Gel Worksheets.
			13. Double click on the worksheet to open the test worksheet builder.
			14. Select **Plate View** and adjust the wells to reflect how the gel should be loaded. Appropriately move patients and controls to move MGMT\_BISULFITE\_CTL to wells A2 and B2.
			15. Click the **Worksheet View** tab.
			16. Verify the control lot number is filled in for the 50\_BP\_Marker.
			17. Select **Print Plate View**. Select **OK**.
			18. Select **Print icon** on the Plate View. Verify the correct printer is highlighted and **Print**.
			19. Save the worksheet.
			20. Select **Back** in the MGMT Gel - Test Worksheet Builder window.
			21. Exit Soft Molecular application.
		13. Remove tubes from thermal cycler at conclusion of PCR run.
		14. Samples are ready to analyze, or they may be stored at 4°C or –20°C.
			1. Analyze by gel electrophoresis:
				1. Add 4ul of loading dye to the PCR tubes and mix with pipette.
				2. Use a 3% (1:1 NuSieveAgarose:Agarose) agarose gel (see Gel Electrophoresis Procedure).
				3. Load 20ul of PCR product into gel.
		15. **Process your worksheet: Gel Loading Action**
			1. Log into Soft Molecular
			2. Open MGMT Gel - Test Worksheet Processing icon on the dashboard.
			3. Scan the MGMTGEL2 Plate View into the Worksheet# field and select **Find**.
			4. Complete the MGMT Gel activity by marking the **Completed** checkbox and selecting **Save**.
			5. Select **Back** in MGMT Gel – Test Worksheet Processing window.
			6. Exit Soft Molecular application.
		16. **Refer to Bio-Rad Gel Doc XR Instrument Procedure to capture and edit gel image.**
		17. **Process your worksheet: Acquire Image**
			1. Log into Soft Molecular
			2. Open MGMT Gel - Test Worksheet Processing icon on the dashboard.
			3. Scan the MGMTGEL2 Plate View into the Worksheet# field and select **Find**.
			4. Verify Worksheet is populated in the Image Type field.
			5. Select **Images** button. On the window that opens, select the **Add File** tab then select the **add file (folder)** icon.
			6. Find and select the file to be added from the Windows Explorer window. Select **Open**.
			7. Choose **Gel Image** from Template dropdown.
			8. Select the **green check** icon to add file(s). Close the window.
			9. Complete the Acquire Image activity by marking the **Completed** checkbox and click the **Tasklist** button.
			10. Select **Yes** to save.
			11. Click **Select All** followed by **Coll/Exp.** Then mark the **Completed** checkbox and select **Save**.
			12. Select **Back** in the MGMT Tasklist Entry window.
			13. Exit Soft Molecular application.
1. **INTERPRETATION:**
	1. Final interpretation of this assay is done by the Director or Pathologist. Methylated DNA should amplify with the methylated primers and unmethylated DNA should amplify with the non-methylated primers. Most tumor specimens are heterogeneous and contain both tumor and non-tumor cells. Because of this, the unmethylated amplicon may be seen in both tumor DNA and normal DNA. The methylated DNA will only amplify in the methylated reaction and this should only be seen in the tumor tissue; however, not all tumor samples will show a positive reaction and this assay is designed to detect the presence of *MGMT* methylation in tumor samples.
	2. Guidelines for interpretation can be seen in the following chart:
		1. Methylated amplicon= 82bp
		2. Unmethylated amplicon= 92bp

|  |  |  |  |
| --- | --- | --- | --- |
| **DNA** | **Amplification****Methylated primers** | **Amplification****Unmethylated primers** | **Result** |
| **Bisulfite treated DNA** | Positive | Negative | Pass QC |
| **NTC** | Negative | Negative | No contamination |
| **Methylated DNA (pos control)** | Positive | Positive/Negative | Methylated |
| **Unmethylated DNA****(neg control)** | Negative | Positive | Unmethylated |
| **In-house FFPE sample Unmethylated DNA (neg control)** | Negative | Positive | Unmethylated |
| **Patient sample** | 4/4 | 3/4 to 4/4 | Methylated |
| **Patient sample** | 3/4 | 3/4 to 4/4 | Methylated |
| **Patient sample** | 2/4 | 3/4 to 4/4 | Partially Methylated |
| **Patient sample** | 1/4 | 3/4 to 4/4 | Partially Methylated |
| **Patient sample** | 0/4 | 3/4 to 4/4 | Unmethylated |

* 1. If both the negative controls show methylation, repeat entire assay.
	2. If the positive control does not show methylation, repeat entire assay.
	3. If less than 3 of 4 unmethylated reactions do not amplify for patient samples, repeat the assay for that patient sample.
	4. See sample results below:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Top Row of Gel (MTL Rxn) |  | Bottom Row of Gel (UMTL Rxn) |  |
|  | Lane | Sample | Result M/U |  | Lane | Sample | Result |  P/F |  |
|  | 1 | 50bp |   |   |  | 23 | 50bp |   |   |  |
|  | 2 | bisulf+ | + |   |  | 24 | bisulf+ |   |   |  |
|  | 3 | NTC | - |   |  | 25 | NTC |   |   |  |
|  | 4 | MTL CTL | + |   |  | 26 | MTL CTL | + | pass |  |
|  | 5 | MTLCTL | + |   |  | 27 | MTLCTL | + | pass |  |
|  | 6 | UMTLCTL | - |   |  | 28 | UMTLCTL | + | pass |  |
|  | 7 | UMTLCTL | - |   |  | 29 | UMTLCTL | + | pass |  |
|  | 8 | Tonsil | - |   |  | 30 | Tonsil | + | pass |  |
|  | 9 | Tonsil | - |   |  | 31 | Tonsil | + | pass |  |
|  | 10 | Patient A(1) | + |   |  | 32 | Patient A(1) | + | pass |  |
|  | 11 | Patient A(1) | + |   |  | 33 | Patient A(1) | + | pass |  |
|  | 12 | Patient A(2) | - |   |  | 34 | Patient A(2) | + | pass |  |
|  | 13 | Patient A(2) | - |   |  | 35 | Patient A(2) | + | pass |  |
|  | 14 | Patient B(1) | - |   |  | 36 | Patient B(1) | + | pass |  |
|  | 15 | Patient B(1) | - | Nonspecific band (wrong size) |  | 37 | Patient B(1) | + | pass |  |
|  | 16 | Patient B(2) | - |   |  | 38 | Patient B(2) | + | pass |  |
|  | 17 | Patient B(2) | - |   |  | 39 | Patient B(2) | + | pass |  |
|  | 18 | Patient C(1) | + |   |  | 40 | Patient C(1) | + | pass |  |
|  | 19 | Patient C(1) | + |   |  | 41 | Patient C(1) | + | pass |  |
|  | 20 | Patient C(2) | + |   |  | 42 | Patient C(2) | + | pass |  |
|  | 21 | Patient C(2) | + |   |  | 43 | Patient C(2) | + | pass |  |
| 1-29-14 gel 28991 mgmt.tif |  |  |  |  |  |  |  |  |
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Patient A partially methylated

Patient B unmethylated 4/4

Patient C methylated 4/4

1. **RESULTS REVIEW**
	1. Log into Soft Molecular
	2. Open My Orders by using the icon on the dashboard.
	3. Click on **Director Review** tab.
	4. Click two times on tasklist.
	5. Click on **No** button.
	6. Enter MGMT Gel Result on the child level. Enter MGMT Final Result on the parent level. Perform this step for both patient samples and controls.
		1. Note: For Controls, Final Result should be Pass/Fail.
	7. Complete the Result Review action by marking the **Completed** checkbox and selecting **Save**.
	8. Select **Yes** when asked to verify and post QC.
	9. Select **Back** in the MGMT Tasklist Entry window.
	10. Exit Soft Molecular application.
2. **SIGN OUT ENTRY**
	1. Log into Soft Molecular
	2. Open My Orders by using the icon on the dashboard.
	3. Verify Molecular Pathologist tab is displayed.
	4. Click two times on order.
	5. Click on **No** button.
	6. Verify whether RBS rules are triggered correctly (Result, Interpretation sections are filled appropriately).
	7. Mark **Completed** checkbox.
	8. Select **Sign Out** button.
	9. Make sure that the information on the report is correct or edit, as appropriate.
	10. Press **Sign out** and the report will populate the screen.
	11. Sign Out Warning may display a warning that controls have not been posted - click **Yes** to continue.
	12. Select **No** for the second control warning message to continue interpretation release.
	13. Make sure that the information on the report is correct.
	14. Complete sign out by selecting **Complete Sign Out** button.
	15. Select **Back** in Sign Out Entry.
	16. Exit Soft Molecular application.
3. **REPEAT TESTING**
	1. During the testing process, testing for some samples must be repeated for a variety of technical or analytical reasons. The specimen can be sent back to one of multiple prior steps in the workflow.
	2. In Soft Molecular, repeating a sample to a prior action can be accomplished from a variety of steps in the test workflow and places in the system. Please see the Soft Molecular Rerun Procedure for the specific steps to perform when requesting rerun testing.
4. **REFERENCES:**
	1. Zymo package insert Ver 1.0.3
	2. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®). Central Nervous System Cancers, Version 1.2015 (NCCN.org)
5. **REVISIONS:**
	1. 3/15/2018: Addition of Bisulfite Conversion instructions, RKO Cell line and reformatting.
	2. 1/6/2020: Updated with steps for Soft Molecular and updated footer with new lab name.
	3. 2/3/2020: Clarified worksheet building and processing steps.
	4. 10/6/2020: Changes in Zymo Kit processing steps and annealing temp for PCR.
	5. 4/22/2021: Clarification on some of the Soft Molecular steps and updated PCR settings for the annealing temperature.
	6. 7/6/2021: Updated the placement of instruction to check on the 50 bp marker.