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| Agena MassARRAY DICER1 Assay  |
| **Purpose** | This procedure provides instructions for preparing samples and reagents, setting up multiplex, polymerase chain reaction (PCR) and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) reactions using the Agena Bioscience MassARRAY DICER1 assay on Formalin-Fixed, Paraffin-Embedded (FFPE) tumor tissue.  |
| **Policy Statements** | This procedure applies to all technical staff performing testing on the Agena MassARRAY instrument.  |
| **Principle and Clinical Significance** | The *DICER1* gene provides instructions for making the Dicer protein which plays a role in regulating the expression of other genes. The Dicer protein aids in the production of a molecule called microRNA (miRNA). MicroRNAs are short lengths of RNA. Dicer cleaves precursor RNA molecules to produce miRNA. MicroRNAs control gene expression by blocking the process of protein production. In the first step of making a protein from a gene, messenger RNA (mRNA) is formed and acts as the blueprint for protein production. MicroRNAs attach to specific mRNA molecules and stop the process by which protein is made. Sometimes, miRNAs break down the mRNA, which also blocks protein production. Through this role in regulating the expression of genes, Dicer is involved in many processes, including cell growth and proliferation and the maturation of cells to take on differentiation.3Mutations in the *DICER1* gene can cause *DICER1* syndrome. People with this condition have an increased risk of developing many types of tumors, particularly certain tumors of the lungs (pleuropulmonary blastoma), kidneys (cystic nephroma), ovaries (Sertoli-Leydig tumors), and thyroid (multinodular goiter). Most of these mutations lead to an abnormally short Dicer protein that is likely unable to produce miRNA. Without regulation by miRNA, genes are expressed abnormally, which could cause cells to grow and divide uncontrollably and lead to tumor formation.3The DICER1 Panel for use on the MassARRAY system, is a multiplex Polymerase Chain Reaction, Matrix-Assisted Laser Desorption Ionization – Time of Flight (PCR/MALDI-TOF) test intended for single nucleotide variant (SNV) and small indel genotyping and somatic mutation analysis of genomic DNA extracted from tumor tissue. Formalin-Fixed, Paraffin-Embedded tissue (FFPE) is the validated clinical sample type. A spin column based nucleic acid extraction on the QIACube Connect instrument is utilized. Following DNA extraction, PCR with iPLEX Pro chemistry then amplifies custom target regions of interest; after the inactivation of unincorporated dNTPs, a sequence-specific primer extension step is performed using the supplied custom set of Extend primers and iPLEX Pro reagents. Sample amplification is carried out on the Applied Biosystems 2720 and Veriti Thermalcyclers following the parameters listed in the iPlex Pro IFU. The extension products (analyte) are desalted, transferred to a SpectroCHIP Array (a chip with pre-spotted matrix crystals) and then loaded into the MassARRAY Analyzer (a MALDI-TOF mass spectrometer). The analyte/matrix co-crystals are irradiated by a laser, inducing desorption and ionization. The positively charged molecules accelerate into a flight tube towards a detector. Separation occurs by time-of-flight, which is proportional to the mass of the individual molecules. After data processing, a spectrum is produced with relative intensity on the y-axis and mass/charge on the x-axis. Data acquired by the MassARRAY analyzer is processed by MassARRAY Typer software, and results then are interpreted.1 The Agena Bioscience DICER1 panel consists of 68 variants in *DICER1*, *DROSHA*, *FOXL2*, *TERT*, and *TP53* genes interrogated by 37 assays divided into 6 primer sets. See **Tables 1** and **2** for details. **Table 1: Panel Variants****Table 2: Panel Assay IDs and Primer Sets** |
| **Test Code** | **DICER1 –** Formalin-Fixed, Paraffin-Embedded (FFPE) tissue. |
| **Sample** | 1. **Acceptable specimens:**
	* Formalin-Fixed, Paraffin-Embedded (FFPE) tissue as scrolls or unstained sections.
2. **Unacceptable specimens:**
	* Improperly labeled or unlabeled samples.
	* <40% tumor cellularity, with or without macrodissection (unless approved by molecular pathologist on case-by-case basis).
	* Decalcified specimens.
3. **Specimen Collection and Transport**:
	* FFPE scrolls or slides received from histology department; room temperature.
	* Refer to [*Lab Test Directory*](https://www.childrensmn.org/References/Lab/)on StarNet
4. **Specimen assessment:**
	* Refer to the policy [*MB1.02v7 Specimen Rejection Criteria for Molecular Biology*](https://starnet.childrenshc.org/References/labsop/molbio/specmgt/mb-1.02-rejection-criteria.pdf)
	* Do NOT discard rejected samples unless approved by molecular pathologist.

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| **Special Safety Precautions** | * Standard precautions. Refer to [MB 2.02 Biohazard Containment](https://starnet.childrenshc.org/References/labsop/molbio/safety/mb-2.02-biohazard-containment.pdf)
* Use of engineering controls: Refer to [MB 3.01 Engineering Controls to Prevent Nucleic Acid Contamination](https://starnet.childrenshc.org/References/labsop/molbio/engctl/mb-3.02-wipe-testing-for-amplicon-or-nucleic-acid-contamination.pdf)

Laboratory staff are subject to occupational risks associated with specimen handling. Refer to the safety policies located in the safety section of the [*Molecular Biology Policy Manual*](https://starnet.childrenshc.org/References/labsop/index.php?view=folder&folder=molbio):1. [*Safe Work Practices in Molecular*](https://starnet.childrenshc.org/References/labsop/molbio/safety/mb-2.01-safe-work-practices-in-molecular.pdf)
* [*Biohazardous Spill in Molecular*](https://starnet.childrenshc.org/References/labsop/molbio/safety/mb-2.03-biohazardous-spills-in-molecular.pdf)
* [*Biohazard Containment*](https://starnet.childrenshc.org/References/labsop/molbio/safety/mb-2.02-biohazard-containment.pdf)
1. Wear appropriate personal protective equipment (PPE) including disposable gloves and lab coats.
2. Handle all samples and waste materials as if they were capable of transmitting infectious agents.
3. Change gloves often when handling reagents or samples.
4. Dispose of materials used in this assay, including reagents and used buffer vials, in biohazardous waste.
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| **Materials** |

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| **Reagents** | **Supplies** | **Equipment** |
| -Sani-Cloth Bleach wipes or 1:10 diluted bleach solution-70% ethanol-100% ethanol-5% Extran-DNA Away-Deionized water-Nuclease Free Water (NFW)-UltraPure Water-Clean Resin-HPLC-grade water-Positive Amplification Control material -QIAamp DNA FFPE kit (REF 56404)Room 1:**MassARRAY DICER1 Panel Kit**-Agena DICER1 Panel (REF 06205) – store at -25 °C to -10 °C:* DICER1 PCR Primers
* DICER1 Panel Extend Primers

-PCR Reagent Set (REF 21327M) - store at -25 °C to -10 °C:* MgCl2, 25mM
* 10x PCR Buffer
* dNTP Mix
* PCR Enzyme

-iPLEX Pro Reagent Set (REF 10212)- store at -25 °C to -10 °C:* 3 Point Calibrant
* iPLEX Termination Mix
* iPLEX Buffer Plus, GPR
* iPLEX Pro Enzyme
* SAP Buffer
* Shrimp Alkaline Phosphatase (SAP)

**NOTE:** The MassARRAY DICER1 Panel Kit can be used up to ten times (freeze/thaw), as long as components are maintained on ice or a cold block during access and returned to storage conditions after use. **NOTE:** Keep all reagents in cold blocks once thawed and only take enzymes out of freezer immediately prior to use. Mark white board for each freeze-thaw cycle.  | -Gloves (powder-free)-Filtered pipette tips, various sizes including 200 uL, extended tips-Sharps disposal container-Microtubes -Clear adhesive plate seals (ThermoFisher, Cat #: AB0558)-96 well semi-skirted, color microtiter plates-0.2 mL PCR strip tubes (8)-Reservoirs -QIAcube kit concumables* 1000 uL tips
* 200 uL tips
* RB Tubes
* CB Tubes
* Rotor adaptors

  | -Plate centrifuge-Vortex-Mini tube centrifuge -Tube centrifuge -Thermocycler-PCR work station with UV irradiation-Multichannel pipettes-Single channel Pipettes -Tube racks -Sealing paddle -SpectroCHIP arrays-10 to -30° C freezers-Laminar flow Hood-Refrigerators 2 – 8° C-BSC BSL-2-70⁰ C freezer-Agena MassARRAY with Chip Prep Module (CPM) 96, including:* Typer software v5.01 or greater
* RT-Workstation v4.1 or greater
* Chip Prep Controller v2.2 or greater
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Record shipment receipts on log: MP 4.02.F1 Agena DICER1 Kit and QC Inventory Log |
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| **Calibration** | Annual instrument function check and maintenance performed by Agena BioScience.  |
| **Quality Control** | **See SOP:** MP 4.02 Agena MassARRAY DICER1 Assay Quality Control**Daily Quality Control:**For extraction quality control, the Nanodrop will be used to assess DNA concentration and purity.**External Quality Control:*** Perform QC using an external positive control on a rotating basis based on primer set and a negative control with **every run**.
* Perform QC using a previously tested positive sample (historical patient sample or positive synthetic control if patient sample is not available) for each primer set (positive TERT assay required for primer set 6), a previously tested negative sample for each primer set, positive QC (PC), and a negative control with **new lot/shipments**.
* Record and file results in the appropriate binder.
* Positive Amplification QC – store at – 70 °C
* Negative/Contamination QC (NTC) – Nuclease-free water

QC Monitors:

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| **Control** | **Control Monitor** |
| Historical Positive and Negative Patient (or synthetic control) Samples | Reagent, instrument, equipment, or supply failure. |
| Positive Amplification Control (PC) | Reagent, instrument, equipment, or supply failure. |
| Negative/Contamination Control (NTC) | Reagent, instrument, equipment, or supply failure. Environmental contamination monitoring. |

Record QC results on worksheet and log:MP 4.02.F1 Agena DICER1 Kit and QC Inventory LogMP 4.02.F2 Agena DICER1 New reagent QC worksheetMP 4.02.F3 Agena DICER1 QC Log* Before reporting patient results, all controls must yield valid results
* If results are invalid, repeat testing

**Negative Control (NTC)**1. Lot number (L/N), expiration date
2. Record lot information in appropriate binder

**Positive Controls (POSC) – Rotating targets** 1. Previous positive patient 2. Synthetic controlsTest controls as you would patient samples.**Record and file results in QC binder**

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| **Control Type** | **QC Status** | **Expected Sample Result** |
| **Historical Positive and Negative Patient (or synthetic control) Samples** | Pass | Match to historical interpreted results |
| **POSC: Positive Amplification Control** | Pass | Match to documented target |
| **NTC: Nuclease-free water** | Pass | No Alleles |

**NOTE:** When reagents are in use, update the information on the reagent run prep worksheet.**NOTE:** Additional external quality control may be performed on an as needed basis if certain circumstances arise. Examples include:* Drift in results (e.g., increasing/decreasing positivity rates)
* Potential contamination (negative control)
* After dramatic instrument maintenance or movement
 |
| **Assay Procedure: Sample Preparation and Extraction (Room 2)** | **NOTE:** Clean hood and supplies with a 1:10 bleach dilution or wipes followed by water and alcohol before and after any sample processing **Testing Preparation – Sample prep and DNA extraction: Room 2**1. Make a manual worksheet for **DICER1**; use this worksheet for sample identification throughout testing.

**NOTE:** Label the worksheet with your Experiment/Run name: “date, DICER1”* 1. e.g. March 3, 2020 = 0**3.03.20 DICER1**

**NOTE:** Base runs off available chip spots in room 3.**Note:** Store the QIAamp UCP MinElute columns at 2-8oC. They can be stored up to 4 weeks at room temperature if necessary. The rest of the kit contents should be stored at room temperature. **Note:** Prior to first time use, prepare Buffer AW1 by adding 25 mL ethanol and Buffer AW2 by adding 30 mL ethanol, dating both bottles and checking the box to indicate they have been prepared. **Note:** Ensure the “2” shaker adapter is in place on the QIAcube for the FFPE protocol (not the S2).1. On the QIAcube, log in with username: Admin and password: Admin. Go to the Tools tab and then the Run Modules tab. Select Heat Shaker and set the temperature to 56oC at 8 rpm for 30 minutes to get the Heat Shaker up to temperature prior to loading the samples.
2. Add the FFPE sample to a Sample Tube RB (990381) and centrifuge briefly. Add 300 uL Deparaffinization solution and vortex for 10 seconds. Centrifuge briefly.
3. On the QIAcube, select Stop to terminate the preheat step. Load sample tubes. Set the temperature back to 56 oC at 8 rpm for 3 minutes and 30 seconds. Select Start.
4. Cool to room temperature. If the solution becomes solid or waxy at room temperature, add another 300 uL deparaffiniazation solution and repeat the incubation. The blue solution should remain liquid at room temperature if it has completely melted the paraffin.
5. On the QIAcube, go to the Tools tab and then the Run Modules tab. Select Heat Shaker and set the temperature to 56oC at 8 rpm for 30 minutes to get the Heat Shaker up to temperature prior to loading the samples.
6. Add 25 uL Buffer FTB, 55 uL ultrapure RNase-free Water, and 20 uL Proteinase K to each sample. Vortex and briefly centrifuge.
7. On the QIAcube, select Stop to terminate the preheat step. Incubate for 1 hour and 3 minutes at 56oC and 1000 rpm onboard the QIAcube.
8. Set the Eppendorf ThermoMixer F1.5 to 90oC by using the arrows to start heating. Insert the thermometer into the glycerin on top of the ThermoMixer.
9. Once the QIAcube incubation has finished, take tubes out and place into the ThermoMixer at 90oC without shaking for 1 hour.
10. After incubation, briefly centrifuge.
11. There should be a complete phase separation. Remove and discard the upper blue phase using a fine-tip disposable pipet. Keep the lower ~100 uL aqueous lysate in the Sample Tube RB. This is the 2 mL Eppendorf Safe-Lock Tube.
12. On the QIAcube, go to DNA tab and select QIAamp DNA FFPE Advanced.
13. Select material: FFPE Tissue and click Next.
14. Select Protocol: DNA from FFPE and click Next.
15. Elution volume: 40 and click Next.
16. Select the correct number of samples and click Next.
17. Load Buffer Bottles with enough volume of each respective reagent as indicated on the screen. NOTE: Elution bottle should be filled with HPLC-grade water instead of Buffer ATE.
18. Click Next.
19. Load Tip Racks and Enzymes. Prepare RNase A and Proteinase K in 1.5 mL elution tubes.
20. Assemble and load Centrifuge Rotor Adaptor with the spin columns and elution tubes in the correct positions. Ensure lids are also in the correct location and pushed all the way down and at a 90o angle.
21. Load the heat shaker with the Sample Tubes RB containing the specimens.
22. Start
23. Once the program is complete, open instrument and take out rotor apparatus, take out the spin column and dispose in a dual waste container and close elution tube. Dispose all reagents and consumables in a dual waster container.
24. Quantify samples on the NanoDrop per the NanoDrop procedure, [MP 2.02 NanoDrop OneC Spectrophotometer Nucleic Acid Quantitation (childrenshc.org)](https://starnet.childrenshc.org/References/labsop/molpath/nanodrop/mp-2.02-nanodrop-onec-spectrophotometer-nucleic-acid-quantitation.pdf)
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| **Assay Procedure: Sample Addition (Room 2)** | **NOTE:** Clean hood and supplies with a 1:10 bleach dilution or wipes followed by water and alcohol before and after any sample processing 1. Prepare Plate Map and Master Mix Calculator.
	1. [G:\Molecular\Molecular Pathology\Agena - Dicer 1\Forms\DICER1 Plate Map and Calculator.xlsx](file:///G%3A%5CMolecular%5CMolecular%20Pathology%5CAgena%20-%20Dicer%201%5CForms%5CDICER1%20Plate%20Map%20and%20Calculator.xlsx)

NOTE: All extracted samples are run in duplicate. Some cases may require running in triplicate and molecular pathologist will notify lab staff of any samples requiring this.1. Label a 96-well PCR plate with Experiment/Run name: “date, DICER1”
	1. e.g. March 3, 2020 = 0**3.03.20 DICER1.** Place in cold block.
2. Divide and mark plate based on Primer Set layout according to plate map.
3. Add 2.0 uL of either sample or control to designated well of the plate according to plate map.

**NOTE:** The NTC and each sample will be pipetted six times, once for each primer set.**NOTE:** Controls are primer set based thus pipetted only once and are routinely rotated. Do not vortex synthetic positive QC. Thaw and quick spin only.**NOTE:** A previously tested positive sample needs to be run for each primer set for new lots/shipments.1. Seal the PCR plate and place in fridge until master mix is prepared.

**NOTE:** Visually inspect the individual wells from the bottom of the reaction plate to confirm uniformity before continuing.  |
| **Assay Procedure: PCR Master Mix Preparation (Room 1):** | 1. Clean hood with 5% Extran followed by 70% ethanol. Prepare Master Mix run worksheet:
	1. [G:\Molecular\Molecular Pathology\Agena - Dicer 1\Forms\DICER1 Plate Map and Calculator.xlsx](file:///G%3A%5CMolecular%5CMolecular%20Pathology%5CAgena%20-%20Dicer%201%5CForms%5CDICER1%20Plate%20Map%20and%20Calculator.xlsx)
		1. **NOTE:** reagent volumes allow for 25% overage
2. Pull the reagents listed in the Table below to thaw. Leave Enzyme in freezer.
3. Print Master Mix Calculator created in “Sample Addition”, Step 1.
4. Take enzyme out of freezer and place in cold block.
5. Vortex all reagents except the enzyme (flick), quick spin and place in cold block.
6. Label seven 1.5 mL tubes, one MMX and the remaining 1-6, place in cold block.
7. Prepare the PCR master mix (cocktail) in the MMX labeled 1.5 mL tube by adding reagents in order, as listed in the table below. Write how many tests are being removed on the white board in room 1. When opening a new box, write the numbers 1 through 5 on top of each vial as corresponds to the table below.
8. Once the PCR (MMX) cocktail has been prepared, evenly distribute into Tubes 1-6 according to the calculator.
9. Add each PCR Primer Set to the corresponding labeled tube.
10. Vortex and spin tubes briefly.
11. Label 0.2 mL PCR strip tubes with designated primer set and place in cold block.
12. Evenly aliquot cocktail from Tubes 1-6 into corresponding labeled strip tube according to the calculator worksheet.
13. Cap tubes and spin briefly.

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| Reagent  | Per Reaction (uL) |
| 1. HPLC-grade Water | 0.80 |
| 2. 10x PCR Buffer | 0.50 |
| 3. MgCl2 | 0.40 |
| 4. dNTP mix | 0.10 |
| 5. PCR Enzyme | 0.20 |
| PCR (MMX) Cocktail Final Volume | 2.00 |
| Split MMX across Tubes 1-6 | PCR (MMX) Cocktail Final Volume/6 |
| 6. PCR Primers (1-6 accordingly) | 1.00 |
| PCR Cocktail (MMX + Primer) Final Volume | 3.00 |
| Sample DNA/Positive Control/Negative Control | 2.00 |
| PCR Total Reaction Volume | 5.00 |

**NOTE:** reagent volumes on the worksheet allow for 25% overage **NOTE:** The worksheet calculates volumes accordingly 1. Bring the Master Mix in the cold block into Room 2 for dispersal.

**NOTE:** If for any reason transfer of the master mix is delayed, place the entire block in the fridge.**NOTE:** Leave the Master Mix Calculator worksheet in Room 1 for SAP and iPLEX Pro Extension Cocktail prep below.  |
| **Assay Procedure: Master Mix Dispersal (Room 2)** | 1. Take the sample plate out of the fridge, carefully remove seal and change gloves.
2. Using a multi-channel pipet, add 3.0 uL of master mix to the sample plate according to the map, changing tips each time.
3. Seal the PCR reaction plate, briefly pulse vortex 1-2 times, then do a quick spin in the centrifuge by bringing up to 1200 rpm.

**NOTE:** Crease all four sides with the paddle. **NOTE:** Visually inspect the individual wells from the bottom of the reaction plate to confirm uniformity before continuing. 1. Place the plate in the cold block and move to Room 3 to start thermal cycling.
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| **Assay Procedure: PCR Amplification (Room 3)** | **NOTE:** Turn on the thermocycler so that the heated cover can come to the correct operating temperature and there is no time delay between the PCR reaction plate being prepped and cycling being started. 1. Place plate in the thermocycler (rotate usage). Run program **DICER-**PCR. See the Table below for program details.

**NOTE:** Total volume: 5.0 uL**NOTE:** Cycle program takes approximately 1 hour and 45 minutes

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| **Step** | **Temperature** | **Time** | **Number of Cycles** |
| **1** | Initialization | 95°C  | 5 minutes | 1 cycle |
| **2** | Denaturation | 95°C  | 30 seconds |   |   |
| **3** | Annealing | 56°C  | 30 seconds | 45 cycles of steps 2-4 |
| **4** | Extension | 72°C  | 1 minute |   |   |
| **5** | Final extension | 72°C  | 5 minutes | 1 cycle |
| **6** | Sample preservation | 4°C  | -- | Hold |

**NOTE:** If not proceeding directly to the next step, the reaction plate should be sealed and stored at 4 °C – 10 °C (if storing for less than 24 hours), or at -20 °C (if storing for more than 24 hours). Do not store for more than 2 weeks.  |
| **Assay Procedure: Shrimp Alkaline Phosphatase (SAP) Cocktail Preparation (Room 1)** | 1. Pull the reagents listed in the Table below to thaw to room temp. Vortex all reagents except enzyme (flick) and quick spin.

**NOTE:** Leave enzymes in the freezer until use, and keep in a cold block when pipetting.1. In a cold block, prepare the SAP cocktail in a labeled 1.5 mL tube by adding reagents in order, as listed in the table below.

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| **Reagent** | **Per reaction (uL)** |
| 1. HPLC-grade water | 1.53 |
| 2. SAP Buffer | 0.17 |
| 3. Shrimp Alkaline Phosphatase (SAP) | 0.30 |
| **SAP Cocktail Final Volume** | 2.00 |

**NOTE:** reagent volumes on the worksheet allow for 25% overage**NOTE:** The worksheet calculates volumes accordingly1. Pulse vortex the tube briefly 3 times and quick spin.
2. Label 0.2 mL PCR strip tubes and place in cold block.
3. Evenly aliquot SAP master mix into strip tubes according to calculator.
4. Immediately bring to Room 3 for dispersal.

**NOTE:** Leave the Master Mix Calculator worksheet in Room 1 for iPLEX Pro Extension Cocktail prep below. |
| **Assay Procedure: SAP Cocktail Addition to Samples (Room 3)** | **NOTE:** If plates were stored frozen prior to this step, make sure they are thawed completely, gently homogenized, spun down, and kept in a cold block**NOTE:** Clean hood and supplies with a 1:10 bleach dilution or wipes followed by water and alcohol before and after adding the cocktail 1. Perform a quick spin of the PCR reaction plate in the centrifuge by bringing up to 1200 rpm.
2. Remove and discard seal. Change gloves.

**NOTE:** Take care when removing seal: do not let it curl back onto the plate or fold into itself. 1. Using a multi-channel pipet, dispense 2.0 uL of SAP cocktail into each well of the reaction plate, changing tips each time.
2. Seal the reaction plate, briefly pulse vortex 1-2 times, then perform a quick spin in the centrifuge by bringing up to 1200 rpm.

**NOTE:** Crease all four sides with the paddle.**NOTE:** Visually inspect the individual wells from the bottom of the reaction plate to confirm uniformity before continuing. 1. Place the plate with compression pad in the thermocycler and run program **DICER-SAP**. See the table below for program details.

**NOTE:** Total volume: 7.0 uL**NOTE:** Cycle program takes approximately 45 minutes

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| **Step** | **Temperature** | **Time** | **Number of Cycles** |
| **1** | Dephosphorylation | 37°C  | 40 minutes | 1 cycle |
| **2** | Enzyme inactivation | 85°C  | 5 minutes | 1 cycle |
| **3** | Sample preservation | 4°C  | -- | Hold |

**NOTE:** If not proceeding directly to the next step, the reaction plate should be sealed and stored at 4 °C – 10 °C (if storing for less than 24 hours), or at -20 °C (if storing for more than 24 hours). Do not store for more than 2 weeks.  |
| **Assay Procedure: iPLEX Pro Extension Cocktail Prep****(Room 1)** | 1. Pull the reagents listed in the Table below to thaw. Vortex all reagents except enzyme (flick) and quick spin.

**NOTE:** Leave enzymes in the freezer until use, and keep in a cold block when pipetting.1. Label 7 1.5 mL tubes, one MMX and the remaining 1-6, place in cold block
2. In a cold block, prepare the iPLEX Pro Extension master mix (cocktail) in the MMX labeled 1.5 mL tube by adding the reagents in order, as listed in the table below. When opening a new box, write the number 1 through 4 on the top of each vial as corresponds to the table below.
3. Once the Extension (MMX) cocktail has been prepared, evenly distribute into Tubes 1-6 according to the calculator.
4. Add each Extend Primer Set to corresponding labeled tube.
5. Vortex and spin tubes briefly.
6. Label 0.2 mL PCR strip tubes with designated primer set and place in cold block.
7. Evenly aliquot cocktail from Tubes 1-6 into appropriately labeled strip tube according to the calculator worksheet.
8. Cap tubes and spin briefly.

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| **Reagent** | **Per reaction (uL)** |
| 1. HPLC-grade water | 0.62 |
| 2. iPLEX Buffer Plus, GPR | 0.20 |
| 3. iPLEX Termination Mix | 0.20 |
| 4. iPLEX Pro Enzyme | 0.04 |
| **Extension (MMX) Cocktail Final Volume** | 1.06 |
| **Split MMX across Tubes 1-6** | Extension Cocktail Final Volume/6 |
| **EXTEND Primers (1-6 accordingly)** | 0.94 |
| **Extension (MMX + Primers) Final Volume** | 2.00 |

**NOTE:** reagent volumes on the worksheet allow for 25% overage**NOTE:** The worksheet calculates volumes accordingly1. Bring the Master Mix in the cold block into Room 3 for dispersal.

**NOTE:** If for any reason transfer of the master mix is delayed, place the entire block in the fridge.  |
| **Assay Procedure: iPLEX Pro Extension Cocktail Addition to Samples (Room 3)** | **NOTE:** If plates were stored frozen prior to this step, make sure they are thawed completely, gently homogenized, spun down, and kept in a cold block**NOTE:** Clean hood and supplies with a 1:10 bleach dilution or wipes followed by water and alcohol before and after adding the cocktail 1. Perform a quick spin of the PCR reaction plate on the centrifuge by bringing up to 1200 rpm.
2. Remove and discard seal. Change gloves.

**NOTE:** Take care when removing seal: do not let it curl back onto the plate or fold into itself. 1. Using a multi-channel pipet, dispense 2.0 uL of extension cocktail into designated wells of the reaction plate according to plate map, changing tips each time.

 1. Seal the reaction plate, briefly pulse vortex 1-2 times, then perform a quick spin on the centrifuge by bringing up to 1200 rpm.

**NOTE:** Crease all sides, corners and between wells with the paddle.**NOTE:** Visually inspect the individual wells from the bottom of the reaction plate to confirm uniformity before continuing. 1. Place the plate along with compression pad in the thermocycler and run program **DICER-Extension**. See the table below for program details.

**NOTE:** Total volume: 9.0 uL**NOTE:** Cycle program take approximately 65 minutes

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| **Step** | **Temperature** | **Time** | **Number of Cycles** |
| **1** | Initialization | 95°C  | 30 seconds | 1 cycle |
| **2** | Denaturation | 95°C  | 5 seconds |   | 40 cycles |
| **3** | Annealing | 52°C  | 5 seconds | 5 cycles |
| **4** | Extension | 80°C  | 5 seconds |
| **5** | Final extension | 72°C  | 3 minutes | 1 cycle |
| **6** | Sample preservation | 4°C  | -- | Hold |

**NOTE:** If not proceeding directly to the next step, the reaction plate should be sealed and stored at 4 °C – 10 °C (if storing for less than 24 hours), or at -20 °C (if storing for more than 24 hours). Do not store for more than 2 weeks.  |
| **Assay Procedure: Water Addition (Room 3)** | **NOTE:** If plates were stored frozen prior to this step, make sure they are thawed completely, gently homogenized, spun down, and kept in a cold block**NOTE:** Clean hood and supplies with a 1:10 bleach dilution or wipes followed by water and alcohol before and after adding the cocktail 1. Perform a quick spin of the reaction plate on the centrifuge by bringing up to 1200 rpm.
2. Discard and remove seal. Change gloves.

**NOTE:** Take care when removing seal: do not let it curl back onto the plate or fold into itself.1. Using a multi-channel pipet, add 41.0 uL HPLC-grade water to each well of the reaction plate, changing tip each time.
2. Seal the plate and perform a quick spin of the reaction plate on the centrifuge by bringing up to 1200 rpm.

**NOTE:** Crease all four sides with the paddle.**NOTE:** If not proceeding directly to the next step, the reaction plate should be sealed and stored at 4 °C – 10 °C (if storing for less than 24 hours), or at -20 °C (if storing for more than 24 hours). Do not store for more than 2 weeks.  |
| **Preparing a run file (sample group)** | 1. Insert flash drive with the run file into the Agena MassARRAY computer.
	* 1. Open the file and select the sample IDs
		2. Copy and paste and IDs into a notepad document and save in the documents folder as the experiment/run name

**NOTE:** remove any extra characters after the last sample **NOTE: file must be saved as a .txt file**  |
| **Creating a plate in the MassARRAY Software**  | **Create a Sample Group** 1. Open the MassARRAY Typer Plate Editor software, log in.
	1. User: charles
	2. Password: darwin
2. Click on the **Sample view** tab.
3. Expand the customer and project. Right-click on the sample project that you want to add the sample group to and select **Add New Sample Group**.
4. Enter the Experiment/Run name and click the folder button in the toolbar to browse to the location of your sample group text file, and click **Open**, then click **OK**. Check sample IDs and QC IDs against plate set up sheet for accuracy.

**NOTE:** properties – select **“Horizontal”** and **“Keep Selected Region True”****Create a Project & Plate**1. In the MassARRAY Typer Plate Editor software, click on the **Plate tab**.
2. Create a plate.
	1. Right-click on the project that you want to add the plate to and select **New Plate**.
	2. Enter the Experiment/Run name and select the plate type (96- or 384-well) and click **OK**.
	3. The new plate will appear in the Plate tab and a plate layout will be created automatically, based on the plate type specified.

**Apply Assays to the Plate**1. In the **Plate tab**, select the plate that was just created.
2. Select the **Assay tab** and in the plate layout, select the wells of interest.
3. Expand the assay design file.
4. Right click on the test tube “1” icon and select add plex 1.

**Apply Samples to the Plate**1. Click on the **Sample tab**.
2. In the plate layout, select the wells of interest.
3. Right-click the sample or sample group that you want to assign to the selected wells and select **Apply Samples from Group** or **Add Sample**.
4. Check plate locations with sample IDs and QC IDs against plate set up sheet for accuracy.
5. Select **File** > **Save** from the toolbar.
 |
| **Assay Procedure: Creating an Input File (Room 3)** | 1. Double-click the **Chip Linker** icon on the desktop.
2. In the dialog box that appears, enter your username, password, and server.
	1. User: charles
	2. Password: darwin
3. Click **Connect**. The Chip Linker window appears.
4. Select a plate in the Chip Linker directory tree.
5. Select **iPLEX** as the terminator chemistry.
6. Select **Allelotype** for the process method.
7. Select **Nanodispenser 96 to 96** as the dispenser method.
8. Enter the Experiment/Run name.
9. Enter the SpectroCHIP Array barcode or other SpectroCHIP Array identifier. Record chip ID on the Run Worksheet.
10. Click **Add**. The input information appears in the Chip Linker table.
11. If a second SpectroCHIP Array will be processed, repeat step 4 to step 10 for the second SpectroCHIP Array.
12. Click **Create** to create an input XML file. This file will be selected for use when you set up the automatic run.
 |
| **Assay Procedure: Preparing the Instrument (Room 3)** | **NOTE:** Ensure the daily maintenance has been performed prior to advancing. 1. In the Status section in the **Run Setup** tab of SpectroACQUIRE check the Waste Tank, System Fluid, and Resin buttons; they should be green/Okay. If any are red (Waste Tank Full, System Fluid Empty, Resin Low or Empty) perform the necessary maintenance.
2. Click **Chip prep module Deck In/Out** at the top of the SpectroACQUIRE window. The deck will extend.
3. If there are SpectroCHIP Arrays in the completed chips position on the deck remove them.

**NOTE:** the completed position is the deck that is closer to you1. Allow the calibrant to equilibrate to room temperature for 5 minutes (if it has been refrigerated) or 10 minutes (if it has been frozen). Quick spin and pipette 75 μL of calibrant into the calibrant vial and place in the calibrant vial holder on the deck.
2. Load the SpectroCHIP Arrays.
	1. Orient the Chip holder so that the beveled corner is at the top right.
	2. Open a new SpectroCHIP Array pouch and insert the new SpectroCHIP Array into the chip holder in position 1 (on the left); orient the SpectroCHIP Array such that the Agena logo and barcode are at the **bottom**. Make sure that the SpectroCHIP Array is properly seated so that the chip holder and SpectroCHIP Array surface are flush.

**NOTE:** Use flat tweezer for transfer and pointed tweezer for any necessary adjustments **NOTE:** Care must be taken to avoid touching the surface**NOTE:** Chips may be sent on counter on top of a kim wipe **NOTE:** Save the SpectroCHIP Array pouch and holder if chip will not be completely used. Write the open date on the pouch. * 1. If you are processing two plates, insert another new SpectroCHIP Array into the chip holder in position 2 (on the right). If you are only processing one plate, place a previously completed SpectroCHIP Array in position 2, as the MassARRAY Analyzer requires both SpectroCHIP Array positions to be filled to function properly.
	2. Place the full chip holder in the chip holder tray, new chips position, on the deck.
1. Load microtiter plates (MTPs). Up to two 96-well microtiter plates of analyte may be loaded.
	1. Quick spin the microtiter plate(s) at ~1200 – 1500g
	2. Remove the seal, change gloves, and place the first MTP on MTP holder 1 (on the left). This corresponds to chip position
		1. Orient the plate such that well A1 is in the front left corner (match to label on instrument loading deck)
	3. If a second MTP is being processed, place it on MTP holder 2 (on the right). This corresponds to chip position 2.
2. Click **Chip prep module Deck In/Out** again to retract the deck.
 |
| **Assay Procedure: Setting up and Starting the Run (Room 3)** | 1. Select the **Run Setup** tab in SpectroACQUIRE.
2. In the Analyzer Setup section:
	1. Select **Tools** > **Load Parameters** on the SpectroACQUIRE toolbar, then select the appropriate parameter file based on the SpectroCHIP type (**iplex\_CPM.par**). Acquisition parameters will automatically populate.
	2. In the Experiment Setup section:
		1. Under MTP 1, click on the browse button next to Experiment Name and select the XML input file created earlier.
		2. In the Wells to Process field, select **Automatic**.
		3. Repeat steps 1 and 2 for MTP 2 if running two plates. Otherwise, select **None** in the Experiment Name and Wells to Process fields for MTP 2.
	3. Make sure all settings are as shown in the Table below.

|  |  |
| --- | --- |
|  | **SpectroCHIP CPM-96** |
| **Use Autotune** | NOT selected |
| **Start Dispense Condition** | 600 |
| **Resin Volume** | 13 |
| **Sample Volume** | n/a |
|  |  |
| **Shots (n)** | 30 |
| **Maximum acquisitions** | 9 |
| **Minimum good spectra** | 5 |
| **Maximum good spectra** | 5 |
| **Turn off HV After Analysis** | Selected |
| **Analyze Calibrant Pads** | Selected |
| **Filter Saturated Shots** | NOT selected |
| **Chip Type** | SpectroCHIP CPM-96 |

1. In the Chip Prep Module Setup section:
	1. Check the **Normal Operation** box.
		1. If NOT running 2 plates: unselect “Transfer Resin to MTP2”

**NOTE:** if rerunning a plate that has already been spotted unselect “Transfer Resin to MTP1 and/or MTP2”* 1. Select **iPLEX** in the Chemistry drop-down menu.
1. In the Temperature Control section:
	1. If the run will sit on the instrument overnight, select the **MTP Cool** box, and check the **Auto** box.
	2. If using a SpectroCHIP CPM-96, check the **Chip Heat** box and enter **30** in the Setpoint field.
2. Click **Start Chip prep module** to start the run.
 |
| **Assay Procedure: Removing plates, calibrant and SpectroCHIP Array when run is complete**  | 1. Once the run is completed, click **Chip prep module Deck In/Out** to move the deck out and remove the MTPs and calibrant vial. Store remaining calibrant refrigerated or frozen for future use.
	1. Pipette remaining calibrant into a 2 mL cryovial labeled with the clibrant lot number, expiration date, and date the vial was created
	2. Rinse calibrant tube with DI water:
		1. Pipette 100 uL DI water and mix 5-8 times
2. Click **Remove Old Chips from MA4** in the Run Setup tab. The instrument will move the completed SpectroCHIP Arrays from the MassARRAY Analyzer to the completed chips position on the Chip Prep Module deck, and then extend the deck so you may remove the SpectroCHIP Arrays from the completed chips position.

**NOTE:** save incompletely used chips in saved holder and pouch with the desiccant. Write the open date and which wells have been used on the outside of the pouch. Seal with tape. 1. Click **Chip prep module Deck In/Out** to move the deck in.
 |
| **Assay Procedure:** **Recalling Chip to Create a Genotype+Area Report**  | 1. Open MassARRAY Typer Analyzer.
	1. Login:
		1. User: charles
		2. Password: Darwin
2. Open Chip Linker:
	1. Settings: iPlex, Genotype+Area, Nanodispenser 96 to 96
	2. Name the experiment exactly as the Allelotype run
	3. Enter Chip ID
3. Click Recall and select the correct chip from the pop-up window
4. Close and re-open Typer to display recalled chip
5. Find run according to date
6. Double click the recalled chip
7. Check the box
8. File -> OncoPanel Reports -> Chmn\_Dicer1.v1.0-v1
9. Name as recalled chip
 |
| **Analyzing Data**  | 1. Open MassARRAY Typer Analyzer.
	1. Login:
		1. User: charles
		2. Password: darwin
2. In the Project Explorer pane double click on the Allelotype (blue font) and Genotype+Area (magenta font) SpectroCHIP Arrays. The SpectroCHIP Arrays will be added to the Chip List.
3. Load the SpectroCHIP Arrays by checking the box next to the SpectroCHIP Array names in the Chip List.
4. Save a picture of the GenotypeArea "traffic light" diagram in an appropriately dated folder under C:MassArray/Typer/DICER1 Research Reports .
	1. Note: If any well is NOT dark green (except for wells with known synthetic controls or NTC) notify the molecular pathologist. Ensure optimal threshold (dark green) is set at 100.

 1. Ensure positive, negative, and no template controls gave expected results.
	1. Note: If any control is not as expected, notify the molecular pathologist and save original plate and chip for potential troubleshooting and/or repeat run.
2. While still on the GenotypeArea chip, select **File > Reports > Typer Reports > GenotypeArea Report-v1** in the MassARRAY Typer Analyzer menu bar.

**NOTE:** When the report is complete, it will automatically open in OpenOffice. Save this file as a .xls in an appropriately dated folder in the same folder as the GenotypeArea traffic light picture above.1. Load the Allelotype chip and select **File > Reports > OncoPanel Reports >** **CHMN\_DICER1\_v1.0-v1** in the MassARRAY Typer Analyzer menu bar. Save these files in the same folder as the GenotypeArea traffic light picture and report as above.
2. Save the dated folder containing the GenotypeArea traffic light picture and report and OncoPanel reports to a USB thumb drive.

 1. Save results to monthly folder located here: G:\LAB\Molecular\Molecular Pathology\Agena - Dicer 1\Results
2. Print traffic light image, "Assay Summary" from GenotypeArea report and OncoPanel "mutationList" report (open as Excel file by dragging .tsv into open blank Excel sheet; save file as .xls, expand columns).
	1. Condense columns where possible
	2. Select the region to print
	3. Select Add All borders
	4. File -> Print
	5. Print: Selection
	6. Portrait Orientation: Landscape
	7. Margins: Narrow
	8. Scaling: Fit all columns on one page
3. Place printed materials in appropriately dated folder and bring to molecular pathologist for sign out. Also notify pathologist of results available for review.

  |
| **Result Interpretation** | Interpretation of the results is performed by the molecular pathologist on service.**Quality control and validity of results**1. One negative patient sample and one no template control contamination controls for each primer set and one positive patient sample (or synthetic positive sample if patient sample is not available; rotating through a positive for one primer set each run) amplification control are processed with each run.

**NOTE:** If any control fails, notify the molecular pathologist and store plates and chips while waiting for further instructions.

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**NOTES on well colors:**There are 6 assays in each well. The color shown in the Typer software relates to the number of targets that were called (AKA: performed). Green = 100% called Light Green = 99% – 50% called Yellow = 49% - 16% called Red = 15% or less called  |
| **Sample Storage** | **Storage and Retention of Test Specimens**1. Store eluates in positive or negative box (based on results) in the -80 °C freezer in Room 2.
2. Store completed and sealed 96-well plates in the -20 °C freezer in Room 3.
3. Discard samples in red biohazard container after molecular pathologist approval.
 |
| **Equipment and Room Decontamination** | **Refer to:** [MB 3.03 Cleaning and Decontamination of Equipment and Work Areas](https://starnet.childrenshc.org/References/labsop/molbio/engctl/mb-3.03-cleaning-and-decontamination-of-equipment-and-work-areas.pdf) |
| **Limitations** | 1. The performance characteristics of the MassARRAY DICER1 panel have been evaluated by Children’s MN Laboratory. This is a RESEARCH USE ONLY assay.
2. All results from this and other tests must be considered in conjunction with the clinical history, epidemiological data, and other data available to the clinician evaluating the patient.
3. Samples must be collected, transported, and stored using appropriate procedures and conditions. Improper collection, transport, or storage of samples may hinder the ability of the assay to detect the target sequences.
4. The performance of the DICER1 Agena MassARRAY Research Panel was established using extracted DNA from formalin fixed, paraffin-embedded tissue samples.
5. This test is a qualitative test and does not provide the quantitative value of detected alleles present.
6. There is a risk of false positive values resulting from:
	1. Non-specific signals in the assay.
	2. Cross-contamination during sample handling or preparation.
	3. Cross-contamination between patient samples.
	4. Sample mix up.
	5. DNA contamination during product handling.
7. There is a risk of false negative values due to:
	1. The presence of sequence variants in the regions of interest of the assay, procedural errors, amplification inhibitors in samples, or inadequate tumor cellularity for amplification.
	2. Improper sample collection/preservation.
	3. Sample mix up.
	4. Degradation of sample DNA during shipping/storage.
	5. The presence of PCR inhibitors.
8. This test cannot rule out potentially clinically relevant variants in parts of the genome not specifically targeted by the assay.
9. Negative results do not preclude low-level clinically relevant variants and should not be the sole basis of a patient management decision.
10. This panel has been evaluated for use with human sample material only.
 |
| **Method Performance Specifications** | **In-house performance (per the assay validation studies): 2x2 Table, FFPE Samples – overall arbitrated results**

|  |  |
| --- | --- |
| Agena | Expected result |
|  | **Positive**  | **Negative** |
| Positive  | 37 | 1 |
| Negative | 0 | 42 |

**Results (95% CI):** **Positive Percent Agreement:** 100% (90.51% - 100.00%)**Negative Percent Agreement:** 97.67% (87.71% - 99.94%)**Overall Percent agreement:** 98.75% (93.23% - 99.97%)**LoD:** 0.02 TCID50/mL |
| **References** | 1. Agena iPLEX Pro and Gold Reagents User Guide, USG-CUS-071 Rev03, San Diego, CA: Agena Bioscience; 2019.2. QIAamp DNA FFPE Tissue Handbook, HB-0353-004, Germantown, Maryland: Qiagen; 20203. MedlinePlus [Internet]. Bethesda (MD): National Library of Medicine (US); [updated 2022 Feb 9]. DICER1 syndrome; [updated 2020 Aug 18; reviewed 2016 May 01; cited 2022 Feb 9]; [about 5 p.]. Available from: <https://medlineplus.gov/genetics/condition/dicer1-syndrome/>.  |
| **Alternate Methods** | 1. Send out test
 |
| **Proficiency Testing** | Not applicable. Research Use Only assay. |
| **Training Plan/ Competency Assessment** | **Training Plan** | **Initial Competency Assessment** |
| 1. Employee must read the procedure.
2. Employee will demonstrate the ability to perform procedure, record results, and document corrective action after instruction by the trainer.
 | 1. Direct observation
 |
| **Historical Record** |  |  |  |  |
|  | **Version** | **Written/Revised by:** | **Effective Date:** | **Summary of Revisions** |
| 1 | Kristi Prokop /Damon Olson | 06/09/2023 | Initial Version |
| **Archived by:** |  | **Archived date:** |  |
|  |  |  |  |  |