RUO

Instructions for Use

NPM1 MRD Assay

For detection of mutations in the nucleophosmin (*NPM1*) gene.

RUO For Research Use Only. Not for use in diagnostic procedures.

invivoscribe

Catalog #	Products	Quantity
REF 14160019	*NPM1* MRD Assay	96 Reactions

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1. Assay Use

The *NPM1* MRD Assay is a research use only (RUO) product intended for PCR-based detection of *NPM1* mutation in people with acute myeloid leukemia (AML). Specifically, the Assay identifies insertions in exon 12 of the *NPM1* gene.

2. Summary and Explanation of the Test

2.1. Summary

The *Nucleophosmin* (*NPM1*) gene encodes for a protein involved in cellular activities that may relate to proliferative and growth-suppressive roles in the cell.[1] As one of the most commonly mutated genes in AML, *NPM1* gene mutations occur in about one-third of the cases of primary AML in adults[2] and lead to increased blast counts, higher extramedullary involvement, increased platelet counts, and abnormal cytoplasmic localization of NPM1 protein.[1] The vast majority of *NPM1* gene mutations result in a four base pair nucleotide insertion at the position encoding the 288th amino acid residue resulting in a frame shift of the C-terminus on chromosome 5.[1]

In 2016, the World Health Organization (WHO), officially recognized AMLs containing an *NPM1* gene mutation as a distinct AML subtype.[3] Subjects with *NPM1* mutations (*NPM1*+), in the absence of a *FLT3* gene mutation, obtained the highest remission rates and have been associated with a higher complete remission[4] compared to cytogenetically normal AML (CN-AML) subjects whom did not have the *NPM1* mutation.[4,5] It has been suggested that the identification of mutations in both *NPM1* and *FLT3* genes allows for the stratification of the CN-AML subjects into three different prognostic groups with the most favorable prognosis associated with *NPM1*+ and *FLT3*-.[4,5]

Utilizing both *NPM1* and *FLT3* mutation status is the most common method in stratification of the CN-AML population. The ability to sensitively and specifically detect measurable residual disease (MRD) in leukemia subjects has proven to be useful in the clinical management of select subtypes of the disease.

2.2. Explanation

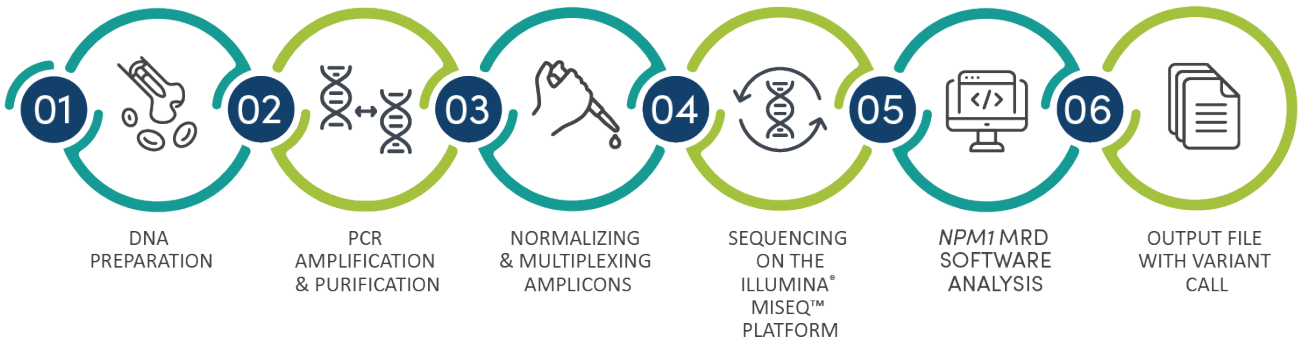
The *NPM1* MRD Assay is designed to detect the presence of four base pair mutations within exon 12 of the *NPM1* gene in a given human DNA sample. The RUO *NPM1* MRD v1.1.1 Software will report mutation variant 'A', 'B', 'D', and 'Other' for mutations other than the aforementioned types. The test is an amplicon-based approach which uses polymerase chain reaction (PCR) to amplify the region of interest and next-generation sequencing (NGS) to detect the region of interest. Primers included in the master mixes are designed with Illumina® adapters containing unique indexes. This method allows for pooling of amplicons from several different samples onto one MiSeq™ flow cell, allowing for up to 24 samples to be analyzed in parallel in a single run.

The assay includes twenty-four dual-indexed PCR master mixes, along with positive and negative controls. The *NPM1* MRD Assay was designed with 24 unique index combinations, labeled as IA or IB.

The associated *NPM1* MRD v1.1.1 Software provides a simple and streamlined method of analysis.

3. Principles of the Procedure

Figure 1: Workflow Summary



3.1. Mutations of *NPM1*

NPM1 mutations are caused by an insertion of four nucleotides in exon 12 of the *NPM1* gene. Primers targeting the area surrounding exon 12 of the *NPM1* gene are used to amplify the DNA. The mutation type and sequence of the *NPM1* PCR product is determined by next-generation sequencing and bioinformatic analysis.

3.2. Next-Generation Sequencing (NGS)

NGS technologies used in this assay rely on the amplification of genetic sequences using forward and reverse primers that include adapter and index tags. Amplicons generated with the *NPM1* MRD Assay are quantified, pooled, and loaded onto a flow cell for sequencing with an Illumina® MiSeq™ sequencing platform. Specifically, the amplified products in the library are hybridized to oligonucleotides on a flow cell and are amplified to form local clonal colonies (bridge amplification). Four types of reversible terminator bases (RT-bases) are added and the sequencing strand of DNA is extended one nucleotide at a time. To record the incorporation of nucleotides, a CCD camera takes an image of the fluorescence as each RT-base is added, and then cleaved to allow incorporation of the next base.

3.3. Sample Multiplexing

This product was designed to allow for two different levels of multiplexing in order to reduce costs and time for laboratories. The first level of multiplexing originates from the multiple indices that are provided with the assays. Each of the 24 dual-indexed master mixes acts as a unique barcode that allows amplicons from individual samples to be pooled together after PCR amplification to generate the sequencing library; the resulting sequences are sorted by the bioinformatic software, which identifies those that originated from an individual sample.

The second level of multiplexing originates from the ability to pool amplicons generated from multiple targets together, such as amplicon generated using Invivoscribe's *FLT3* ITD MRD Assay ((REF 14120019), to generate the library to be sequenced on a single flow cell (it is extremely important that each sample in the pool must have a unique index). **When multiplexing amplicons of different gene targets it is important to use the appropriate sequencing chemistry. The number of sequencing cycles must be sufficient to sequence the largest amplicon in the multiplex.** For example, when multiplexing a combination of *NPM1* MRD Assay and *FLT3* ITD MRD Assay amplicons together, use the MiSeq™ Reagent Kit v3 (600 cycle).

The number of samples that can be multiplexed onto a single flow cell is also dependent on the flow cell that is utilized. Illumina's standard flow cells (MiSeq™ Reagent Kit v3) can generate 22-25 million reads. To determine the number of reads per sample, divide the total number of reads for the flow cell by the number of samples that will be multiplexed.

3.4. Measurable Residual Disease Evaluation

The *NPM1* MRD is an NGS-based deep sequencing assay that can reliably identify DNA sequences specific to previously identified mutations at an allelic sensitivity level of 5×10^{-5}.

4. Reagents and Materials

NOTE: The *NPM1* MRD Assay Kit, Table 1, is usable until the labeled kit expiration date when stored as described:

Reagents provided within this kit may incur up to 5 freeze-thaw cycles without measurable loss of performance. To reduce the number of freeze-thaws, aliquot the Positive and Negative Controls appropriately.

Table 1: Reagent List of the *NPM1* MRD Assay Kit (REF 14160019)

Catalog Number	Reagent Name	Storage Temperature	Unit Quantity	Vials/ Kit
REF 24160009	***NPM1* MRD IA01**		75 µL/Vial	1
REF 24160019	***NPM1* MRD IA02**		75 µL/Vial	1
REF 24160029	***NPM1* MRD IA03**		75 µL/Vial	1
REF 24160039	***NPM1* MRD IA04**		75 µL/Vial	1
REF 24160049	***NPM1* MRD IA06**		75 µL/Vial	1
REF 24160059	***NPM1* MRD IA07**		75 µL/Vial	1
REF 24160069	***NPM1* MRD IA08**		75 µL/Vial	1
REF 24160079	***NPM1* MRD IA09**		75 µL/Vial	1
REF 24160089	***NPM1* MRD IA10**		75 µL/Vial	1
REF 24160099	***NPM1* MRD IA11**		75 µL/Vial	1
REF 24160109	***NPM1* MRD IA12**		75 µL/Vial	1
REF 24160119	***NPM1* MRD IA25**		75 µL/Vial	1
REF 24160129	***NPM1* MRD IB13**		75 µL/Vial	1
REF 24160139	***NPM1* MRD IB14**		75 µL/Vial	1
REF 24160149	***NPM1* MRD IB15**		75 µL/Vial	1
REF 24160159	***NPM1* MRD IB16**		75 µL/Vial	1
REF 24160169	***NPM1* MRD IB17**		75 µL/Vial	1
REF 24160179	***NPM1* MRD IB18**		75 µL/Vial	1
REF 24160189	***NPM1* MRD IB19**		75 µL/Vial	1
REF 24160199	***NPM1* MRD IB21**		75 µL/Vial	1
REF 24160209	***NPM1* MRD IB22**		75 µL/Vial	1
REF 24160219	***NPM1* MRD IB23**		75 µL/Vial	1
REF 24160229	***NPM1* MRD IB24**		75 µL/Vial	1
REF 24160239	***NPM1* MRD IB26**		75 µL/Vial	1
REF 40880048	***NPM1* MRD POS (+)**		500 µL/Vial	2
REF 40920048	***NPM1* MRD NEG (-)**		500 µL/Vial	2

Table 2: Additional Reagents, Materials, and Equipment Required (Not Provided)

Reagent/Material	Suggested Reagents / Materials and Suppliers	Catalog No.	Notes
Taq Polymerase	Invivoscribe • FalconTaq™ DNA Polymerase	60970130	N/A
Molecular Biology Grade Water	N/A	N/A	Sterile, RNase/DNase -free
PCR Purification	Beckman Coulter • AMPure® XP Reagent for PCR Purification	N/A	N/A
Amplicon Quantification	N/A	N/A	N/A

Table 2: Additional Reagents, Materials, and Equipment Required (Not Provided)

Reagent/Material	Suggested Reagents / Materials and Suppliers	Catalog No.	Notes
Lab-grade Ethanol (200 Proof)	N/A	N/A	N/A
1N NaOH	N/A	N/A	N/A
PhiX	Illumina® • PhiX Sequencing Control v3	FC-110-3001	N/A
Calibrated Pipettes	N/A	N/A	Must be able to accurately measure volumes between 0.5 µL and 1000 µL.
Filter Barrier Pipette Tips	N/A	N/A	Sterile, RNase/DNase/Pyrogen-free
PCR Plates or Tubes	N/A	N/A	Sterile, Skirted Plates
PCR Strip Caps or Foil Seal	N/A	N/A	N/A
DNA Low-Binding Tubes	N/A	N/A	DNA, DNase, RNase and PCR inhibitor free
Vortex Mixer	N/A	N/A	N/A
Microcentrifuge	N/A	N/A	N/A
Thermal Cycler	Thermo Fisher Scientific • Veriti™ 96-Well Fast Thermal Cycler	N/A	N/A
Magnetic Plate	Life Technologies • Magnetic Stand 96	N/A	N/A
MiSeq™	Illumina® • MiSeq™ Reagent Kit v3 (600-cycle)/ • MiSeq™ Reagent Kit v3 Box 1/ • MiSeq™ Reagent Kit v3 Box 2	MS-102-3003	Standard flow cell
MiSeq™ Software	MiSeq™ Control Software (MCS)	N/A	v2.6.2.1 or later

5. Software

5.1. *NPM1* MRD v1.1.1 Software

The *NPM1* MRD v1.1.1 Software (REF 14160029) will be provided via a universal serial bus (USB) where the customer will download and install the software in their local hardware infrastructure. The customer is provided the download and end-user instructions for the installation and execution of the software.

5.1.1. Required Equipment

5.1.1.1. A Linux server with the following minimum requirements:

- Processor: Minimum of 16 cores required
- Hard Drive: At least 50 GB of free disk space
- RAM: 64 GB required; 128 GB or more recommended
- Operating System: Ubuntu release 18.04+
- All *NPM1* MRD v1.1.1 Software dependencies must be installed:
  - Docker installed on host machine

6. Warnings and Precautions

Please read the Instructions for Use carefully prior to starting the assay procedure and follow each step closely.

- RUO This product is for Research Use Only.
- The assay is only validated for use on the MiSeq™ with MCS. The assay must be used as a system. Do not substitute other manufacturers' reagents.
- Dilution, reducing amplification reaction volumes, or other deviation in this protocol may affect the performance of this test and/or nullify any limited sublicense that comes with the purchase of this testing kit.
- Do not mix or combine reagents from kits with different lot numbers.
- Materials are stable until the labeled expiration date when stored and handled as directed. Do not use kits beyond their expiration date.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.
- Track the number of freeze thaw cycles.
- Perform all laboratory procedures with standard personal protective equipment (gloves, laboratory coats, and protective eye wear). Follow good laboratory practices and universal precautions when working with specimens. Do not pipette by mouth. Do not eat, drink, or smoke in laboratory work areas. Wash hands thoroughly after handling specimens and assay reagents. Handle specimens in approved biological safety containment facilities and open only in certified biological safety cabinets.
- Due to the analytical sensitivity of this test, use extreme care to avoid the contamination of reagents or amplification mixtures with samples, controls, or amplified materials. Use fresh, aerosol-resistant pipette tips between samples and between dispensing reagents. Closely monitor all reagents for signs of contamination (e.g., negative controls giving positive signals). Discard reagents suspected of contamination.
- To minimize contamination, wear clean gloves when handling samples and reagents and routinely clean work areas and pipettes prior to performing PCR.
- Autoclaving does not eliminate DNA contamination. Follow uni-directional work flow in the PCR laboratory between separate work areas; begin with specimen preparation, then to amplification, and finally to sequencing. Do not bring amplified DNA into the areas designated for specimen preparation.
- Dedicate all pipettes, pipette tips, and any equipment used in a particular area to that area of the laboratory.
- Use sterile, disposable plastic ware whenever possible to avoid RNase, DNase, or cross-contamination.
- All instruments and equipment must be maintained and calibrated per the manufacturers' recommendations.

6.1. Cyber Security Precautions

- Computers and networks are susceptible to security risk if not secured and actively updated. Proper computer and network security help ensure data is not compromised, lost, or damaged due to preventable cyber risks. Equip all computers with up to date and active antivirus software.
- Filter and secure network traffic with a firewall.
- Keep data on local computers to reduce cyber security risks that may be present in transferring sensitive data over a network.
- Install software only for the local user to prevent unauthorized use of the software.
- Ensure Windows and Adobe Acrobat Reader are always updated to the latest available security patches.
- Ensure the default PDF reader in Windows is set to Adobe Acrobat Reader. Opening sample and run reports in an internet browser may lead to cybersecurity risks of subject data.

7. Specimen Collection and Preparation

7.1. Precautions

- Biological specimens from humans may contain potentially infectious materials. Handle all specimens according to your institute's Bloodborne Pathogen program and/or Biosafety Level 2.

7.2. PCR-Interfering Substances

- Divalent cation chelators
- Low retention pipette tips
- EDTA (not significant at low concentrations)

7.3. Specimen Requirements and Stability

- This assay tests genomic DNA (DNA) from the following sources:
  - Peripheral blood or bone marrow in EDTA or sodium heparin
- Specimens can be stored at 4°C or at Room Temperature for up to 7 days after the collection date prior to DNA isolation.

7.4. Sample Preparation

7.4.1. Extract DNA from peripheral blood or bone marrow within 7 days of collection.

7.4.2. Quantify DNA samples using a fluorescence-based method specific to double-stranded DNA (dsDNA).

NOTE: Use a quantification method appropriate for dsDNA. If DNA concentration is <20 ng/µL, refer to troubleshooting guidelines in section 10. Troubleshooting.

7.4.3. Dilute an aliquot of the stock DNA to 20 ng/µL in molecular grade water using low-binding surface tubes. using Equation 1.

Equation 1: Equation to Normalize DNA

$$V_i = \frac{\left(V_f \, x \, 20 \frac{ng}{\mu L}\right)}{C_i}$$

- C_i = DNA concentration from microvolume fluorometer reading
- V_i = volume of undiluted DNA to dilute
- V_f = final volume of diluted DNA
- $V_f - V_i$ = amount of molecular grade water to add to V_i

8. Assay Procedure

8.1. PCR Setup

Perform all thermal cycler steps including installation, operation, calibration, cleaning and maintenance procedures according to the manufacturer's instructions unless stated otherwise below.

NOTE: Minimize the amount of time Taq DNA Polymerase is out of -30°C to -15°C storage.

- A Positive Control, Negative Control, and No Template Control must be included in each run. Up to 21 samples (in singlicate) may be included in one run.
- The No Template Control must be run with each PCR step using molecular grade water to verify absence of amplicon contamination.

8.1.1. Remove Control tubes (*NPM1* MRD POS (+) and *NPM1* MRD NEG (-)) from appropriate storage and thaw at room temperature.

8.1.2. Pipette 35 µL of normalized sample DNA and Controls at 20 ng/µL to a 96-well PCR plate in a staggered layout, as shown in Figure 2. For No Template Control, pipette 35 µL of molecular biology grade water.

Figure 2: Example of Staggered Samples

Key		1	2	3	4	5	6	7	8	9	10	11	12
Sample(s)	A	*NPM1* MRD IA01											
NPM1 MRD POS (+)	B		*NPM1* MRD IA06										
NPM1 MRD NEG (-)	C	*NPM1* MRD IA02											
NTC	D												
	E	*NPM1* MRD IA03											
	F												
	G	*NPM1* MRD IA04											
	H												

8.1.3. Allow the *NPM1* MRD Master Mixes to thaw; then gently vortex to mix and centrifuge.

8.1.4. Perform the remaining PCR plate setup on a cold block.

8.1.5. Cover all of the wells except the working column, i.e. column 1, with a foil seal.

- To prevent accidental cross contamination of the indexes, the remaining **PCR reagents must be added one column at a time, one reagent at a time.** All reagents will be added per column, and the column sealed before moving onto the next.

8.1.6. Opening only one Master Mix at a time, for each exposed PCR reaction, pipette 15 µL of the respective PCR Master Mix.

- This will yield a total volume of 50 µL per well. Use a Master Mix with a unique sequencing index for each DNA sample or Control in the run.

NOTE: It is very important to only have one Master Mix tube open at a time to prevent cross contamination.

8.1.7. Pipette 1 µL of Taq DNA Polymerase to each exposed well.

8.1.8. Cap the working column with 8-well strip caps, ensuring a tight seal over each well.

8.1.9. Repeat starting from section 8.1.5 for the remaining plate columns.

8.1.10. Gently vortex the capped PCR plate and quickly centrifuge to gather all droplets to the bottom of the wells.

- When vortexing try to keep the reaction in the bottom of the tube and off the strip caps.

8.1.11. Place the PCR plate in a pre-heated thermal cycler and close the lid.

8.1.12. Amplify the PCR plate according to the PCR program in Table 3.

- If not immediately continuing onto the next steps, the PCR product plate may be stored at 4°C for up to 72 hours.

Table 3: PCR Amplification Thermal Cycler Program

Step	Temperature	Time	Cycles
1	95°C	7 minutes	1
2	95°C	45 seconds	25x
3	60°C	45 seconds	
4	72°C	90 seconds	
5	72°C	10 minutes	1
6	4°C-15°C	∞	Hold
Ramp rate: 2.9°C/second			

NOTE: Set the heated lid to 105°C and the reaction volume to 50 µL

8.2. First PCR Product Purification (0.8x Cleanup)

NOTE: AMPure® XP Reagent for PCR purification is viscous and needs to be pipetted slowly. Ensure beads are homogenized prior to pipetting.

8.2.1. Remove AMPure® XP Reagent from storage and allow to equilibrate to room temperature for at least 30 minutes before use.

8.2.2. Transfer the appropriate volume of AMPure® XP Reagent needed to a new reservoir to minimize the risk of contamination by pipette tips.

8.2.2.1. The required volume of AMPure® XP Reagent = (n + 2) × 40 µL (n is number of samples/controls to be purified).

8.2.3. Prepare 80% ethanol using 100% ethanol and molecular biology grade water (0.5 mL for each sample/control to be purified).

8.2.4. Remove the PCR product plate from the thermal cycler or 4°C storage and centrifuge to ensure all liquid is at the bottom of the wells.

8.2.5. Pipette 40 µL of the aliquoted, room temperature AMPure® XP Reagent to each PCR product to be purified. Mix by pipetting up and down 10 times. The color of the mixture should appear homogenous after mixing.

8.2.6. Incubate 5 minutes at room temperature.

8.2.7. Place the mixed samples/controls on a 96-well plate magnet and allow the magnetic particles to separate for 2 minutes.

- Keep the plate on the magnetic stand at all times during this procedure, until step 8.2.15.

8.2.8. Aspirate 85 µL of the clear supernatant and discard.

- Avoid removing any magnetic particles.

8.2.9. Keeping the plate on the magnetic stand, add 200 µL of 80% ethanol to each sample/control well without mixing.

8.2.10. Incubate at room temperature on the magnetic stand for 30 seconds.

8.2.11. Aspirate 200 µL of the ethanol and discard.

8.2.12. Repeat steps 8.2.9 through 8.2.11, for a total of 2 washes.

8.2.13. Using a fine-tipped pipette, aspirate and discard any excess ethanol.

8.2.14. With the plate still on the magnet stand, allow the magnetic particles to air-dry for 5 minutes.

8.2.15. Remove the plate from the magnet stand and add 50 µL of 10 mM Tris-HCl, pH 8.0 buffer.

- Mix by pipetting until homogeneous.
- Ensure all magnetic particles are in solution.

8.2.16. Incubate at room temperature for 2 minutes.

8.2.17. Place the plate on the magnet stand for 2 minutes or until the supernatant has cleared.

8.2.18. Transfer 47 µL of eluate to a fresh plate. If continuing onto the next section, mix by pipetting 3 times.

8.2.19. Centrifuge to ensure all of the solution is at the bottom of the wells.

8.2.20. If not immediately continuing to the next step, the purified amplicons can be stored at 4°C for up to 72 hours or at -20°C for up to 2 weeks.

8.3. Second PCR Product Purification (0.7x Cleanup)

8.3.1. If not already at room temperature, remove AMPure® XP Reagent from storage and allow to equilibrate to room temperature for at least 30 minutes before use.

8.3.2. Transfer the appropriate volume of AMPure® XP Reagent needed to a new reservoir to minimize the risk of contamination by pipette tips.

8.3.2.1. The required volume of AMPure® XP Reagent = (n + 2) × 32.9 µL (n is number of samples/controls to be purified).

8.3.3. Prepare 80% ethanol using 100% ethanol and molecular biology grade water (0.5 mL for each sample/control to be purified).

8.3.4. If purified amplicon was stored at -20°C, remove from storage and thaw. Centrifuge the PCR product plate.

8.3.5. Pipette 32.9 µL of the aliquoted, room temperature AMPure® XP Reagent to each PCR product to be purified. Mix by pipetting up and down 10 times. The color of the mixture should appear homogenous after mixing.

8.3.6. Incubate 5 minutes at room temperature.

8.3.7. Place the mixed samples/controls on a 96-well plate magnet and allow the magnetic particles to separate for 2 minutes.

- Keep the plate on the magnetic stand at all times during this procedure, until step 8.3.15.

8.3.8. Aspirate 75 µL of the clear supernatant and discard.

- Avoid removing any magnetic particles.

8.3.9. Keeping the plate on the magnetic stand, add 200 µL of 80% ethanol to each sample/control well without mixing.

8.3.10. Incubate at room temperature on the magnetic stand for 30 seconds.

8.3.11. Aspirate 200 µL of the ethanol and discard.

8.3.12. Repeat steps 8.3.9 through 8.3.11, for a total of 2 washes.

8.3.13. Using a fine-tipped pipette, aspirate and discard any excess ethanol.

8.3.14. With the plate still on the magnet stand, allow the magnetic particles to air-dry for 5 minutes.

8.3.15. Remove the plate from the magnet stand and add 50 µL of 10 mM Tris-HCl, pH 8.0 buffer.

- Mix by pipetting until homogeneous.
- Ensure all magnetic particles are in solution.

8.3.16. Incubate at room temperature for 2 minutes.

8.3.17. Place the plate on the magnet stand for 2 minutes or until the supernatant has cleared.

8.3.18. Transfer 47 µL of eluate to a fresh plate. Mix by pipetting 3 times.

8.3.19. Centrifuge to ensure all of the solution is at the bottom of the wells.

- If not immediately continuing to the next step, the purified amplicons can be stored at 4°C for up to 72 hours or at -20°C for up to 2 weeks.

8.4. Quantification and Validity of Purified PCR Products

Use an appropriate method that assesses both concentration and sizing of purified dsDNA PCR products between 200 bp – 1000 bp. Perform all quantification steps including installation, operation, calibration, cleaning and maintenance procedures according to the manufacturer's instructions.

8.4.1. Verify control and sample validity per Table 4.

Table 4: Control and Sample Quantification Validity

Validity Requirement	Acceptance Criteria
NPM1 MRD POS (+), *NPM1* MRD NEG (-), Sample	≥1.00 nM
No Template Control	≤1.00 nM

8.4.2. If a control or sample molarity (nM) value does not meet the acceptance criteria, refer to section 10. Troubleshooting.

8.4.3. If not immediately continuing to the next step, the amplicons can be stored at 4°C for up to 2 weeks.

8.5. Pooling and Quantification of the Library

Accurate quantification and dilution of the pool loaded onto the MiSeq™ flow cell is critical for generating optimal cluster density on the flow cell and obtaining high-quality data in a sequencing run.

8.5.1. Determine the desired library pool's target molarity (nM) value between 2-10 nM.

8.5.2. Normalize the Positive Control, Negative Control, and sample to the target molarity (nM) value using 10 mM Tris-HCl, pH 8.0.

8.5.3. Combine equal volumes of the normalized controls and sample in a volume appropriate tube. Include an equal volume of No Template Control.

8.5.4. Gently vortex the tube to thoroughly mix and quickly centrifuge.

8.5.5. Quantify the library pool using a method that is appropriate for both concentration and sizing of purified dsDNA PCR products between 200 bp – 1000 bp.

8.5.6. If not immediately continuing to the next step, the library pool can be stored at -20°C. Avoid unnecessary freeze-thaw cycles.

8.6. Preparation of the Library Pool for the MiSeq™ Sequencing Run

The concentration of the final library pool to be loaded onto the MiSeq™ Reagent v3 Cartridge is 14 pM.

8.6.1. If stored at -20°C, thaw library pool. Gently vortex the tube to thoroughly mix and quickly centrifuge.

8.6.2. Create a 20 µL denatured library pool at a target concentration of 1.75 nM by first calculating the volume of final library, X, using information in Table 5 and Equation 2. Use the final library molarity (nM) value determined in section 8.5.5 as the denominator in Equation 2.

Table 5: Library Denaturation

Reagent	Volume (µL)
Final Library	*X* *(use Equation 2)*
Diluent (10mM Tris-HCl, pH 8)	*Y* *(use Equation 3)*
10 nM PhiX (12.5%)	0.5
1N NaOH	2
Total Reaction Volume	**20**

Equation 2: Equation to Calculate Final Library Volume

$$X = \frac{(Target\ nM\ x\ Total\ Reaction\ Volume\ (\mu L))}{Final\ Library\ nM}$$

$$X = \frac{1.75\ nM\ x\ 20\ \mu L}{Final\ Library\ nM}$$

8.6.3. Second, calculate the volume of diluent, Y, to create a 20 µL denatured library pool at 1.75 nM using information in Table 5 and Equation 3.

Equation 3: Equation to Calculate Diluent

$$Y = Total\ \text{Reaction}\ Volume\ (\mu L) - Volume\ of\ PhiX\ (\mu L) - Volume\ of\ NaOH\ (\mu L) - Volume\ of\ Final\ Library\ (\mu L)$$

$$Y = 20\ \mu L - 0.5\ \mu L - 2.0\ \mu L - X\ \mu L$$

8.6.4. Add the calculated volume of diluent (*Y*) into a 1.5 mL low binding tube.

8.6.5. Add the calculated volume of library (*X*) into the tube.

8.6.6. Add 0.5 µL 10 nM PhiX into the tube.

8.6.7. Add 2 µL of 1N NaOH into the tube. Mix well by flicking the tube 5 times. Briefly centrifuge the tube.

8.6.8. Incubate the tube at room temperature for 5 minutes to denature the double-stranded DNA into single-stranded DNA.

8.6.9. Immediately upon the completion of the 5-minute incubation, add 980 µL of pre-chilled HT1 buffer to the reaction.

8.6.10. Vortex the tube to thoroughly mix and quickly centrifuge. The denatured Library Pool reaction is now at 40 pM (Library Pool pM + PhiX pM).

8.6.11. Combine 350 µL of the 40 pM denatured Library Pool and 650 µL of pre-chilled HT1 to create a Final Library Pool at 14 pM. Vortex the tube to thoroughly mix and quickly centrifuge.

8.7. Preparation of the MiSeq™ Reagent Cartridge

Prepare reagent cartridge according to the following sections of the *MiSeq System Guide* (REF 15027617).

- Thaw Reagent Cartridge
- Inspect the Reagent Cartridge

8.8. MiSeq™ Sample Sheet Creation

The MiSeq™ sample sheet will be provided via USB (REF 14160029).

NOTE: Do not make any modifications to the sample sheet other than those instructed below. Doing so may inhibit analysis.

8.8.1. Using the provided sample sheet, ensure the following parameters are correct:

- Workflow: GenerateFASTQ
- Application: FASTQ Only
- Assay: Nextera
- Chemistry: Amplicon
- Reads 1 and 2: 251

8.8.2. Do not edit or delete lines 22 and 23 on the provided sample sheet.

8.8.3. Input *Sample_ID*, *Sample_Name*, *I7_Index_ID*, *index*, *I5_index_ID*, *index2*, and a *Description* beginning on line 24 on the provided sample sheet.

- *Sample_ID* and *Sample_Name* column requirements:
  - Underscores and blank spaces are not allowed; use a dash "-" if needed. Use only these characters: A/a through Z/z, 0 through 9, -
  - Use the Master Mix ID or sample index, e.g. IA01
  - *Sample_ID* and *Sample_Name* must be identical
  - Example: NPM1-MRD-IA01-Pos
- *I7_Index_ID* and *I5_Index_ID* column requirements:
  - Correlate *I7_Index_ID* and *I5_index_ID* to the Master Mix ID, e.g., if Master Mix ID is IA01, input N701 and N501, per Table 6
  - Index ID pairs must be unique and not shared across multiple samples on the same sequencing run
- *index* and *index2* column requirements:
  - Enter a unique index sequence
  - Index sequence pairs must be unique and not shared across multiple samples on the same sequencing run
- *Description* column requirements:
  - Must contain DNA amount in ng, e.g. 700ngInput. It is necessary to include "ng" with the numerical value and exclude space between "ng" and "Input."

Table 6: Indexes

Master Mix ID	I7_Index_ID	I7_Index Sequence	I5_index_ID	I5_index Sequence
IA01	N701	TAAGGCGA	N501	TAGATCGC
IA02	N702	CGTACTAG	N503	TATCCTCT
IA03	N703	AGGCAGAA	N505	GTAAGGAG
IA04	N704	TCCTGAGC	N507	AAGGAGTA
IA06	N706	TAGGCATG	N504	AGAGTAGA
IA07	N707	CTCTCTAC	N506	ACTGCATA
IA08	N708	CAGAGAGG	N508	CTAAGCCT
IA09	N709	GCTACGCT	N501	TAGATCGC
IA10	N710	CGAGGCTG	N503	TATCCTCT
IA11	N711	AAGAGGCA	N505	GTAAGGAG
IA12	N712	GTAGAGGA	N507	AAGGAGTA
IA25	N705	GGACTCCT	N502	CTCTCTAT
IB13	N702	CGTACTAG	N502	CTCTCTAT
IB14	N703	AGGCAGAA	N504	AGAGTAGA
IB15	N704	TCCTGAGC	N506	ACTGCATA
IB16	N705	GGACTCCT	N508	CTAAGCCT
IB17	N706	TAGGCATG	N501	TAGATCGC

Table 6: Indexes

Master Mix ID	I7_Index_ID	I7_Index Sequence	I5_index_ID	I5_index Sequence
IB18	N707	CTCTCTAC	N503	TATCCTCT
IB19	N708	CAGAGAGG	N505	GTAAGGAG
IB21	N710	CGAGGCTG	N502	CTCTCTAT
IB22	N711	AAGAGGCA	N504	AGAGTAGA
IB23	N712	GTAGAGGA	N506	ACTGCATA
IB24	N701	TAAGGCGA	N508	CTAAGCCT
IB26	N709	GCTACGCT	N507	AAGGAGTA

8.8.4. Save the CSV file, explicitly named "SampleSheet.csv," to a folder accessible by the MiSeq™ instrument.

8.9. MiSeq™ Reagent Cartridge Loading

Load 600 µL of the 14 pM Final Library Pool onto a MiSeq™ Reagent v3 Cartridge according to the following sections of the *MiSeq System Guide* (REF 15027617).

- Load Sample Libraries

8.10. MiSeq™ Run Start

8.10.1. Start the MiSeq™ run according to the *MiSeq System Guide* (REF 15027617).

8.10.2. When selecting the run, review run parameters and verify the cycling conditions.

NOTE: To reduce the occurrence of run-to-run cross-contamination, it is recommended to alternate sequencing runs with IA or IB master mixes, or alternatively, run a non-*FLT3* ITD MRD Assay run between *FLT3* ITD MRD Assay sequencing runs.

8.11. Acceptance Criteria

8.11.1. Verify sequencing run validity in Table 7.

Table 7: Run Validity

Validity Requirement	Acceptance Criteria
MiSeq™ Cluster Density	≥500 K/mm^2
Total MiSeq™ Run Q30 Score	≥60%
MiSeq™ Reads Pass Filter	≥10 Million
NPM1 MRD POS (+)	Determined to be "Detected" and a VRF of ≥1.2x10^{-7}
NPM1 MRD NEG (-)	"Not Detected"
No Template Control	≤1,000 reads and is determined to be "Undetermined"

8.11.2. If the sequencing run does not meet all acceptance criteria, see section 10. Troubleshooting.

9. Data Analysis

9.1. *NPM1* MRD v1.1.1 Software

NOTE: If using Windows 10, the formatting of the MiSeq™ samplesheet.csv output may be impacted by the MiSeq Control Software. When executing software analysis, ensure proper formatting of the SampleSheet.csv by using the sample sheet created in section 8.8.

9.1.1. Use the *NPM1* MRD v1.1.1 Software (REF 14160029) for data analysis.

9.1.2. Refer to the following README files included in the software package to understand software execution via command-line or via the "DREW" rest-API interface:

9.1.2.1. DREW_README.md

9.1.2.2. Distributable_README.txt

9.2. Sample Interpretation

9.2.1. A minimum of 3 variant supporting reads is necessary for a positive call.

9.2.2. Minimum reads required for 95% confidence in negative call at $5x10^{-5}$, given 700 ng DNA input, is 157,600.

9.2.2.1. A negative sample with fewer than 157,600 reads is reported as Undetermined.

9.3. Mutation and Insertion Variant Detection

9.3.1. The software will report mutation variant 'A', 'B', 'D', or 'Other' for mutations other than the aforementioned types.

9.3.2. The number of variant-supporting reads is provided in the "READS" column.

9.3.3. The observed variant read frequency (VRF) is provided in the "VRF" column.

9.3.4. The software can detect insertions ≥4 base pairs and ≤20 base pairs between bases 13 and 25 of *NPM1* exon 12.

9.4. Sequence Annotations Provided in Final Output TSV File

9.4.1. The transcript name is provided in the "REFSEQ" column.

9.4.2. The coding sequence (CDS) name is provided in the "CDS" column.

9.4.3. The amino acid change is provided in the "AA" column.

9.4.4. The Chromosomal location is provided in the "HG19_COORDINATES" column and was determined using the GRCh37/hg19 Human Genome Reference Build.

9.4.5. Sequence contig information is provided in the "CONTIG_SEQ" column.

10. Troubleshooting

Table 8: Troubleshooting Guide

General Failure Category	Description of Failure	Action
Sample and Reagent Preparation	Sample DNA quantity is < 20 ng/µL by a fluorescence-based method	1. Reprocess the primary specimen (peripheral blood) one time (if within the validated stability claim). 2. If outside the stability claim, request an additional specimen collection.
Quantification of Sample	Concentration of NTC is >1.00 nM	1. If the NTC is between 1.00 nM – 2.00 nM, manually check to confirm no peak is present between 200 – 1000 bp range to pass.
		2. If NTC >2.00nM, sample contamination is likely; therefore, identify if there is enough starting material to re-amplify all samples. If so, repeat protocol from the beginning.
	If undiluted amplicon is <1.00 nM	1. Re-quantify with a new dilution. 2. If a greater value is obtained, continue with corrected value. 3. If same value is obtained, re-purify and repeat the protocol from the beginning for that sample. After re-amplification, it may be pooled with the amplicons from the first run. If there is not enough sample to repeat amplification, continue through the end of the protocol pooling the max volume (10-40 µL) of sample.
MiSeq™ Run	MiSeq™ Run Q30 is <60%	1. Re-quantify the pooled library and re-sequence.
MiSeq™ Run	MiSeq™ Reads Pass Filter is <10 Million	1. Re-quantify the pooled library and re-sequence.

11. References

1. Kelemen, K. (2022). The Role of Nucleophosmin 1 (*NPM1*) Mutation in the Diagnosis and Management of Myeloid Neoplasms. *Life*. 12(1), 109.
2. Falini, B. et al., (2005). Cytoplasmic Nucleophosmin in Acute Myelogenous Leukemia with a Normal Karyotype. *N Engl J Med*. 352:254-266.
3. https://www.cancer.org/cancer/acute-myeloid-leukemia/detection-diagnosis-staging/how-classified.html. Accessed 16 November 2022
4. Döhner K. et.al. (2005) Mutant nucleophosmin (*NPM1*) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics:interaction with other gene mutations. *BLOOD*. 106:3740-3746.
5. Rau, R and Brown, P. (2009) Nucluophosmin (*NPM1*) mutations in adult and childhood acute myeloid leukemia: Towards definition of a new leukemia. *Hematol Oncol*. 27(4):171-181.

- *MiSeq System Guide* (REF 15027617).
- http://www.illumina.com

12. Technical and Customer Service

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13. Symbols

The following symbols are used in Invivoscribe NGS product labeling.

REF	Catalog Number		Expiration Date
VOL	Reagent Volume	RUO	Research Use Only
LOT	Lot Number		Manufacturer
	Storage Conditions		Consult Instructions for Use

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