Chapter 34

Assessment of Cell-Free microRNA by NGS Whole-Transcriptome Analysis in Cutaneous Melanoma Patients' Blood

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Abstract

MicroRNAs (miRs) are small RNA molecules (18–22 nucleotides) that regulate the transcriptome at a post-transcriptional level by affecting the expression of specific genes. This regulatory mechanism is critical to maintain cell homeostasis and specific functions. Aberrant expression of miRs have been associated with pathobiological processes including cancer. There are few technologies available that are able to profile whole-genome miR expression using minimal amounts of blood samples and without the need for time-consuming extraction steps. Here, we describe the HTG EdgeSeq miR Whole-Transcriptome Assay (WTA) in serum and plasma samples. To identify specific cell-free miR (cfmiR) patterns we have first focused on the analysis of normal donor samples and have then compared these to patients with cutaneous melanoma. The identification of specific cfmiR for melanoma patients will allow for better patient surveillance during targeted and/or checkpoint inhibitor immunotherapy (CII) treatment.

Key words miRNA, Metastatic melanoma, Serum, Plasma, miR Whole Transcriptome Assay

1 Introduction

Metastatic melanoma is a rapidly progressing cancer that can metastasize to distant organs such as the liver, lung, and brain [1]. Early detection of melanoma metastasis remains a key factor for treatment decisions such as targeted therapy, CII, and radiological or surgical interventions [2]. In the past few years, new CII have significantly improved survival outcomes for metastatic melanoma patients and have become the standard of care [2]. However, there is still a need for biomarker assay development as this would allow for the evaluation of disease progression in melanoma patients undergoing treatment [2].

Detection assays of cell-free nucleic acids (cfNA) found in metastatic cutaneous melanoma patient samples have improved in

recent years, particularly in using circulating cell-free DNA (cfDNA) to target specific mutations [3–5]. However, the detection of cfDNA in the blood is still limited due to degradation. On the contrary, microRNAs (miRs) are short stable RNA molecules (18–22 nucleotides) that can control gene expression posttranscriptionally [6, 7]. Specific miRs are found in both the serum and plasma of cutaneous melanoma patients, as our group and others have demonstrated [7–12].

Several diagnostic and prognostic cfmiRs have been proposed as biomarkers, both individually and in combination, for assessment of cutaneous melanoma patients [7]. However, the specificity of diagnostic and prognostic cfmiRs identified in melanoma and other solid tumors is of major concern. Single cfNA biomarkers are not reliable for detection due to specificity and frequency. This is because most of the cfmiRs are associated with normal physiological functions and/or are associated with other diseases [7]. To increase the specificity of cfmiRs as biomarkers, we have focused on the identification of cfmiRs signatures in melanoma patients compared to normal cancer-free blood samples. In this regard, we have used the HTG WTA probe-based and Next-Generation Sequencing (NGS)-based detection platform. HTG WTA is designed to detect 2083 miRs with minimal sample input requirements, reduced preprocessing isolation steps, and high reproducibility.

Using HTG WTA, we have described the profiling of serum and plasma samples taken from normal healthy donors to identify cfmiR signatures, and we have performed a comparative analysis of these signatures with those obtained from melanoma patients. With this approach, we have found that specific cfmiRs are differentially expressed in normal (cancer-free) donor plasma and serum samples (Fig. 1) and that specific cfmiRs are found in plasma samples from
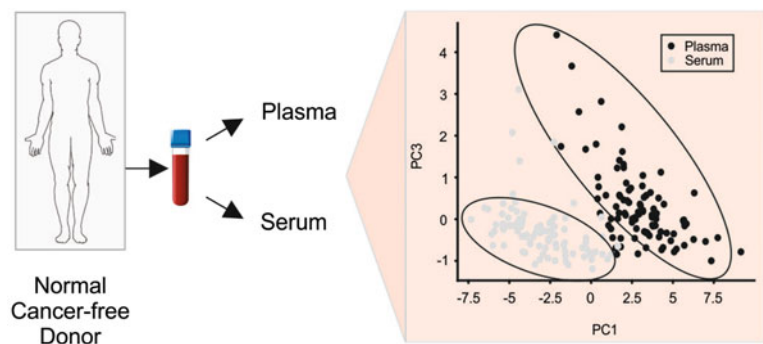

Fig. 1 cfmiR comparison between normal serum and plasma samples. Shown is a principal component analysis (PCA) plot for the top 100 cfmiRs DE in serum compared to plasma samples of normal (cancer-free) donors. The figure shows that the top 100 cfmiRs may distinguish serum from plasma samples in this analysis
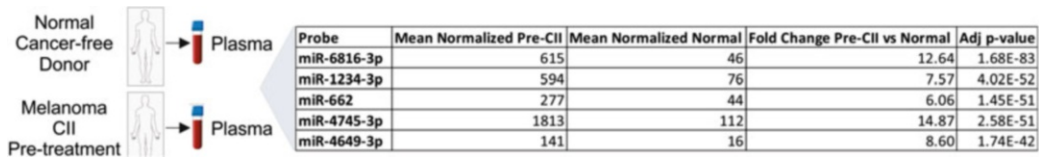
Probe	Mean Normalized Pre-CII	Mean Normalized Normal	Fold Change Pre-CII vs Normal	Adj p-value
miR-6816-3p	615	46	12.64	1.68E-83
miR-1234-3p	594	76	7.57	4.02E-52
miR-662	277	44	6.06	1.45E-51
miR-4745-3p	1813	112	14.87	2.58E-51
miR-4649-3p	141	16	8.60	1.74E-42

Fig. 2 cfmiRs serve as a marker for cutaneous melanoma treatment. The top 5 differentially expressed cfmiRs in pretreatment samples of melanoma patients receiving CII compared to plasma samples of normal (cancer-free) healthy donors. This is a list of cfmiRs that were found DE in the pretreatment blood samples of melanoma patients receiving CII. The approach described herein will allow evaluation of these cfmiRs over time and may identify cfmiR biomarkers that can be used to monitor therapeutic efficacy and tumor response during the course of treatment

pretreatment melanoma patients receiving CII treatment when compared to plasma samples obtained from normal (cancer-free) donors (Fig. 2). This technology may therefore lead to the identification of cfmiR biomarkers that can be evaluated over time to monitor therapeutic efficacy and tumor response during the course of treatment.

2   Materials

2.1   Serum Isolation

1. Corvac™ red/gray mottled stopper serum separator tubes (referred to as tiger-top tubes).
2. BD Vacutainer™ Venous Blood Collection Tubes: SST™ Serum Separation Tubes: Conventional Stopper (referred to as red-top tubes).
3. Serum Filter Fisherbrand™ 16 mm OD X 2–3/4 in.
4. 2 mL transfer pipettes.
5. 2 mL sterile cryovials.

2.2   Plasma Isolation

1. BD Vacutainer™ Glass Blood Collection Tubes: Buffered Sodium Citrate (referred to as blue-top tubes).
2. 15 mL conical polypropylene centrifuge tubes.
3. Glass or plastic transfer pipettes.
4. 2 mL sterile cryovials.

2.3   Library Preparation and Cleanup

1. HTG EdgeSeq instrument (HTG Molecular).
2. HTG Edge System software (HTG Molecular).
3. HTG EdgeSeq miRNA sample prep pack containing biofluids lysis buffer, plasma lysis buffer, and proteinase K (HTG Molecular).
4. HTG EdgeSeq assay reagent pack and HTG EdgeSeq plate pack containing sample plate and stop plate (HTG Molecular).

5. Pipette tip pack.

6. Parafilm®.

7. Human brain total RNA.

8. 10 mM dNTP.

9. Hemo Klentaq® reaction buffer (5×): When diluted to 1× with other reaction reagents, this buffer contains 60 mM Tricine, 5 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 6% glycerol, pH 8.7 (at 25 °C).

10. Hemo Klentaq® DNA polymerase.

11. Molecular biology grade water.

12. HTG EdgeSeq sequencing tag pack containing forward and reverse primers (HTG Molecular).

13. AMPure XP beads.

14. DynaMag™-96 Side Skirted magnet.

15. 200 proof ethanol.

16. 10 mM Tris–HCl buffer, pH 8.0.

17. 1.5 mL LoBind tubes.

18. Twin.tec® PCR plate 96, unskirted, clear.

19. Microseal 'B' seal.

20. T100™ Thermal cycler or any modern equivalent thermal cycler.

21. Orbital shaker with planar mixing orbit for 1.5 mL centrifuge tubes.

2.4 Library Quality Check

1. High Sensitivity (HS) D1000 ScreenTape.

2. HS D1000 sample buffer (Agilent Technologies Inc., proprietary buffer).

3. HS D1000 DNA ladder.

4. 2200 TapeStation Instrument (Agilent Technologies Inc.).

5. 8-strip optical tube.

6. 8-strip optical Cap.

7. Loading tips (1pk).

8. KAPA library Quant kit (Illumina) universal qPCR mix kit.

9. Multiplate® PCR plates™ 96-well.

10. Tween 20.

11. 1 M Tris–HCl buffer, pH 8.0.

12. DNA dilution buffer: 10 mM Tris–HCl, pH 8.0 + 0.05% Tween 20.

13. Modern day thermocycler Real-Time PCR Detection System.

2.5 Next-Generation Sequencing	1. HTG EdgeSeq Library Calculator Input Template (HTG Molecular).

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2. HTG EdgeSeq RUO Library Calculator (HTG Molecular).

3. MiSeq™ instrument (Illumina).

4. NextSeq™ 550 instrument (Illumina).

5. MiSeq® V3 (150 Cycle) kit (Illumina).

6. NextSeq 500/550 Mid Output kit v2.5 (75 cycles) (Illumina).

7. NextSeq Accessory Box v2 (Illumina).

8. *PhiX* Control v3 Library (Illumina).

9. 10 mM Tris–HCl buffer, pH 8.5.

10. 200 mM Tris–HCl buffer, pH 7.0.

11. 2 N NaOH.

12. 2 N HCl.

13. HTG EdgeSeq ILM Seq primer kit containing ILM MiSeq primer, HTG ILM NextSeq Index primer, and ILM NextSeq Read1 primer (HTG Molecular).

14. HTG EdgeSeq Parser software (HTG Molecular).

3 Methods

3.1 Serum Isolation

1. Collect blood in tiger-top tubes and centrifuge at $1300 \times g$ at room temperature (RT) for 10 min (*see* **Notes 1** and **2**).

2. Obtain a red-top tube. Remove the red-top cap while keeping the 7 mL vacutainer inside the sterile biological safety cabinet. Discard the red-top cap.

3. Using a transfer pipette, transfer serum from the spun-down serum separator tube to the 7 mL red-top vacutainer. Avoid poking the gel layer with the transfer pipette.

4. Discard transfer pipette and serum separator tube.

5. Obtain a serum filter. Avoid touching the bottom filter portion.

6. Insert the serum filter into the 7 mL vacutainer and push it down without covering the entire mouth of the serum filter. Be careful not to over-flow the serum filter.

7. Use a new transfer pipette to transfer the filtered serum from the serum filter to a 2 mL sterile cryovial (*see* **Note 3**).

8. Discard the serum filter, 7 mL vacutainer, and transfer pipette.

9. Store all 2 mL serum cryovials at $-80\,^\circ$C for long-term storage.

3.2 Plasma Isolation

1. Centrifuge the blue-top tube at $1300 \times g$ for 10 min at RT (*see* **Note 2**).

2. Gently remove the blue-top tube from the centrifuge to a tube rack.

3. Slowly transfer the plasma from the blue-top tube to a 15 mL conical polypropylene centrifuge tube (*see* **Note 4**).

4. Centrifuge the 15 mL conical centrifuge tube containing plasma at $1300 \times g$ for 10 min at RT (*see* **Note 2**).

5. Transfer the clarified plasma from the 15 mL conical centrifuge tube to a 2 mL sterile cryovial.

6. Store all 2 mL plasma cryovials at $-80\ ^\circ$C.

3.3 Library Preparation and Cleanup

1. Remove cryovials from freezer. Thaw isolated serum/plasma on ice until completely defrosted. Pipet to mix the samples thoroughly to obtain a homogenous sample (*see* **Notes 5** and **6**).

2. Thaw biofluids lysis buffer (for serum) and/or plasma lysis buffer (for plasma) by placing the tubes on a 50 °C heat block for 30 min to ensure complete solubility of any buffer precipitates.

3. Aliquot 15 μL of serum/plasma into a separate 1.5 mL LoBind tube.

4. Prepare to lyse the samples by adding 15 μL of biofluids lysis buffer or plasma lysis buffer into each serum or plasma sample, respectively. Add 3 μL of proteinase K to each sample and pipet to mix thoroughly. Quick spin for 5 s to collect the residual lysis buffer at the bottom of the tube.

5. Lyse the samples by incubating on a preheated 50 °C orbital shaker set to 500 rpm for 3 h (*see* **Note 7**).

6. Aliquot 25 μL of lysate per well into an HTG sample plate (*see* **Note 8**). Load the HTG EdgeSeq Instrument with sample plate, stop plate, HTG WTA reagent cartridge, and pipette tips according to the schematic provided by the HTG Edge System software. Initiate the run according to the HTG Edge-Seq user manual. Allow the HTG WTA to perform automated probe capture for 20 h.

7. Following the 20-h processing period, remove the samples now located in the stop plate and store them at $-20\ ^\circ$C until they are ready for library preparation or move onto library preparation steps immediately (*see* **Note 9**). Clean the instrument according to the HTG EdgeSeq User Manual.

8. Carefully mix samples in the stop plate with a pipette set to 10 μL to obtain a homogenous aqueous mixture (*see* **Note 10**).

9. Prepare the PCR mixture on ice in a twin.tec® PCR plate using 21 μL of master mix (0.6 μL of 10 mM dNTP, 6 μL of 5× Hemo *Klentaq*® Reaction buffer, 2.4 μL of Hemo *Klentaq*® DNA Polymerase, and 12 μL of molecular biology grade water), 6 μL of primers from HTG EdgeSeq sequencing tag pack (3 μL of forward primer, 3 μL of reverse primer) and 3 μL of sample (*see* **Note 11**). Seal plate with a Microseal® 'B' seal.

10. On a preheated thermal cycler, run PCR using the following settings.
    (a) 95 °C for 4 min.
    (b) 95 °C for 15 s, 56 °C for 45 s, 68 °C for 45 s (repeat for 16 total cycles).
    (c) 68 °C for 10 min.
    (d) Hold at 4 °C.

11. Perform library cleanup by thoroughly pipetting to mix 37.5 μL of AMPure XP beads with 15 μL of PCR-amplified libraries in a new twin.tec® PCR Plate. Incubate for 5 min at RT.

12. In the meantime, prepare 400 μL of 80% ethanol per sample (*see* **Note 12**).

13. Place plate on a DynaMag™ 96 Side Skirted Magnet for 2 min or until the supernatant becomes completely clear.

14. While still on the magnetic plate, remove supernatant without disturbing the library-bound beads and add 200 μL of 80% ethanol to wash. Incubate at RT for 30 s (*see* **Note 13**).

15. Repeat 80% ethanol wash.

16. Remove supernatant (ethanol) and let the beads dry on the magnet for 2 min (*see* **Note 14**).

17. Remove the plate from the magnet and thoroughly resuspend the beads with 40 μL of 10 mM Tris–HCl, pH 8.0 to elute libraries.

18. Incubate at room temperature for 2 min.

19. Place the plate back on the magnet for 2 min or until the supernatant becomes completely clear.

20. Transfer the supernatant (containing libraries) without disturbing the beads (*see* **Note 15**) and store in a new plate.

21. Store at −20 °C for up to 2 weeks, or −80 °C for longer storage until ready to sequence.

3.4 Library Quality Check

1. Perform library fragment-size analysis by first allowing the Agilent HS D1000 ScreenTape and reagents to equilibrate to RT for 30 min. Vortex and quick spin the Agilent HS D1000 sample buffer and HS D1000 DNA ladder for at least 5 s.

2. Pipet and mix 2 μL of each library and 2 μL of sample buffer into each well of the 8-strip optical tube. Briefly vortex and quick spin for 5 s.

3. Load Agilent 2200 TapeStation Instrument with 8-strip tubes, loading tips, and equilibrated HS D1000 ScreenTape. Initialize instrument program as specified in the Agilent 2200 TapeStation System User Manual.

4. Confirm the library fragment size is between 150 and 170 base pairs (bp).

5. To quantitate the libraries, perform qPCR with the KAPA Library Quant kit Universal qPCR mix kit.

6. Thaw Universal qPCR master mix and standards in a 4 °C refrigerator (*see* **Note 16**).

7. Prepare 30 mL of DNA dilution buffer (10 mM Tris–HCl, pH 8.0 + 0.05% Tween 20) by mixing 29.55 mL of molecular-grade water, 300 μL of 1 M Tris–HCl, pH 8.0, and 150 μL of 10% Tween 20 (*see* **Note 17**).

8. Prepare a 10,000× dilution series for each sample by diluting the samples by 100× (2 μL of sample + 198 μL of DNA dilution buffer) twice in a twin.tec® PCR plate. Carefully mix the samples in between dilutions.

9. Prepare 16 μL of master mix (12 μL of qPCR master mix and 4 μL of molecular-grade water) per sample, standard, and nontemplate control (NTC, negative control) (*see* **Note 18**).

10. Aliquot 16 μL of master mix into each well of the Multiplate® PCR plates™. Add 4 μL of library, standard, or DNA dilution buffer (NTC) into respective wells.

11. Perform qPCRs under the following conditions.

    (a) 95 °C for 5 min.

    (b) 95 °C for 30 s, 60 °C for 45 s (repeat for 35 total cycles).

3.5 Library Normalization, Pooling, and Next-Generation Sequencing

1. Remove MiSeq® V3 (150 Cycle) kit or NextSeq 500/550 Mid Output kit v2.5 (75 cycles) from −20 °C storage and place reagent cartridge and HT1 buffer in a water bath at RT to thaw before beginning preparations for NGS (*see* **Note 19**). Remove HTG ILM MiSeq primer (MiSeq) or HTG ILM NextSeq index primer and ILM NextSeq Read1 primer (NextSeq) to thaw on ice.

2. Fill out the HTG EdgeSeq Library Calculator Input Template with the sample concentrations as determined by the qPCR results. Select the correct sequencing instrument (MiSeq or NextSeq) and input the intended final loading concentration into the HTG EdgeSeq RUO Library Calculator software. Upload the input template into the software and generate the pooling and denaturing protocol.

3. Quantitated libraries are diluted, normalized, pooled, and denatured according to the protocol generated by the HTG EdgeSeq RUO Library Calculator software. The following steps are performed with calculated volumes provided in the protocol generated by the HTG EdgeSeq RUO Library Calculator software.

4. Dilute libraries with 10 mM Tris–HCl, pH 8.5.

5. Pool diluted libraries together.

6. Denature the normalized pool of libraries with 2 N NaOH for 8 min at RT.

7. Add cold HT1 buffer. Invert the tube to mix.

8. Neutralize the NaOH with 2 N HCl. Vortex to mix well and quick spin for 5 s.

9. Dilute and denature stock *PhiX* Control v3 libraries by adding 5 μL of 0.2 N NaOH to 5 μL of 4 nM *PhiX*. Vortex to mix and quick spin for 5 s.

10. Incubate at RT for 5 min.

11. Add 5 μL of 200 mM Tris–HCl, pH 7.0 to neutralize the NaOH.

12. Dilute *PhiX* to 20 pM by adding 985 μL of prechilled HT1 buffer (*see* **Note 20**).

13. Dilute 20 pM *PhiX* to 12.5 pM by adding 32.5 μL of 20 pM *PhiX* to 19.5 μL of HT1 buffer.

14. Spike denatured libraries with 12.5 pM *PhiX* control libraries at the volume specified by the HTG-generated protocol.

15. Heat-denature pooled libraries and *PhiX* at 98 °C for 4 min.

16. Immediately chill on ice for a minimum of 5 min.

17. Load the entire denatured library pool and custom primer into the reagent cartridges of the respective MiSeq® V3 (150 Cycle) kit or NextSeq 500/550 Mid Output kit v2.5 (75 cycles) as instructed in the HTG user manual (*see* **Note 21**).

18. Load sequencer as instructed in the respective Illumina system guide (*see* **Notes 22** and **23**).

19. After sequencing, generate FASTQ files by uploading the sequencing run files and sample sheet into the Illumina Local Run Manager module (*see* **Note 24**).

20. Upload FASTQ files and sample sheet (*see* **Note 23**) into the HTG EdgeSeq Parser software and begin parsing and alignment. Save the generated .xls file containing the final counts for 2083 miRs per each sample for downstream data analysis.

3.6	*Data Analysis*	Data analysis will depend on the specific needs and experimental goals of the user. In general, though, data analysis for the sequenced miRs includes the following steps: (1) raw data processing and quality check, (2) mapping miRs to 2083 reference miRs, (3) acquiring read counts for each miR respectively, (4) identification of differentially expressed (DE) miRs, and (5) performing exploratory analysis for comparisons of DE miRs. The HTG platform accounts for the first four steps of this analysis. Exploratory analysis using datasets obtained from the HTG Reveal software can be performed in the interactive platform Jupyter (https://jupyter.org). Some of the python libraries/packages used for this analysis include: scikit-learn (https://scikit-learn.org/) for data classification; Matplotlib (https://matplotlib.org/) for generating 2D plots; Seaborn (https://seaborn.pydata.org/) for creating hierarchically clustered heatmaps; NumPy (https://numpy.org/) and SciPy (https://www.scipy.org/) for statistical analysis.

4   Notes

1. Allow blood specimens to sit for 1 h at RT before processing them for serum isolation. Process blood under a clean HEPA-filtered laboratory hood (BSL2 or higher level) to avoid contamination.

2. During centrifugation, label cryovials with the specimen number, protocol ID, and the date the blood was drawn.

3. Cap tightly and do not exceed the 1.8 mL volume of each cryovial.

4. Avoid disturbing/pipetting the solid blood layer near the top surface of the plasma.

5. Turn on and set up the HTG Instrument according to the HTG EdgeSeq user manual before processing the blood specimens.

6. Designate the human brain total RNA to be the experimental positive control. Leave one well empty on the sample plate to load the positive control.

7. A heat block can be used instead of the orbital shaker, but samples must be mixed every hour.

8. There are three formats of HTG plates (8, 24, or 96-well sample plates) and selection is made according to the number of samples.

9. Take care not to invert the stop plate. If done, it will spill the samples and risk cross-contamination. If storing at -20 °C, parafilm the lid to prevent frost from entering the plate.

10. The HTG EdgeSeq instrument performs a denaturation oil overlay on the samples in the stop plate. Mix the samples while taking care to avoid aspirating the oil overlay. Mixing the oil into the aqueous sample may inhibit downstream PCR amplification.

11. Arrange forward and reverse primers to ensure unique barcode combinations for each sample. For example, for 24 samples, 3 forward X 8 reverse primers allow for 24 unique forward-reverse primer combinations.

12. Prepare extra ethanol accounting for pipetting errors to ensure there is enough for all samples, including the standard and NTC.

13. Use a multichannel pipette when working with more than 8 samples at a time to prevent the beads from drying while working.

14. Do not allow beads to over-dry. Look for a matte finish on the beads. Cracks are indicative of over-drying and will lead to lower library yields.

15. Use a 20 μL pipette to reduce bead carryover.

16. Add 1 mL of 10× primer mix to 5 mL of qPCR master mix upon first use of the qPCR with the KAPA Library Quant kit Universal qPCR mix kit. Also, qPCR master mix is light-sensitive.

17. Prepare 10% Tween 20 by diluting 15 μL of stock Tween 20 with 135 μL of molecular-grade water.

18. Perform qPCR on samples, standards, and NTC in at least duplicates.

19. For 24 samples, use MiSeq™ instrument and reagents. For 96 samples, use NextSeq™ 550 instrument. For NextSeq kits, HT1 buffer is provided in the NextSeq accessory box v2.

20. 20 pM denatured *PhiX* may be stored at −20 °C for up to 2 weeks. Save and store extra *PhiX* for other HTG WTA sequencing runs. *PhiX* is the positive sequencing control.

21. For MiSeq runs, load custom primer ILM MiSeq primer. For NextSeq runs, load HTG ILM NextSeq index primer and ILM NextSeq Read1 primer.

22. Set-up sequencing parameters to be 1×50 bp read length for either instrument. MiSeq® V3 kit (150 cycle) is the lowest cycle kit validated with HTG WTA.

23. The sample sheet for either the MiSeq or NextSeq runs is located in the "Reports" tab of the HTG Edge System software. This sample sheet may be used for setting up MiSeq (required) or NextSeq (optional) runs.

24. The MiSeq™ instrument has automatic FASTQ generation. Local Run Manager is only required for NextSeq sequencing. Alternatively, if the user has an Illumina BaseSpace account, FASTQ generation may occur automatically for NextSeq runs.

Acknowledgments

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