# Sequencing set-up guidelines for NGS libraries prepped with Agilent NGS kits

## I. Illumina Instruments (SureSelect$^{XT}$/XT2/QXT, SurSelect RNA-Seq and HaloPlex/HaloPlex$^{HS}$)

1. HiSeq1000/2000 and HiSeq1500/2500
    1) Planning a run using IEM (Illumina Experiment Manager)
    2) Starting a run in the HCS (HiSeq Control Software) user interface
2. MiSeq
    1) Planning a run using IEM
    2) Starting a run in the MCS (MiSeq Control Software) user interface
3. NextSeq
    1) Planning a run
        A. Using IEM (standalone mode)
        B. Using BaseSpace
    2) Starting a run in the NCS (NextSeq Control Software) user interface

## II. ION Instruments (SureSelect$^{XT}$ and HaloPlex/HaloPlex$^{HS}$)

1. Planning a run and reanalyzing a run using the Torrent Server (applies to PGM, Proton and S5)
2. Starting a run on an ION Instrument

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## III. Appendices

1. bcl to fastq conversion for Illumina sequencing
2. Example sample sheet and other template csv files
    A. Example sample sheet
        a XT/XT2/RNA-Seq/HaloPlex
        b. QXT without adapter trimming
        c. QXT with adapter trimming
        e. QXT with custom primer and adapter trimming options
        e. HaloPlex$^{HS}$
    B. Library prep kit template csv file for BaseSpace
        a XT/XT2/RNA-Seq/HaloPlex
        b. HaloPlex$^{HS}$
        c. QXT without adapter trimming
        e. QXT with custom primer and adapter trimming options
3. How to add Agilent library prep kits as custom Library Prep Kit types in IEM
4. Illumina sequencing reagent kit selection guide
5. Recommended seeding concentrations for Illumina sequencing
6. How to convert an Agilent BED file into a manifest file
7. Considerations for PhiX spike-in

**ION SEQUENCING-RELATED**

8. ION reagent kits used by Agilent for template preparation and sequencing
9. Seeding concentration for ION sequencing and ION sphere QC
10. How to modify an Agilent BED file to be uploaded to the Ion Server

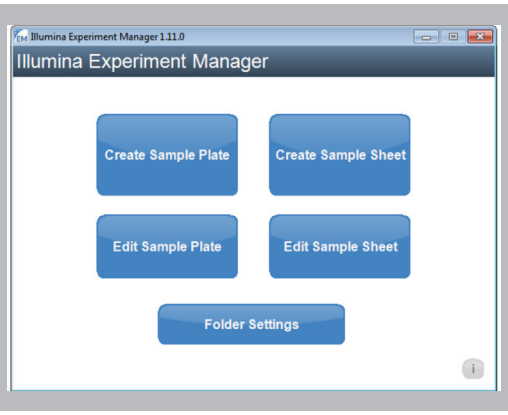
# Sequencing set-up guidelines for NGS libraries prepped with Agilent NGS kits

## Important notes for HaloPlex$^{HS}$ (Illumina):

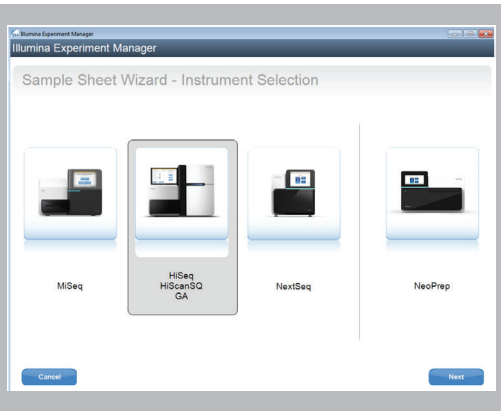
1. Only works with standalone machines and cannot be set up through BaseSpace
2. When using the MiSeq, the MiSeq Reporter software can be reconfigured to generate the additional I2 fastq file e.g. the molecular barcode file. Please refer to the "Additional MiSeq Platform sequencing run setup requirements" in the HaloPlex$^{HS}$ protocol. In any other situation, the HaloPlex$^{HS}$ data will need to be converted offline using the bcl2fastq software or Picard tool. Please refer to Appendix 1B for details.

* Versions of software demonstrated in this document:
  IEM v1.11.0
  HiSeq Control Software v2.2.68
  MiSeq Control Software v2.6.2.1
  NextSeq Control Software v2.0.2
  Torrent Server v5.0.4

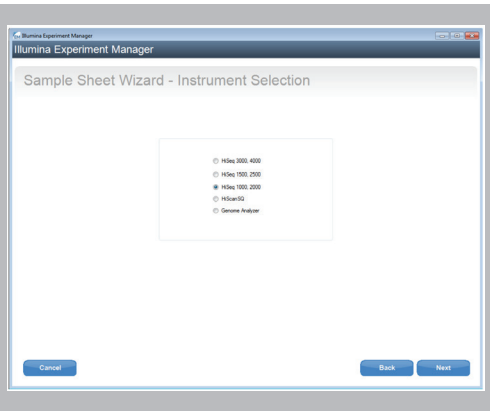
# I.1.1. Planning a run on the HiSeq 2000/2500 using IEM for Agilent NGS libraries



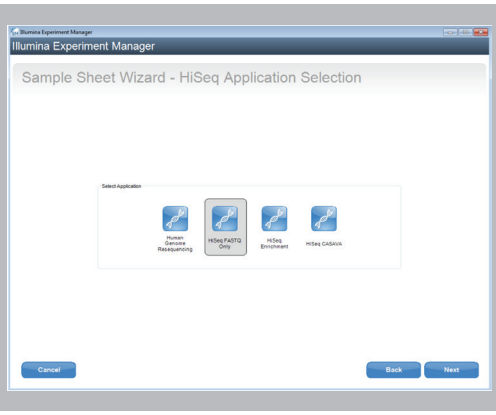

Click “Create Sample Sheet.”




Choose “HiSeq/HiScanSQ/GA.”

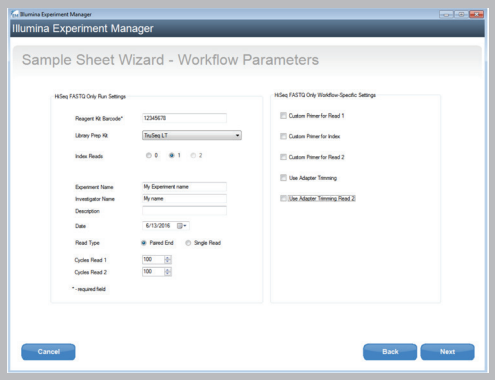



Choose the sequencer that will be used.




Choose “HiSeq FASTQ Only.”

# I.1.1. Planning a run on the HiSeq 2000/2500 using IEM for Agilent NGS libraries




**Fill in the fields for: Reagent Kit Barcode, Experiment Name, Investigator Name, and Description. Paired End sequencing is recommended for all Agilent NGS libraries.**

## For SureSelect$^{XT}$/XT2/RNA-Seq: Choose TruSeq LT as the Library Prep Kit.

Choose TruSeq LT as the Library Prep Kit. If you are following the shearing size of 150-bp to 200-bp and using a 100-bp or shorter read length, adapter trimming is not necessary. If you are using shorter shearing size or longer read length and prefer to have Illumina software do the trimming, check the adapter-trimming options. Otherwise, uncheck these two options and trim adapters later using Agilent SureCall software or the AGeNT toolkit.

## For HaloPlex: Choose TruSeq LT, but uncheck the adapter-trimming option and perform trimming in SureCall or using the AGeNT toolkit.

## For QXT: Choose Nextera XT and either:

(1) leave "Use Adapter Trimming" checked or (2) uncheck this option and trim the adapter using SureCall or the AGeNT toolkit. Trimming with SureCall or the AGeNT toolkit is recommended.

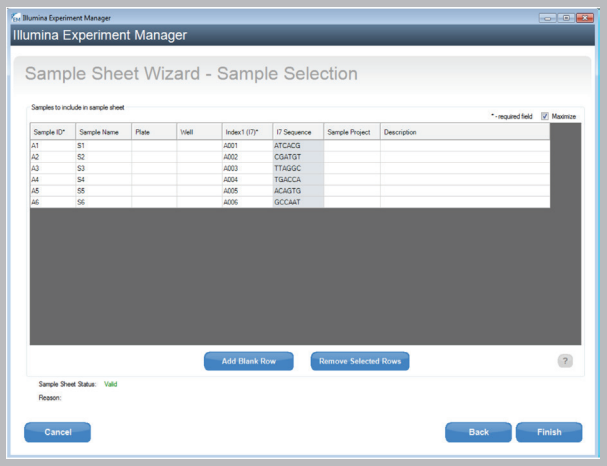
If you choose the "Use Adapter Trimming" option, you will need to enter the consensus sequence for the QXT adapters provided in the QXT protocol (CTGTCTCTTGATCACA), when you edit the sample sheet later.

Leave the three "Custom Primer" options unchecked; spike the Agilent read primers into the Illumina read primers; and place the primer mixtures in their original positions.

**Note:** Because of possible reagent fill-volume variabilities, we recommend pipetting the appropriate volumes of the Illumina read primers out of their original tubes and mixing them with required volumes of the respective QXT read primers in separate tubes. Refer to the QXT protocol/custom primer-preparation tables for details.) Then place the primer mixtures at positions corresponding to the relevant Illumina read primers on the reagent rack.

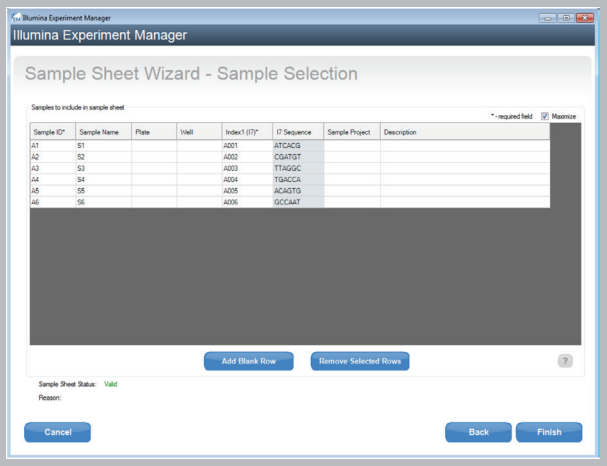
## For HaloPlex$^{HS}$: Choose Nextera XT and uncheck "Use Adapter Trimming."

# I.1.1. Planning a run on the HiSeq 2000/2500 using IEM for Agilent NGS libraries



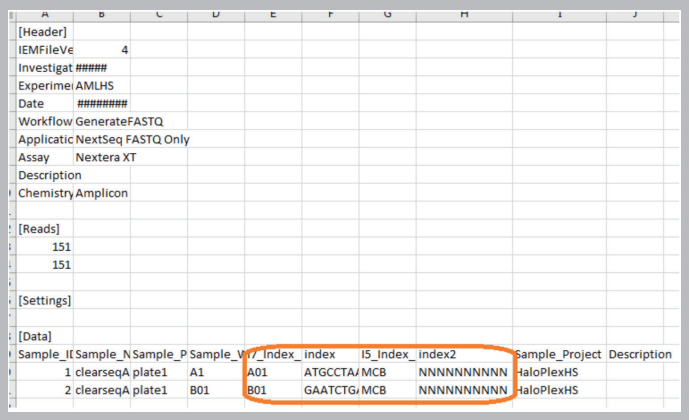

## Click "Add Blank Row" for each sample.

Fill in the fields for: Sample ID and Sample Name. It does not matter what you select for the index at this time, because you will edit the sample sheet to enter the correct index sequences. Click "Finish" to save the sample sheet. The screen below is single-indexed for demonstration purposes only.




## When asked, "Would you like to view your sample sheet in Excel?" click "Yes."

Then enter the correct sample index names and sequences and save the sample sheet. Copy or move the sample-sheet csv file to the appropriate location on your HiSeq, if you ran IEM on a different computer. Refer to Appendix 2A for example sample sheets of respective library types.



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| Experime | AMLHS | | | | | | | | |
| Date | ######## | | | | | | | | |
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| Applicatic | NextSeq FASTQ Only | | | | | | | | |
| Assay | Nextera XT | | | | | | | | |
| Description | | | | | | | | | |
| Chemistry | Amplicon | | | | | | | | |
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| [Settings] | | | | | | | | | |
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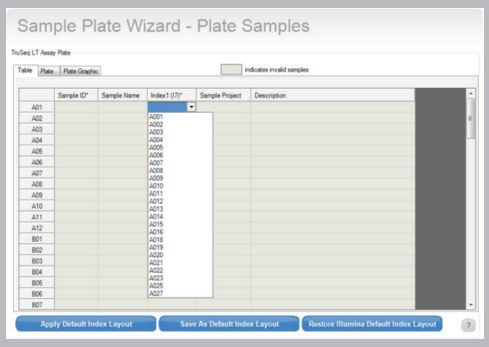
| [Data] | | | | | | | | | |
| Sample_ID | Sample_N | Sample_P | Sample_W | I7_Index_ | index | I5_Index_ | index2 | Sample_Project | Description |
| 1 | clearseqA | plate1 | A1 | A01 | ATGCCTA/ | MCB | NNNNNNNNNN | HaloPlexHS | |
| 2 | clearseqA | plate1 | B01 | B01 | GAATCTG/ | MCB | NNNNNNNNNN | HaloPlexHS | |

## Note for HaloPlex$^{HS}$:

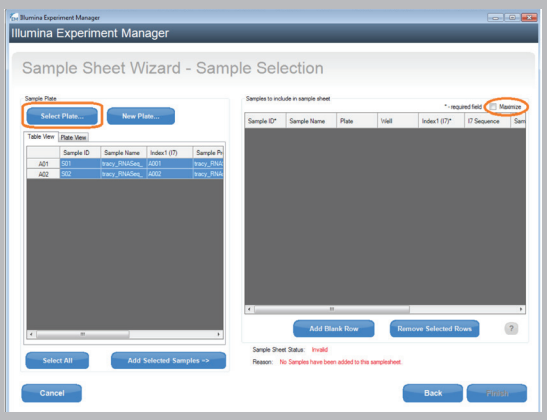
Index 2 is the molecular barcode and is not used for demultiplexing. Use 10 "N"s for index 2 for all samples in the sample sheet. You will need to perform offline bcl-to-fastq conversion to demultiplex using only index 1. In the meantime, generate a fastq file for index 2; the fastq file will be needed for HaloPlex$^{HS}$ data analysis. Please refer to Appendix 1 for more instructions.

# I.1.1. Planning a run on the HiSeq 2000/2500 using IEM for Agilent NGS libraries

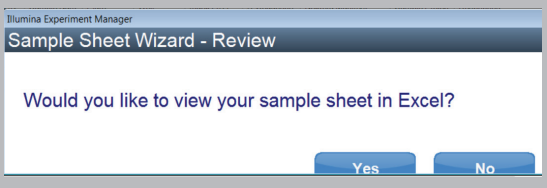



Alternatively, you may create a sample plate first and then use the sample plate to generate a sample sheet.

- Open IEM and click "Create Sample Plate."
- On the Library Prep Kit Selection page, choose a Library Prep Kit for your Agilent Libraries as stated above, e.g., "TruSeq LT" for the single-indexed SureSelect$^{XT}$/XT2/RNA-Seq and HaloPlex, and "Nextera XT" for the dual-indexed QXT and HaloPlex$^{HS}$.
- Enter the plate name and choose the appropriate index configuration.
- Enter your sample information from the "Table" view. Similarly, choose any index from the drop-down list for now, and the correct index will be entered when you edit the sample sheet later (as described above).




- After sample information has been entered in the Table view, the Plate and Plate Graphic views will automatically populate. Click "Finish."
- Go back to the IEM root page and click "Create Sample Sheet" > "HiSeq/HiScanSQ/GA" > your sequencer> "HiSeq FASTQ Only."
- Follow the instructions for the Sample Sheet Wizard – Workflow Parameters page (see above).
- On the Sample Selection page, uncheck the "Maximize" option to bring the sample-plate view and sample-sheet view onto the same page, and then click "Select Plate" to navigate to a sample plate that you created. Note: If you don't see the plate file that you intend to import, it might be because the Library Prep Kit does not match the one chosen when creating the sample plate.
- You can also use the "New Plate" option to create a sample plate here. Either highlight part of the sample plate or click "Select All" and then click "Add Selected Samples" to move the samples to the sample sheet. Click "Finish" and save the sample sheet.



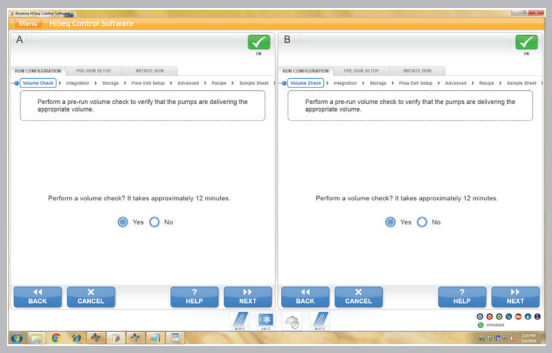

Click "Yes" when you are prompted with the below question, and edit the index names and sequences.

# I.1.2. Starting a run in the HiSeq Control Software user interface

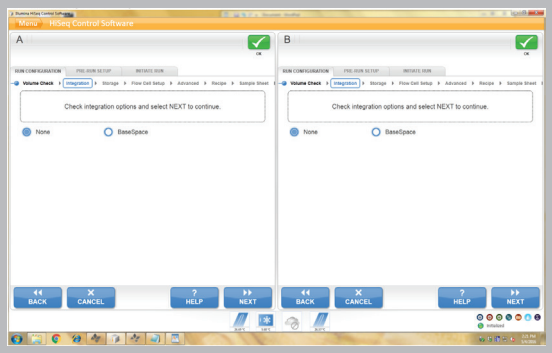
**Note:** For QXT, refer to the SureSelectQXT protocol for custom-primer spike-in guidelines.

**For HiSeq 2500/1500 models that support Rapid Mode, the user interface bifurcates at the beginning for High Output Mode and Rapid Mode. The below screen captures are based on Rapid Mode on the HiSeq 2500.**


Start the Rapid Mode workflow. Note that the example on the left has clusters generated on a cBot, and the one on the right represents on-board cluster generation.

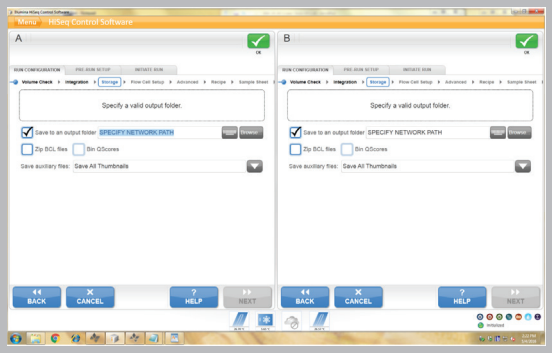



Perform volume check.

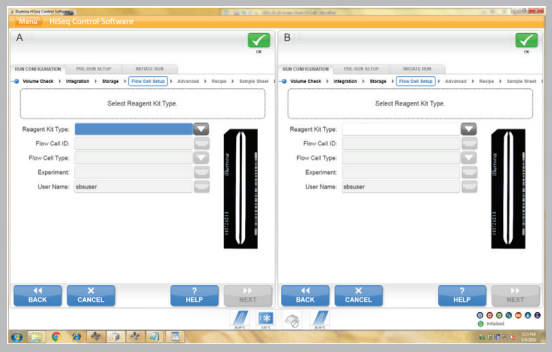



Check integration options.

# I.1.2. Starting a run in the HiSeq Control Software user interface

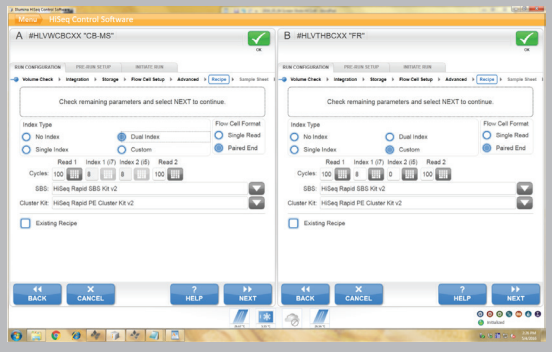



Choose storage options.



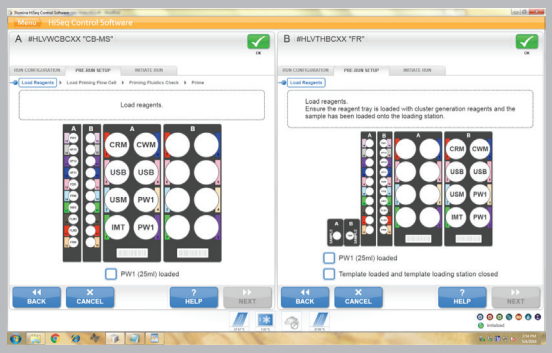

Flow-cell setup.


Advanced settings.



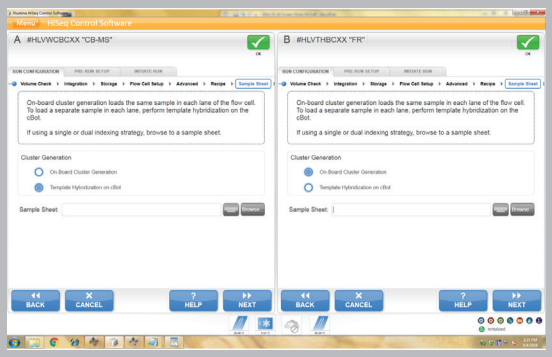

Fill in the recipe. Index type and number of cycles must align with the libraries to be sequenced. The examples in the below screen are QXT on the left and XT on the right.

# I.1.2. Starting a run in the HiSeq Control Software user interface

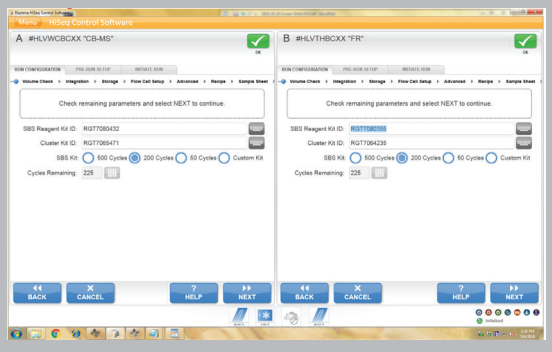



Choose the cluster-generation option and select the sample sheet.


Enter SBS Kit ID and Cluster Kit ID.



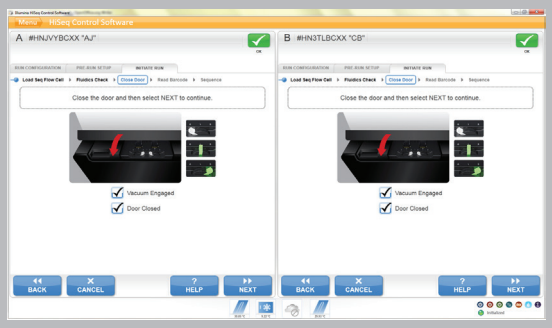

Load reagents.



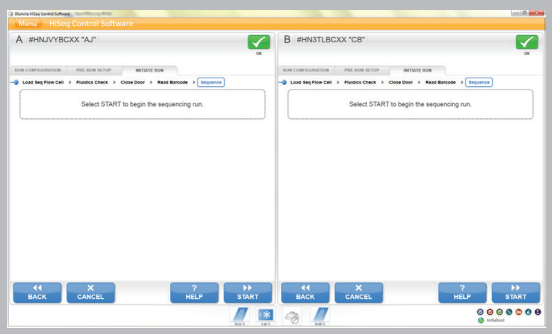

Load the flow cells, e.g., the priming flow cell on the left for clusters generated on a cBot and the sequencing flow cell on the right for on-board cluster generation.

# I.1.2. Starting a run in the HiSeq Control Software user interface


Fluidics check.



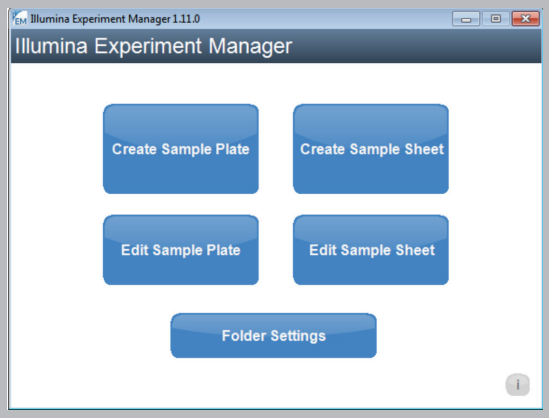

Close the door and allow the machine to read the flow-cell barcode.




Start the run.


The run starts.

# I.2.1. Planning a run on the MiSeq using IEM for Agilent NGS libraries



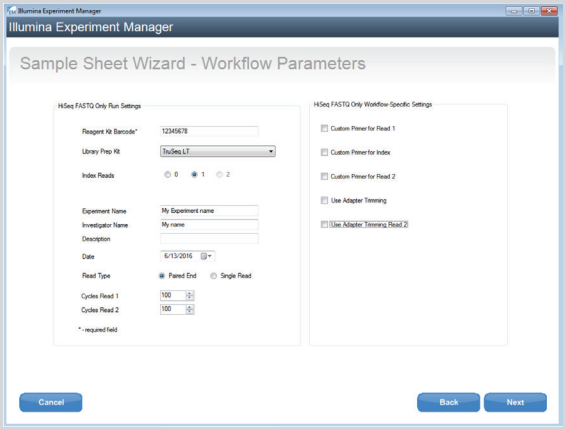

Click "Create Sample Sheet."


On the next screen (Instrument Selection), choose "MiSeq."


On the MiSeq Application Selection page, we recommend selecting "Other" as the category and "FASTQ Only" as the application.

# I.2.1. Planning a run on the MiSeq using IEM for Agilent NGS libraries

**On the next screen, fill in the fields for: Barcode, Experiment Name, Investigator Name, and Description. Specify selections for Library Prep Kit, Read Type, Cycles Read 1, and Cycles Read 2. You may set Cycles Read 1 and Cycles Read 2 to values based on your application needs and cartridge capacity.**




**For SureSelect$^{XT}$/XT2/RNA-Seq: Choose TruSeq LT as the Library Prep Kit.**

If you are following the shearing size of 150-bp to 200-bp, and using 100-bp or shorter read length, adapter trimming is not necessary. If you are using shorter shearing size or longer read length and prefer to have Illumina software do the trimming, check the adapter-trimming option. Otherwise, uncheck these two options and trim adapters later using Agilent SureCall software or the AGeNT trimming tool.

**For HaloPlex: Choose TruSeq LT, but uncheck the adaptor-trimming option and perform trimming in SureCall or using the AGeNT toolkit.**

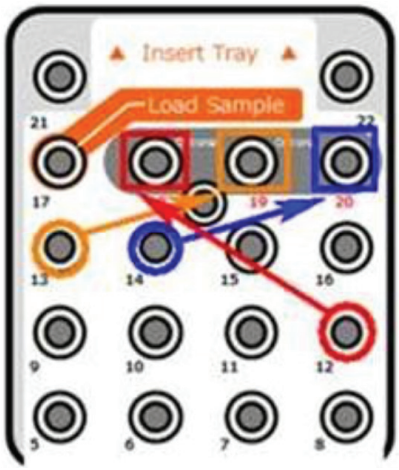
**For QXT: Choose Nextera XT and either:**

(1) leave "Use Adapter Trimming" checked or (2) (recommended) uncheck this option to trim the adapter using SureCall or the AGeNT toolkit.

If you choose the "Use Adapter Trimming" option, you will need to enter the consensus sequence for the QXT adapters provided in the QXT protocol (CTGTCTCTTGATCACA) when you edit the sample sheet.

# I.2.1. Planning a run on the MiSeq using IEM for Agilent NGS libraries

**You have two options for placing the QXT read-primer mixtures, and this determines whether you need to check the "Custom Primer" options on this screen.**




## OPTION 1

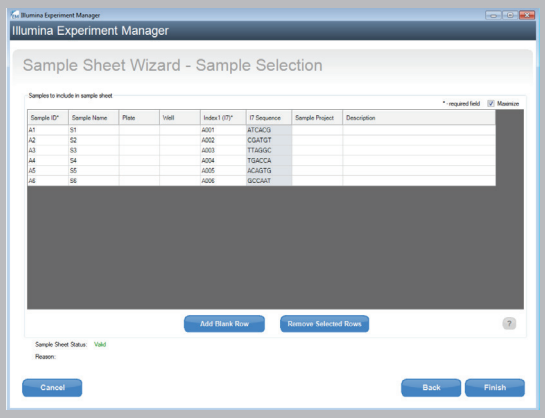
If you combine the Agilent QXT read primers with the Illumina read primers from wells 12, 13, and 14 and then move the primer mixtures to wells 18, 19, and 20 in the cartridge (see QXT protocol for instructions), you should check all three "Custom Primer" options. This instructs the MiSeq sippers to draw primers from the custom primer wells, e.g., 18, 19, and 20. Please note that the well positions do not match the Illumina names (i.e., HP10 is in position 12).

## OPTION 2

You can draw the Illumina read primers out and mix with the Agilent QXT read primers in clean tubes, place the primer mixtures back in the respective original Illumina read primer positions, and leave the three "Custom Primer" options unchecked.

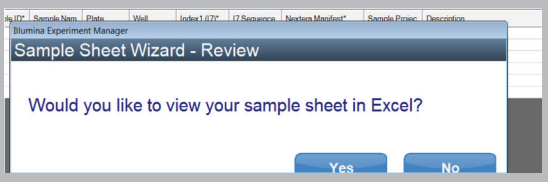
For HaloPlex$^{HS}$: Choose Nextera XT and uncheck the Use Adaptor Trimming option.

# I.2.1. Planning a run on the MiSeq using IEM for Agilent NGS libraries

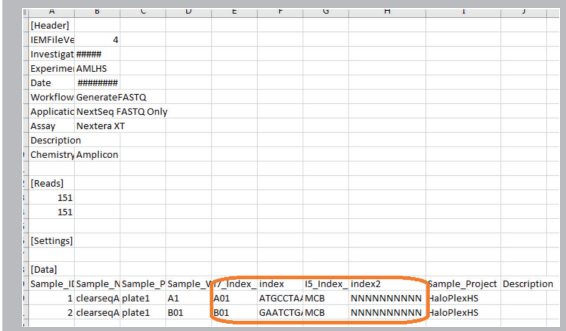



**Click "Next" to proceed to the Sample Selection screen.** Add a blank row for each sample. Fill in the SampleID, Sample Name, and Plate fields. At this point, you can specify any index and then change it when you edit the sample sheet later. The screen below is single-index for demonstration purposes only.

- Click "Finish" to save the sample sheet.



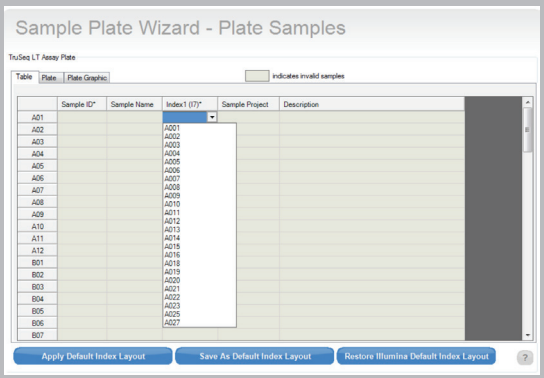

**When prompted, if you wish to open the sample sheet in Excel, click "Yes."** Then enter the correct sample index names and sequences and save the sample sheet. Copy or move the sample-sheet csv file to the appropriate location on your HiSeq, if you ran IEM on a different computer. Refer to Appendix 2A for example sample sheets of respective library types.




**Note for HaloPlex$^{HS}$:**

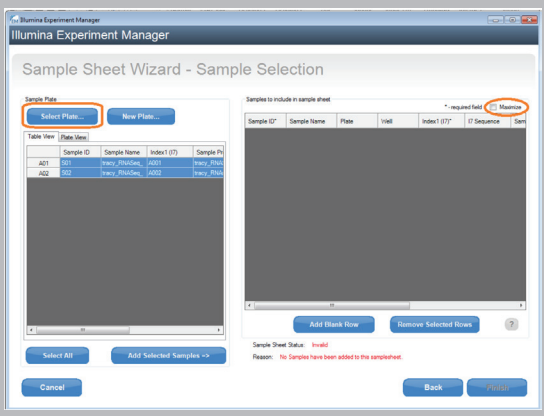
Index 2 is the molecular barcode and is not used for demultiplexing. Use 10 "N"s for index 2 for all samples in the sample sheet. You would demultiplex HaloPlex$^{HS}$ sequencing data using only index 1 and would need to generate the index 2 fastq files in addition to the paired-end read fastq files for data analysis. To do this, you can either modify the configuration of the MiSeq Reporter software or perform offline bcl-to-fastq conversion. Please refer to Appendix 1 for instructions.

# I.2.1. Planning a run on the MiSeq using IEM for Agilent NGS libraries

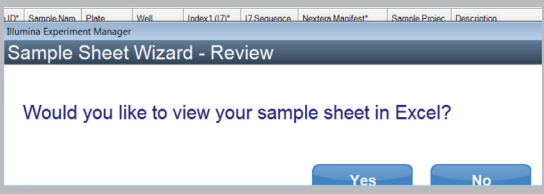



**Alternatively, you may create a sample plate first and then use the sample plate to generate a sample sheet.**

- Open IEM and click "Create Sample Plate."
- On the Library Prep Kit Selection page, choose a kit for your Agilent Libraries, e.g., "TruSeq LT" for the single-indexed SureSelect$^{XT}$/XT2 and HaloPlex, and "Nextera XT" for the dual-indexed QXT and HaloPlex$^{HS}$.
- Enter the plate name and choose the appropriate index configuration.
- Enter your sample information from the Table view. Similarly, choose any index from the drop-down list for now, and the correct index will be entered when you edit the sample sheet later (as described above).




- After sample information has been entered in Table view, the Plate and Plate Graphic views will automatically populate. Click "Finish."
- Go back to the IEM root page and click "Create Sample Sheet" > "MiSeq" > "FASTQ Only."
- Follow the instructions for the Sample Sheet Wizard – Workflow Parameters page (see above).
- On the Sample Selection page, uncheck the "Maximize" option to bring the sample-plate view and sample sheet view onto the same page, and then click "Select Plate" to navigate to a sample plate that you created.




**Note for HaloPlex$^{HS}$:**

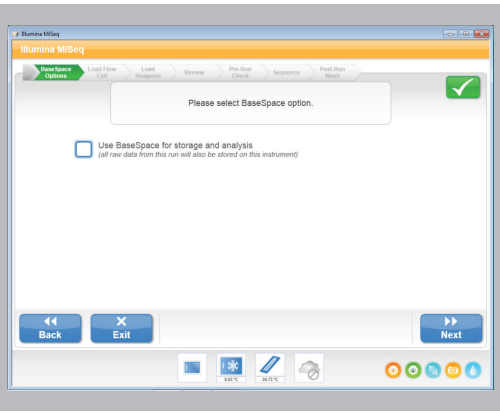
If you don't see the plate file that you intend to import, it might be because the Library Prep Kit does not match the one chosen when creating the sample plate.

- You can also use the "New Plate" option to create a sample plate. Either highlight part of the sample plate or click "Select All" and then click "Add Selected Samples" to move the samples to the sample sheet. Click "Finish" and save the sample sheet.
- Click "Yes" when you are prompted with the below question, and edit the index names and sequences.

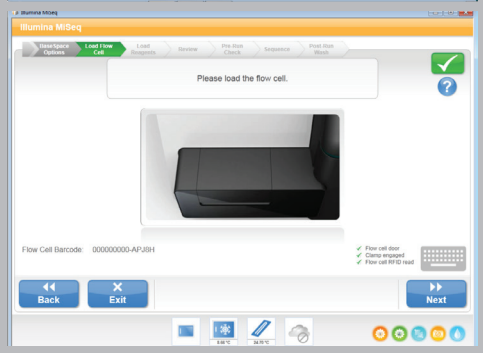
# I.2.2. Starting a run in the MiSeq Control Software user interface

**Note: For QXT, refer to the SureSelectQXT protocol for custom-primer spike-in guidelines.**


Click “SEQUENCE.”



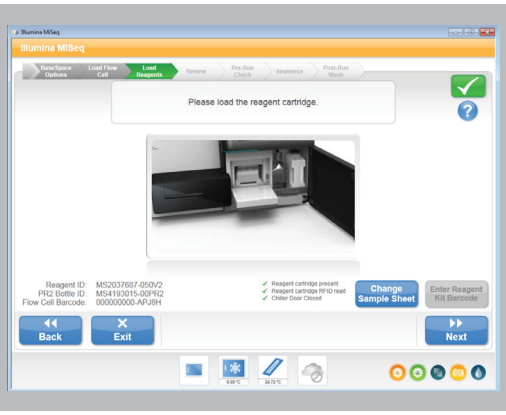

Choose or leave unchecked the “BaseSpace” option and follow the workflow provided by the software.

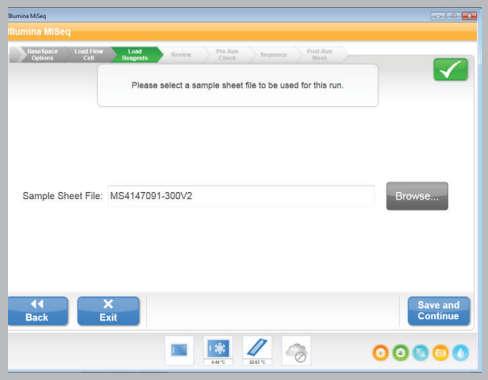

Load the flow cell as guided.

# I.2.2. Starting a run in the MiSeq Control Software user interface


Load the waste bottle.



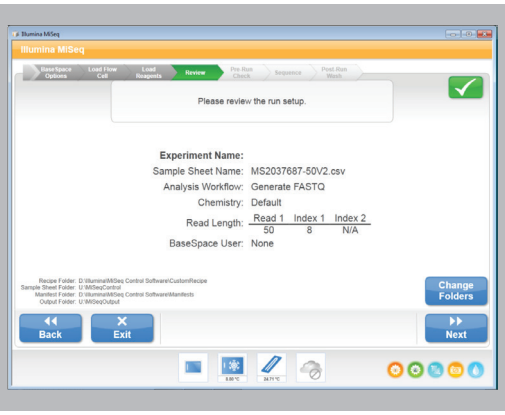

Load the reagent cartridge.



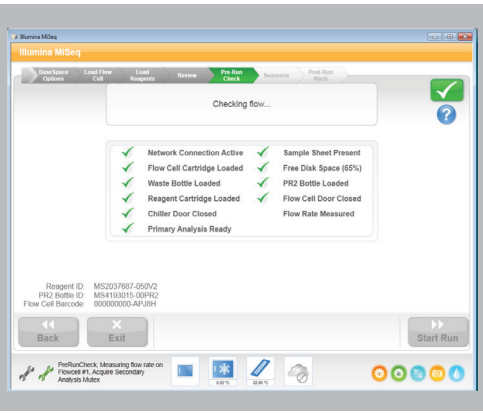

When you click "Change Sample Sheet" on the "Please load the reagent cartridge" screen, it brings you to the screen below.

Navigate to the sample sheet (csv file) by clicking "Browse." After clicking on "Save and Continue," you are brought back to the previous screen ("Please load the reagent cartridge."). Click "Next."

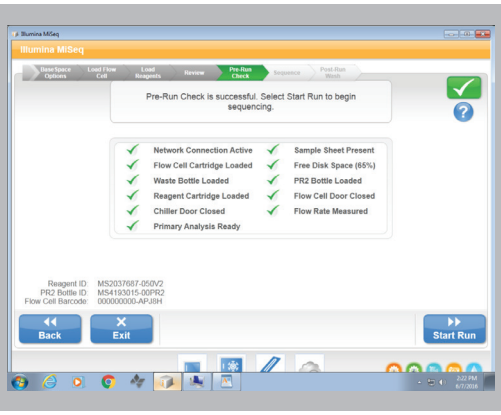
# I.2.2. Starting a run in the MiSeq Control Software user interface




Review the run setup.




The instrument performs a pre-run check.

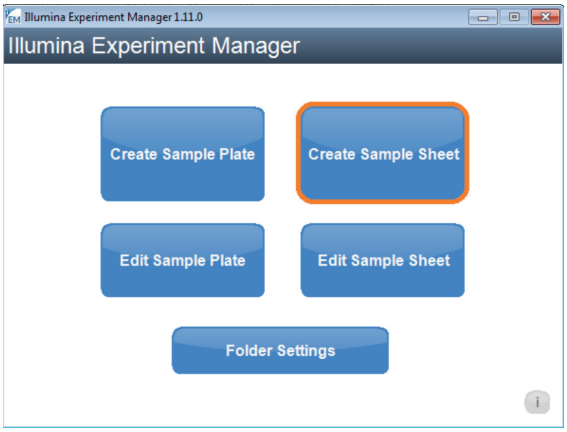



The pre-run check completes.

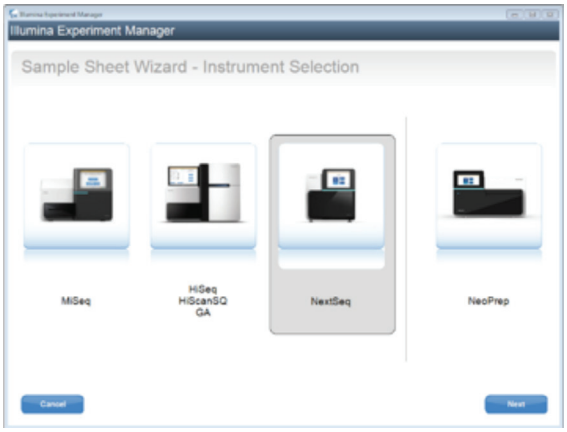

The run starts.

# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries

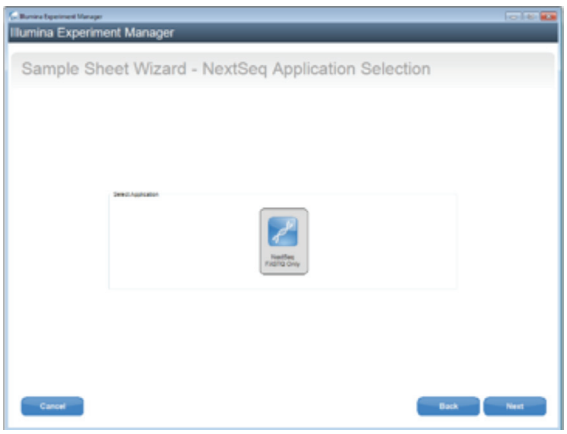
## Planning a run using IEM




**Open IEM, click "Create Sample Sheet"**

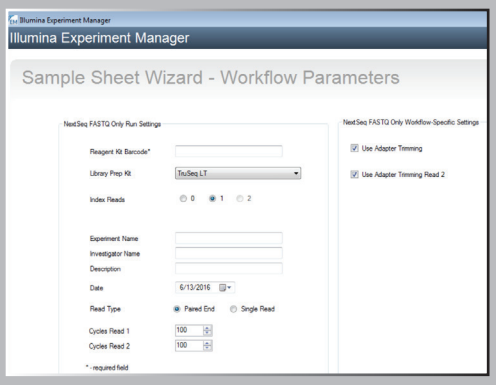



**"NextSeq"**




**"FASTQ Only."**

# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries




**On the Sample Sheet Wizard - Workflow Parameters page, enter Reagent Kit Barcode, Experiment Name, Investigator Name, and Description. Paired-end sequencing is recommended for all Agilent NGS libraries.**

**For XT/XT2/RNA-Seq, choose TruSeq LT as the Library Prep Kit.**

If you are following the shearing size of 150-bp to 200-bp and using 100-bp or shorter read length, adapter trimming is not necessary. If you are using shorter shearing size or longer read length and prefer to have Illumina software do the trimming, check the adapter-trimming option. Otherwise, uncheck these two options and trim adapters later using Agilent SureCall software or the AGeNT trimming tool.

**For HaloPlex: Choose TruSeq LT, but uncheck the adaptor trimming options and perform trimming in SureCall or using the AGeNT toolkit.**

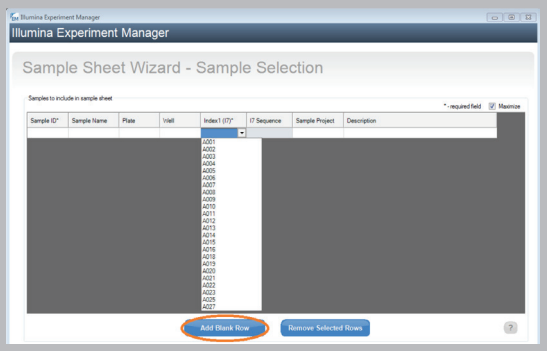
**For QXT: Choose Nextera XT and either:**

(1) leave "Use Adapter Trimming" checked or (2) uncheck this option to trim the adapter using SureCall or the AGeNT toolkit. Trimming with SureCall or the AGeNT toolkit is recommended.

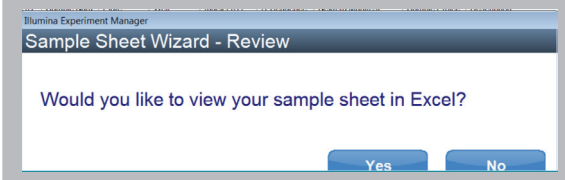
If you choose the "Use Adapter Trimming" option, you will need to enter the consensus sequence for the QXT adapters provided in the QXT protocol (CTGTCTCTTGATCACA), when you edit the sample sheet.

**For HaloPlex$^{HS}$: Choose Nextera XT and uncheck "Use Adaptor Trimming."**

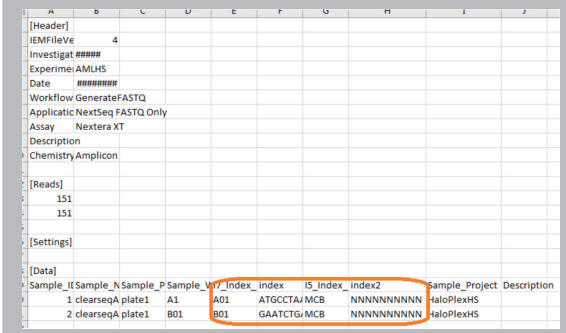
# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries




**On the Sample Selection page, click "Add Blank Row" to add rows and fill in the sample information.** You may choose any index from the drop-down list (same for dual-indexed samples) at this time. The correct indexes will be entered when you edit the sample sheet later. The screen below is single-indexed for demonstration purposes only.



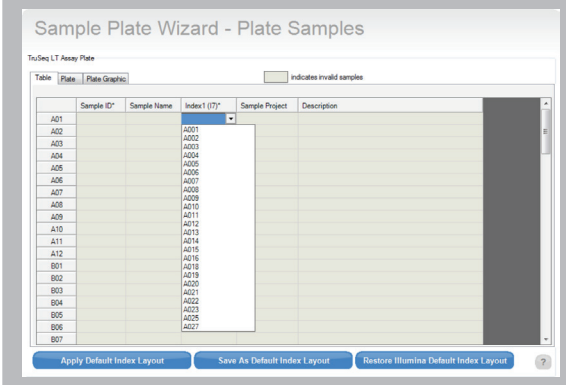

**When all sample information is entered, click "Finish" and save the sample sheet.** When prompted with the below question, click "Yes." When the sample sheet opens, enter the actual index names and sequences used. Save the edited sample sheet to the original location.



| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| [Header] | | | | | | | | | |
| IEMFileVe | 4 | | | | | | | | |
| Investigat | ##### | | | | | | | | |
| Experime | AMLHS | | | | | | | | |
| Date | ########## | | | | | | | | |
| Workflow | GenerateFASTQ | | | | | | | | |
| Applicatic | NextSeq FASTQ Only | | | | | | | | |
| Assay | Nextera XT | | | | | | | | |
| Description | | | | | | | | | |
| Chemistry | Amplicon | | | | | | | | |
| | | | | | | | | | |
| [Reads] | | | | | | | | | |
| 151 | | | | | | | | | |
| 151 | | | | | | | | | |
| | | | | | | | | | |
| [Settings] | | | | | | | | | |
| | | | | | | | | | |
| [Data] | | | | | | | | | |
| Sample_ID | Sample_N | Sample_P | Sample_Well | I7_Index_ | index | I5_Index_ | index2 | Sample_Project | Description |
| 1 | clearseqA | plate1 | A1 | A01 | ATGCCTA/ | MCB | NNNNNNNNNN | HaloPlexHS | |
| 2 | clearseqA | plate1 | B01 | B01 | GAATCTG/ | MCB | NNNNNNNNNN | HaloPlexHS | |

**Note: For HaloPlex$^{HS}$,** index 2 is the molecular barcode and will not be used for demultiplexing. Use 10 "N"s for index 2 for all samples in the sample sheet. You will need to perform offline bcl-to-fastq conversion to demultiplex using only index1. In the meantime, generate a fastq file for index 2; the fastq file will be needed for HaloPlex$^{HS}$ data analysis. Please refer to Appendix 1 for more instructions. **For QXT,** refer to the P5 index sequence table specific to NextSeq in the QXT protocol, as the index sequences must be entered as the reverse complement of those used for the HiSeq 2000/2500 and the MiSeq.

# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries




**Alternatively, you may create a sample plate first and then use the sample plate to generate a sample sheet.**

- Open IEM and click "Create Sample Plate."
- On the Library Prep Kit Selection page, choose a Library Prep Kit for your Agilent Libraries as stated above, e.g., "TruSeq LT" for the single-indexed SureSelect$^{XT}$/XT2/ RNA-Seq and HaloPlex, and "Nextera XT" for the dual-indexed QXT and HaloPlex$^{HS}$.
- Enter the plate name and choose the appropriate index configuration.
- Enter your sample information from the Table view. The columns with * are required fields. Similarly, choose any index from the drop-down list for now, and the correct index will be entered when you edit the sample sheet later (as described above).
- After sample information has been entered in Table view, the Plate and Plate Graphic views will automatically populate. Click "Finish."
- Go back to the IEM root page and click "Create Sample Sheet" > "NextSeq" > "FASTQ Only."


**Follow the instructions for the "Sample Sheet Wizard - Workflow Parameters page" (see above).**

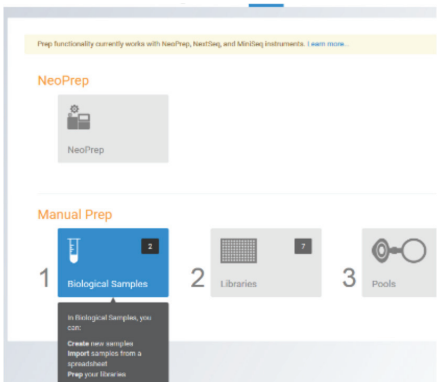
- On the Sample Selection Page, uncheck the "Maximize" option to bring the sample-plate view and sample-sheet view onto the same page, and then click "Select Plate" to navigate to a sample plate that you created. Note: If you don't see the plate file that you intend to import, it might be because the Library Prep Kit does not match the one chosen when creating the sample plate.
- You can also use the "New Plate" option to create a sample plate. Either highlight part of the sample plate or click "Select All" and then click "Add Selected Samples" to move the samples to the sample sheet. Click "Finish" and save the sample sheet.
- Click "Yes" when you are prompted with the question "Would you like to view your sample sheet in Excel?" and then edit the index names and sequences.

# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries

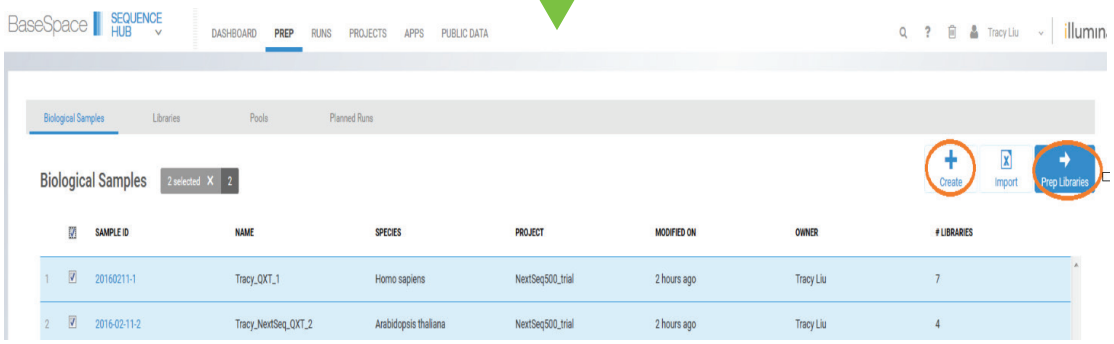
## Planning a run using BaseSpace

*You can either add samples and then add library information (e.g., plan for index usage on the samples) or import prepared libraries in one step.

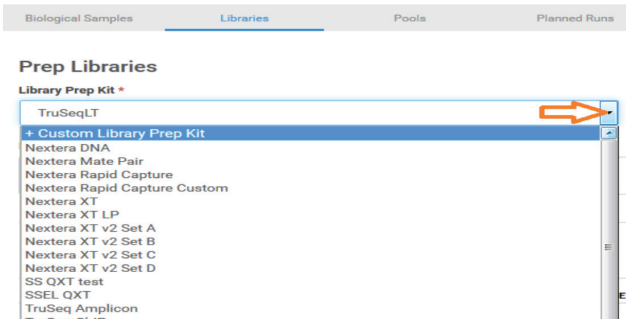
### TO ADD SAMPLES INDIVIDUALLY




**Log into BaseSpace and then go to PREP > Biological Samples > Create.**



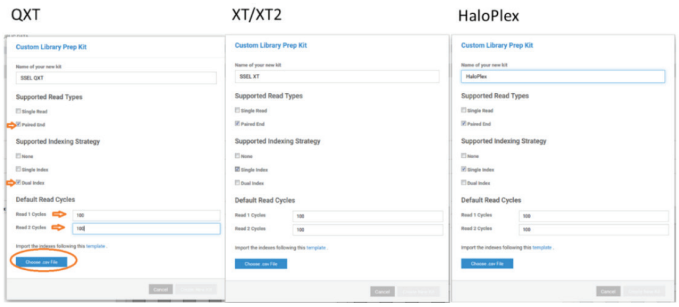

**Enter biological samples one by one, and then choose/check the samples to process with "Prep Libraries."**




**Click "+ Custom Library Prep Kit."**

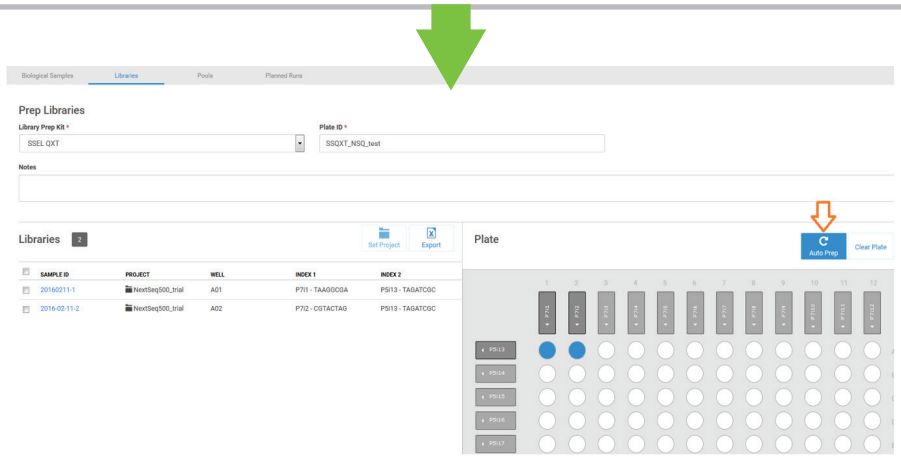
# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries

## TO ADD SAMPLES INDIVIDUALLY (cont.)

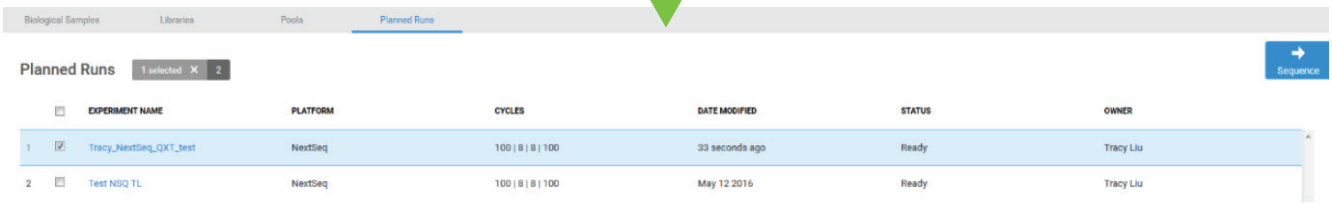



**Describe the new Custom Library Prep Kit in the pop-up window. Upload the QXT, XT/XT2/RNA-Seq, or HaloPlex LibraryPrepKit template .csv file (available in the appendices). This will generate a corresponding Library Prep Kit in the "Library Prep Kit" drop-down list. Note 100-bp read length is shown for demonstration only.**

**Note for QXT:** Because BaseSpace automatically converts the P5 indexes to reverse complement sequences, the P5 index sequences in the QXT LibraryPrepKit template file that we provide are not reverse complement.



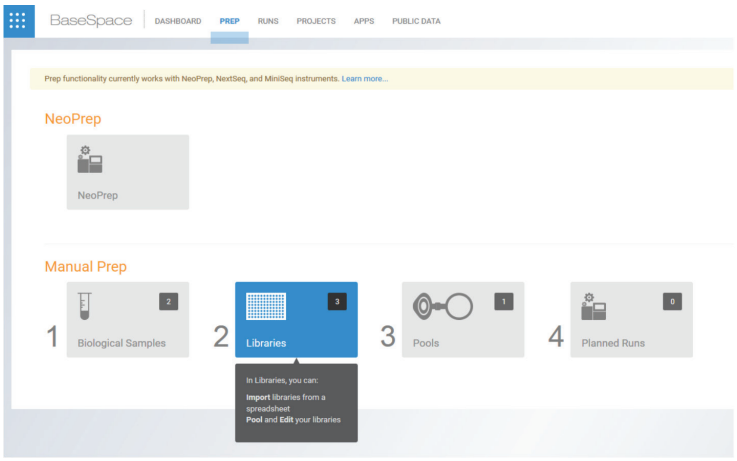

**Choose the desired Library Prep Kit in the drop-down list, enter a Plate ID, and click "Auto Prep" to populate the wells and assign indexes to each sample. You can also check the boxes in front of a sample and drag it to a well.**



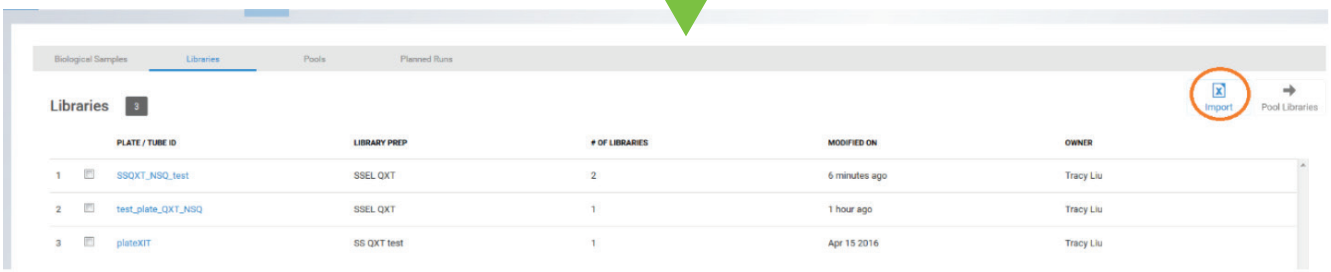

**Proceed to "Pool Libraries" and "Plan Run" following the BaseSpace workflow. A new run will appear in the list of "Planned Runs." Check the run you want to sequence, and click "Sequence." This run will be ready for sequence and can be retrieved in NCS.**

# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries

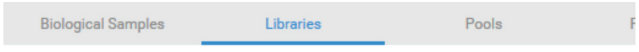
## TO ADD A SAMPLE LIBRARY FILE (e.g. with indexes added/assigned)




**Go to PREP > Libraries.**




**Import the sample library csv file corresponding to your library type (XT/XT2, QXT, or HaloPlex). You can download a template from here. Note: The name given in the "LibraryPrepKit" field in the csv file must exactly match an existing Library Prep Kit (either by Illumina default or created by you), and the [Header] line cannot be blank.**




### Import Sample Libraries

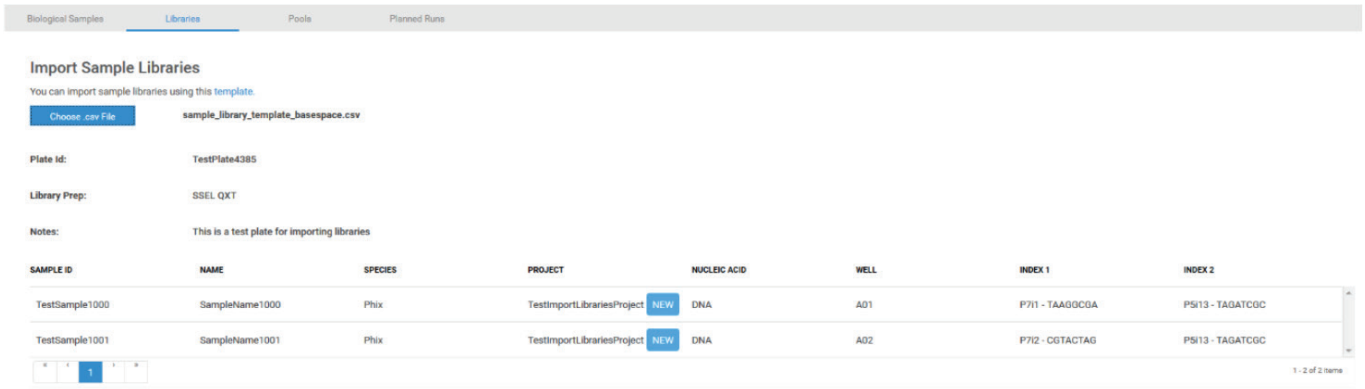
You can import sample libraries using this template.

| | A | B | C | D | E |
|---|---|---|---|---|---|
| 1 | [Header] | | | | |
| 2 | FileVersion | 1 | | | |
| 3 | | | | | |
| 4 | [Data] | | | | |
| 5 | UserSampleID | Name | Species | Project | NucleicAcid |
| 6 | TestSample123 | SampleA | Bos_taurus | Project_A | DNA |
| 7 | RealSample456 | SampleB | PhiX | Project_B | RNA |

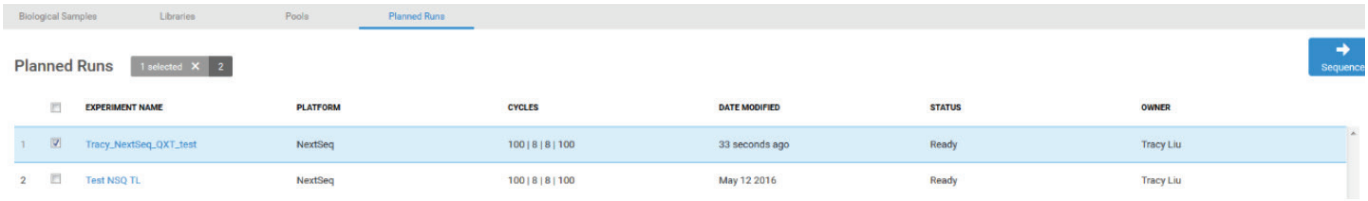
**A link to download a template is provided in the import window.**

# I.3.1. Planning a run on the NextSeq for Agilent NGS libraries

## TO ADD A SAMPLE LIBRARY FILE (cont.)




**The samples in the csv file will populate in a plate.**




**Proceed to "Pool Libraries" and "Plan Run" following the BaseSpace workflow. A new run will appear in the list of "Planned Runs." Check the run you want to sequence, and click "Sequence." This run will be ready for sequencing and can be retrieved in NCS.**

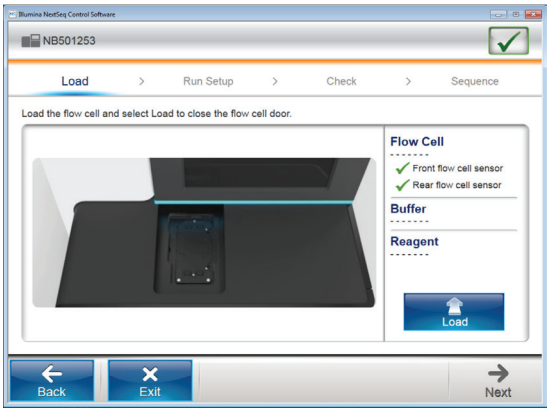
# I.3.2. Starting a run in the NextSeq Control Software user interface

## Note:

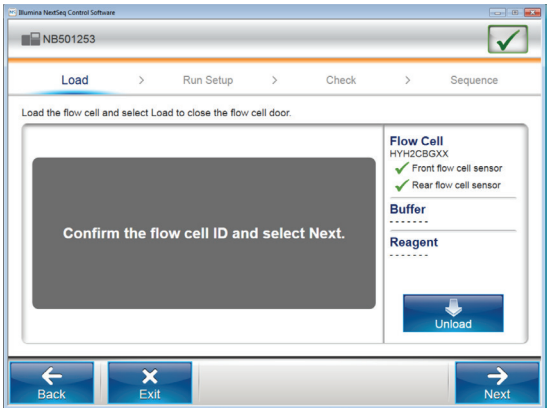
1. The screen shots shown in this section are from a run on a stand-alone machine.

2. For QXT, refer to the SureSelect$^{QXT}$ protocol for custom-primer spike-in guidelines.


**Click "SEQUENCE" and follow the workflow provided by the software.**

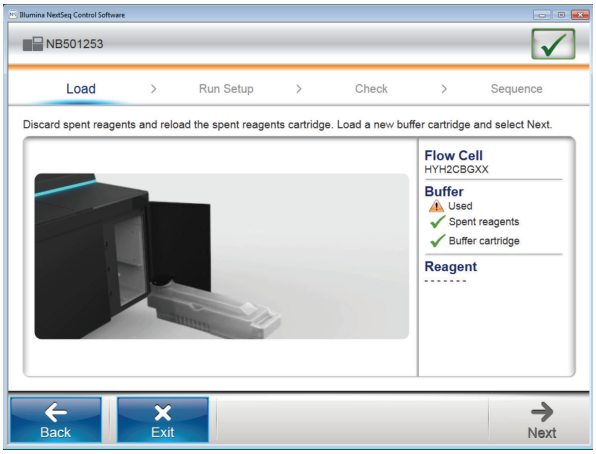



**On BaseSpace- and BaseSpace Onsite-configured machines, you will log into BaseSpace after clicking "Sequence." Standalone machines proceed to "Load."**

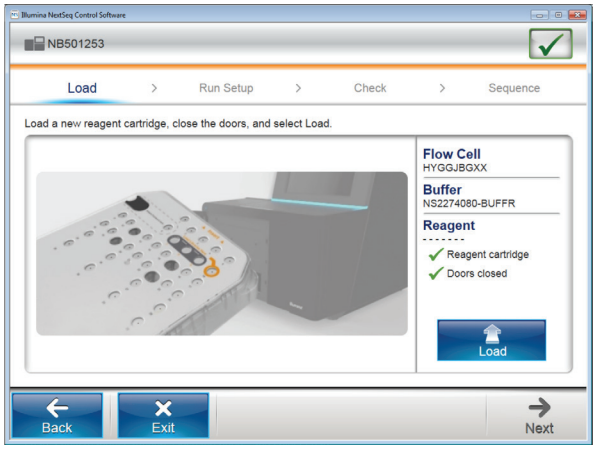



**The instrument scans the flow-cell ID and checks the flow-cell sensor.**

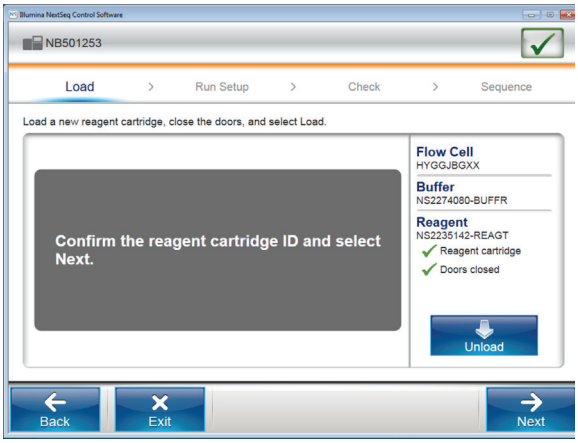
# I.3.2. Starting a run in the NextSeq Control Software user interface




**Empty the spent reagent cartridge and load the buffer cartridge.**




**Load the reagent cartridge.**



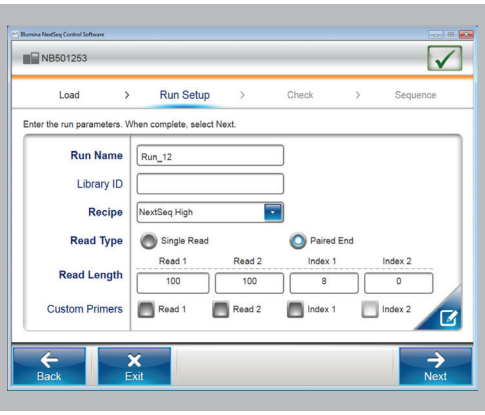

**Confirm the reagent cartridge ID.**

# I.3.2. Starting a run in the NextSeq Control Software user interface

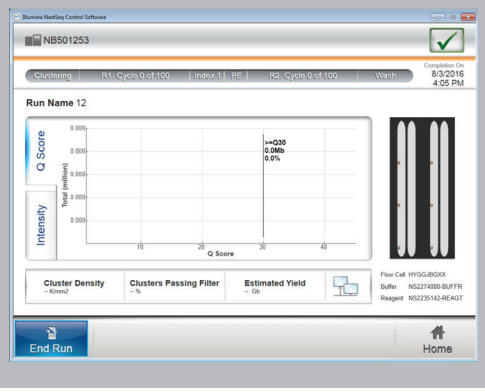
**On BaseSpace- and BaseSpace Onsite-configured machines, you will be provided with a screen where you can select a run planned on BaseSpace at the "Run Setup" step before the run parameter screen shown below.**

| | Read Type | Index Type | Read Length | Index 1 Read Length | Index 2 Read Length | Custom Primers |
|---|---|---|---|---|---|---|
| XT/XT2 | Paired End | single | 2x 75/2x100/2x150 bp | 8 | 0 | No |
| QXT | Paired End | dual | 2x 75/2x100/2x150 bp | 8 | 8 | Check all 3 |
| HaloPlex | Paired End | single | 2x100 or 2x150 bp depending on design | 8 | 0 | No |
| HaloPlex$^{HS}$* | Paired End | dual | 2x100 or 2x150 bp depending on design | 8 | 10 | No |

Use the parameters in this table for your Agilent NGS libraries.




*HaloPlex$^{HS}$ can only be set up on stand-alone machines.



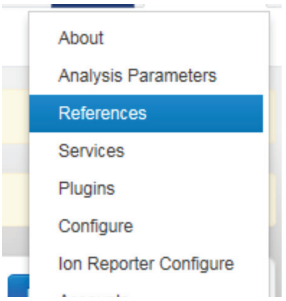

The run starts.

# II.1. Planning a run and reanalyzing a run in the Torrent Server for Agilent$^{XT}$ and HaloPlex/HaloPlex$^{HS}$ libraries

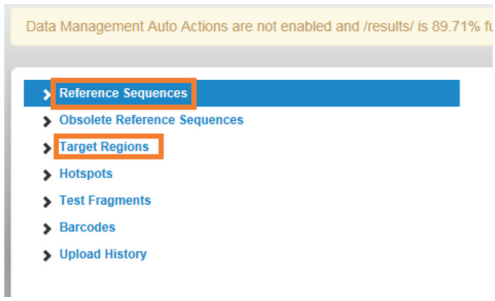
**Notes:**

- The demonstration of Torrent Server is based on v5.0.4.
- This section should apply to PGM, Proton, and S5, although these settings had not been validated on the S5 when this document was completed.
- We recommend analyzing sequencing data from the Torrent Server and associated BAM files with Agilent SureCall software.
- HaloPlex$^{HS}$ data must be reanalyzed to enable modified adapter trimming.

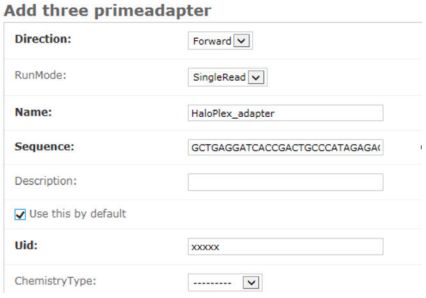
## PLANNING A RUN ON THE TORRENT SERVER
### Add genome and ROI




**Go to the Reference page by clicking on the gear icon in the upper right corner.**




**Add reference genome and target region BED file (the covered.BED file of your design).**

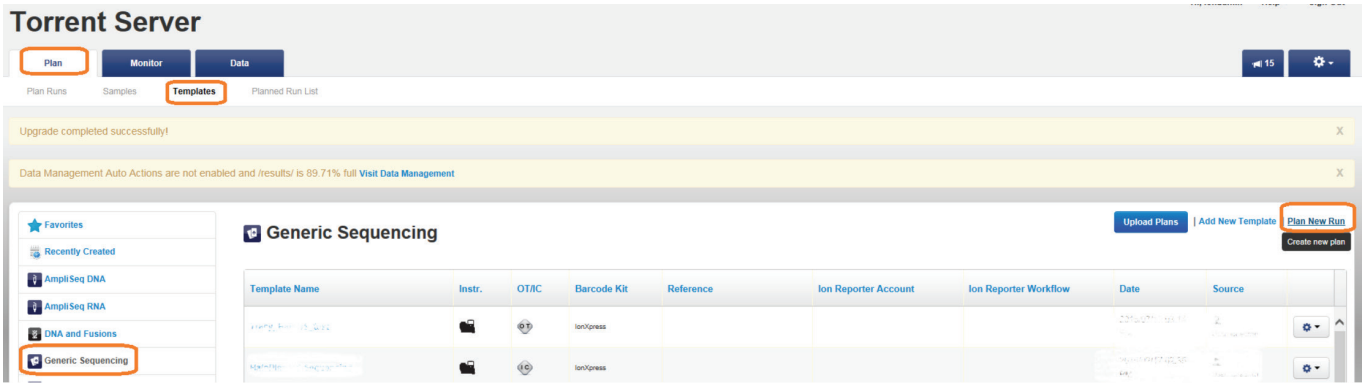



**When first sequencing HaloPlex or HaloPlex$^{HS}$ libraries on the ION instrument, define the HaloPlex-specific 3'-adapter sequence as a custom 3'-adapter on the Torrent Server.**

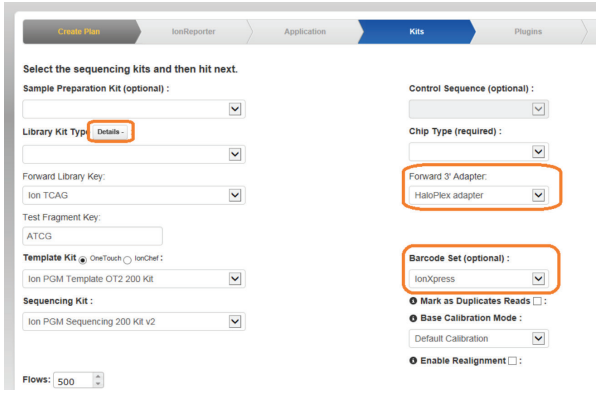
Go to http://ionserver/admin/rundb/threeprimeadapter/add/ and add GCTGAGGATCACCGACTGCCCATAGAGAGGCTGAGA in the "Sequence" field. Name it as you see fit, and save.

# II.1. Planning a run and reanalyzing a run in the Torrent Server for Agilent$^{XT}$ and HaloPlex/HaloPlex$^{HS}$ libraries

## PLAN A RUN

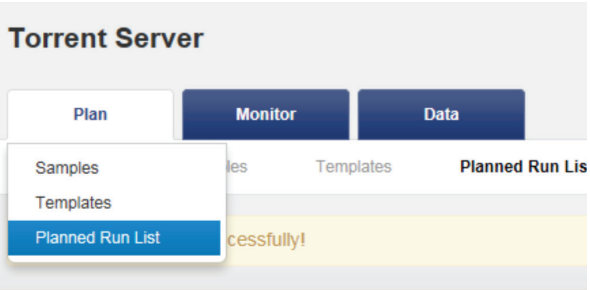



**Go to the "Plan" tab, click "Templates" > "Generic Sequencing" > "Plan New Run."**




- **Use default settings under the IonReporter and Application tabs.**
- **Specify kits, 3' adapter, and barcode set under the Kits tab.**

**Leave the Sample Prep Kit and Library Prep Type fields blank. For HaloPlex/HaloPlex$^{HS}$, click "Details," and change the 3' Adapter to the one that you defined above (A.1). For SureSelect, use the default 3' Adapter ION P1B. Specify the "Chip Type," "Template Kit," and "Sequencing Kit" you are using, and choose "IonXpress" barcode set for all Agilent kits.**




- **Plug-ins are optional. We currently have not validated any pre-installed plug-ins and recommend that you run without plug-ins and use SureCall for analysis.**
- **Complete the Projects and Plan sections. This will create a new run under "Planned Run List" and can be navigated from the touch screen of the sequencer linked to your Torrent Server. The Monitor tab lets you monitor the status on the Ion Chef or the associated sequencing instrument under "Runs in Progress."**

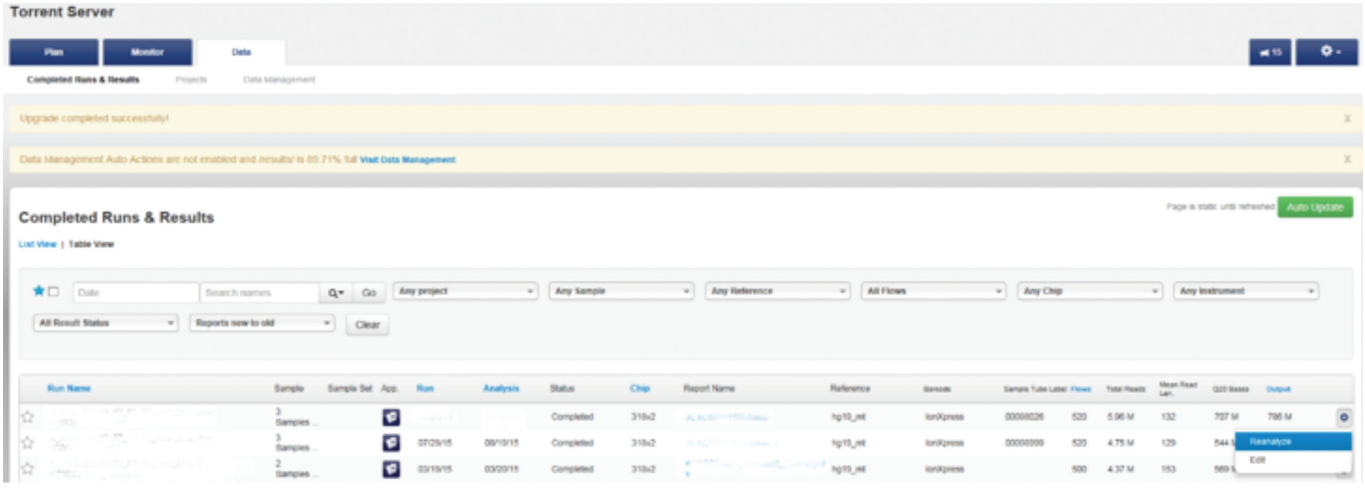
# II.1. Planning a run and reanalyzing a run in the Torrent Server for Agilent$^{XT}$ and HaloPlex/HaloPlex$^{HS}$ libraries

## Reanalyzing a run on the Torrent Server

A completed run can be reanalyzed and analysis parameters can be modified when reanalyzing.

**Note: HaloPlex$^{HS}$ data must be reanalyzed to trim an additional 15 bases from the 3 prime.**

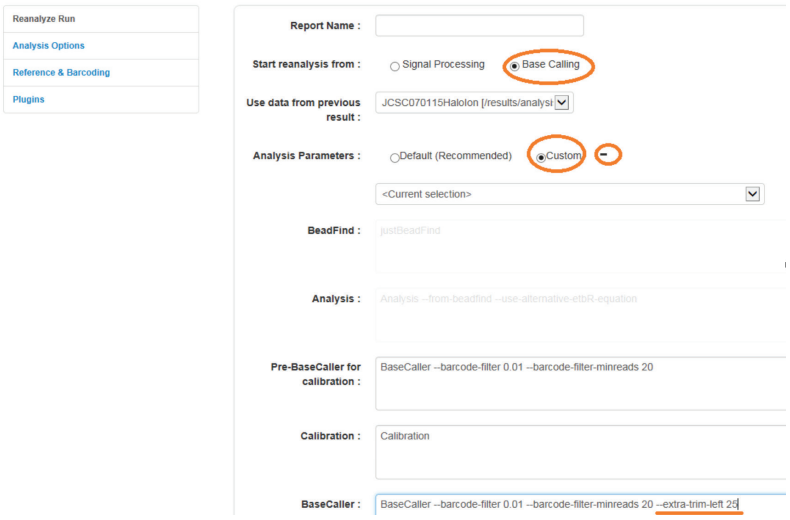
### PLAN A RUN



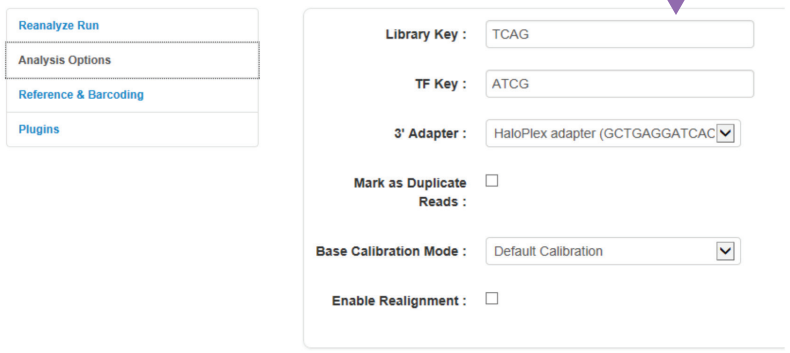

**Go to "Data" > "Completed Run & Results." Click the gear icon (far right) of the run you want to reanalyze and choose "Reanalyze."**

# II.1. Planning a run and reanalyzing a run in the Torrent Server for Agilent$^{XT}$ and HaloPlex/HaloPlex$^{HS}$ libraries

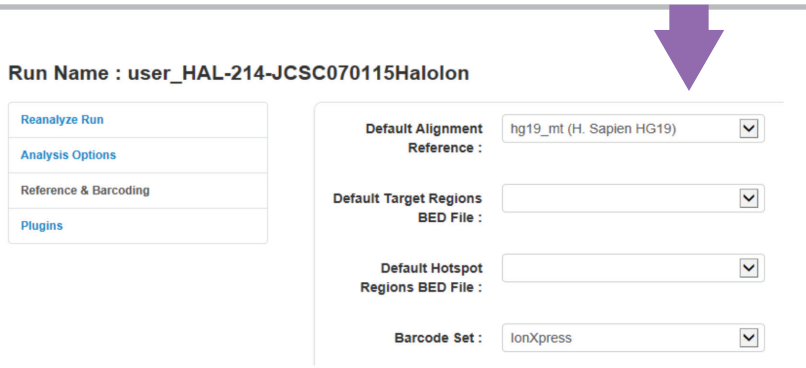
## PLAN A RUN




**For HaloPlex$^{HS}$, choose "Base Calling" and "Custom." Click the plus sign next to "Custom" to bring the detailed custom settings. Add text "—extra-trim-left 25" in the BaseCaller field, as shown below.**




**Specify the 3' Adapter in the Analysis Options section. Use the HaloPlex custom 3' Adapter that was added as described above (see A. 1) for HaloPlex or HaloPlex$^{HS}$ data, and ION P1B for SureSelect. No other changes are needed in "Analysis Options."**

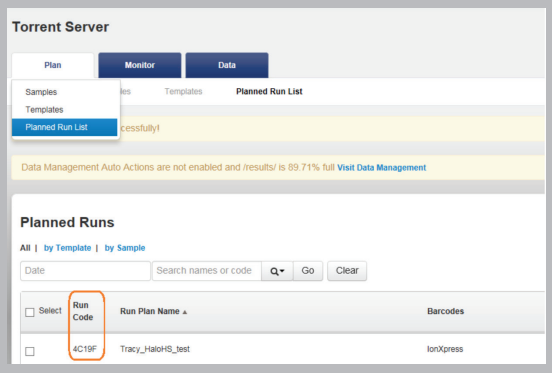



**Specify reference genome, regions BED file, and barcode set in the Reference & Barcoding section. Use IonXpress as the barcode set for all Agilent libraries.**

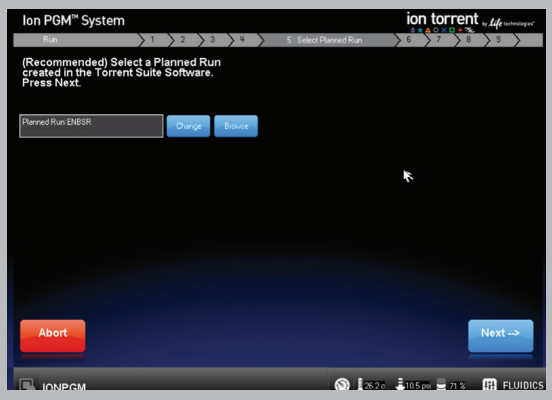
**Plug-ins are optional. We currently have not validated any pre-installed plug-ins and recommend that you run without plug-ins and use SureCall for analysis. Click "Start."**

# II.2. How to start a run on ION sequencing instruments

**Note:** There isn't anything specific to NGS libraries prepped with the Agilent kit when launching a planned run on ION sequencing instruments. Follow the ION documentation to prepare the sequencer and the chip. When a chip is loaded properly, call the planned run from the touch screen of the sequencer. Below is an example of launching a run on the ION PGM.*




**After a run is planned on the Torrent Server, the run will appear in the Planned Run List with a unique, 5-digit Run Code.**




**After the sequencer is properly initialized and the chip is loaded, click "Browse," next to "Planned Run" in the screen's header section 5: Select Planned Run, and then select the run. Alternatively, enter the Run Code in the Planned Run field. Click "Next."**


**The digital protocol associated with the selected run will populate in the Run Info section. Verify the settings and continue, following the software-guided workflow to start the run.**

*The ION PGM touch screen images are adapted from the ION PGM sequencing 200 kit V2 user guide (Publication Number MAN0007273).

# III.1. Options for bcl-to-fastq conversion for Illumina sequencing

**Bcl-to-fastq conversion, along with demultiplexing, typically is done on the instrument. There are occasions when offline conversion may be needed.**

**A. When a run was not set up properly and lacks critical parameters, such as when the Library Prep Kit was not specified. This should be a rare case, and the conversion can be performed with the Illumina bcl2fastq software in a standard manner. Download the software from the Illumina website and consult with Illumina if you need assistance.**

# III.1. Options for bcl-to-fastq conversion for Illumina sequencing

## B. HaloPlex$^{HS}$ users will likely need to do offline conversion to retrieve the I2 fastq file, which contains the molecular barcode reads. Currently, there are three possible ways to obtain the I2 fastq file while generating demultiplexed fastq files of the paired-end reads.

**1. When sequencing on a MiSeq, you may modify the MiSeq Reporter software configuration for this purpose; do so before the sequencing run, if the software is installed on a MiSeq workstation. Or you may reanalyze the data using stand-alone MiSeq Reporter (this way the change will not affect subsequent sequencing runs).**

The instructions on how to modify the MiSeq Reporter configuration can be found in the HaloPlex$^{HS}$ ILM protocol under "MiSeq platform sequencing run setup guidelines."

Basically, modify the MiSeq Reporter configuration file for the instrument that directs the sequencer to generate a third fastq file for the molecular barcodes in index 2. The configuration XML file is usually found at: C:/Illumina/MiSeq Reporter/MiSeq/Reporter.exe.config.

```
<?xml version="1.0"?>CRLF
<configuration>CRLF
  <appSettings>CRLF
    <add key="Repository" value="D:\Illumina\MiSeqAnalysis" />CRLF
    <add key="GenomePath" value="C:\Illumina\MiSeq Reporter\Genomes" />CRLF
    <add key="TempFolder" value="D:\Illumina\MiSeqAnalysis\Temp" />CRLF
    <add key="EnableHTTPService" value="1"/>CRLF
    <add key="DefaultRedirectPath" value="Default.htm"/>CRLF
    <add key="ClientSettingsProvider.ServiceUri" value="" />CRLF
    <add key="CopyToRTAOutputPath" value="1"/>CRLF
    <add key="MaximumHoursPerProcess" value="72"/>CRLF
    <add key="CreateFastqForIndexReads" value="1"/>CRLF
  </appSettings>CRLF
  <startup>CRLF
    <supportedRuntime version="v4.0" sku=".NETFramework,Version=v4.0" />CRLF
```

**The file must be edited with a text editor and not with Excel. The syntax of the line must precisely match the line shown in the green box, including the < and > brackets and quotation marks.**

The characters shown in black (CR LF) stand for carriage return and linefeed; they may not be visible in your editor. Do not change any other lines. You may cut and paste from the line below into a blank line you've inserted into the file:

**<add key="CreateFastqForIndexReads" value="1" />**

Save the file and restart the instrument for this change to take effect.

**NOTE:** This change will remain in place for all subsequent runs for all assays. If you are running an assay other than HaloPlex$^{HS}$, you may edit the file and change the "1" to a "0."

# III.1. Options for bcl-to-fastq conversion for Illumina sequencing

**2. Use Illumina's bcl2fastq conversion software.**

- **Use a base mask parameter ("---use-bases-mask Y*,I8,Y10,Y*"), where * represents the read length**
- **When using v2.17 or a newer version, run it with "--mask-short-adapter-reads 0" so that it doesn't delete the index2 read sequence**
- **Modify the sample sheet to have only the sample index (and not the molecular barcode index) by clearing the content in the "I5_index_ID" and "index2" columns. The software cannot handle "N" characters in a barcode sequence.**

**3. Customers can use Picard tools from the Broad Institute to do bcl-to-fastq conversion, if functional Illumina software is not available. Below are the commands (using Picard tools 2.4.1 and java 8). Picard can be downloaded here: http://broadinstitute.github.io/picard/**
**The tool documentation is here: http://broadinstitute.github.io/picard/command-line-overview.html**

Two tools are needed:
ExtractIlluminaBarcodes (to find the barcodes)
IlluminaBasecallsToFastq (to generate the fastq files based on the output of the first command)

Here are the commands that have been used at Agilent:
nohup java -jar picard.jar ExtractIlluminaBarcodes BASECALLS_DIR=<sequencing_run_directory>/Data/Intensities/BaseCalls/ OUTPUT_DIR=<barcode_output_dir_name> LANE=1 READ_STRUCTURE=<read_structure> BARCODE_FILE=<barcode_file> METRICS_FILE=<metric_file_name> NUM_PROCESSORS=<n> &

nohup java -jar picard.jar IlluminaBasecallsToFastq BASECALLS_DIR=<sequencing_run_directory>/Data/Intensities/BaseCalls/ LANE=1 BARCODES_DIR=<barcode_output_dir_name> READ_STRUCTURE=<read_structure> FLOWCELL_BARCODE=<FCID> MACHINE_NAME=<machine_name> RUN_BARCODE=<run_number> ADAPTERS_TO_CHECK=PAIRED_END

NUM_PROCESSORS=<n> READ_NAME_FORMAT=CASAVA_1_8 COMPRESS_OUTPUTS=true MULTIPLEX_PARAMS=<multiplex_params_file> IGNORE_UNEXPECTED_BARCODES=true TMP_DIR=<temp_directory_location> &

# III.1. Options for bcl-to-fastq conversion for Illumina sequencing

**Variables explained (which is also found in the Picard tool documentation):**

- sequencing_run_dir: The sequencing raw data directory
- barcode_output_dir_name: Files with indexes of barcodes will be placed here. (Files will be named by lane, and tile number with "_barcode.txt" at the end.)
- read_structure: A string explaining which cycle in the read corresponds to which part. T=template, B=sample barcode, M=molecular barcode, S=skip (i.e., a 2x150 run with 8-bp sample barcode and 10-bp molecular barcode would be: 150T8B10M150T). The cycle numbers for reads have to match those in the RunInfo.xml file.
- barcode_file: File listing the barcodes expected (for a run with single sample barcode, columns are: "library_name" "barcode_sequence_1" and "barcode_name." File is tab-delimited and those names have to be in the first row. Library_name must be unique, as does the sample barcode).
- FCID, machine_name, and run_number are used to populate the read name info when using READ_NAME_FORMAT=CASAVA_1_8
- multiplex_params: Similar to barcode file, but the columns are just "OUTPUT_PREFIX" and "BARCODE_1." I used the "library_name" from the other file as the "output_prefix," for example. File is also tab-delimited.
- temp_directory_location: Make sure that the tmp directory is large enough to hold a lot of files for sorting. The default tmp dir on my server doesn't have enough space, so I have to explicitly tell Picard to use the other drive.

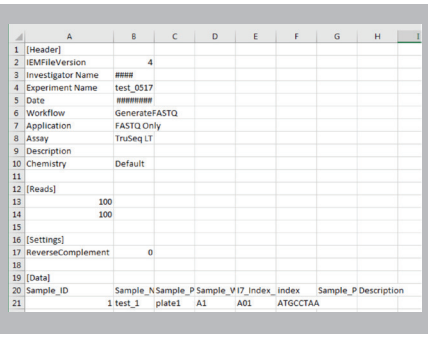
Picard tools ran in roughly the same amount of time as running with CASAVA. File sizes are correct. If they want to mimic the default bcl2fastq filtering, they should also set INCLUDE_NON_PF_READS=false (defaults to true for Picard).

# III. Appendices

## 1. Offline bcl-to-fastq conversion for Illumina sequencing. See separate file.

## 2. Example sample sheet and other template csv files (Read length is shown for demonstration purposes only.)

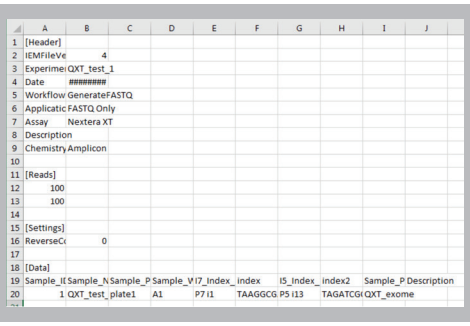
A. Example sample sheet, also available as template csv files upon request



| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| 1 | [Header] | | | | | | | |
| 2 | IEMFileVersion | 4 | | | | | | |
| 3 | Investigator Name | #### | | | | | | |
| 4 | Experiment Name | test_0517 | | | | | | |
| 5 | Date | ######## | | | | | | |
| 6 | Workflow | GenerateFASTQ | | | | | | |
| 7 | Application | FASTQ Only | | | | | | |
| 8 | Assay | TruSeq LT | | | | | | |
| 9 | Description | | | | | | | |
| 10 | Chemistry | Default | | | | | | |
| 11 | | | | | | | | |
| 12 | [Reads] | | | | | | | |
| 13 | 100 | | | | | | | |
| 14 | 100 | | | | | | | |
| 15 | | | | | | | | |
| 16 | [Settings] | | | | | | | |
| 17 | ReverseComplement | 0 | | | | | | |
| 18 | | | | | | | | |
| 19 | [Data] | | | | | | | |
| 20 | Sample_ID | Sample_N | Sample_P | Sample_W | I7_Index_ | index | Sample_P | Description |
| 21 | 1 | test_1 | plate1 | A1 | A01 | ATGCCTAA | | |



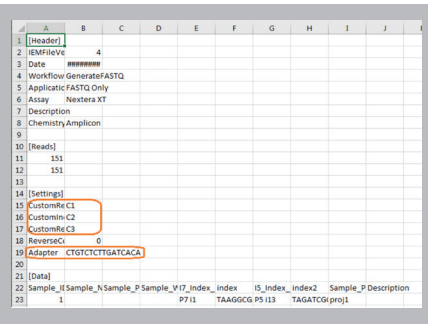
XT/XT2/RNA-seq/HaloPlex



| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | [Header] | | | | | | | | | |
| 2 | IEMFileVe | 4 | | | | | | | | |
| 3 | Experime | QXT_test_1 | | | | | | | | |
| 4 | Date | ######## | | | | | | | | |
| 5 | Workflow | GenerateFASTQ | | | | | | | | |
| 6 | Applicatic | FASTQ Only | | | | | | | | |
| 7 | Assay | Nextera XT | | | | | | | | |
| 8 | Description | | | | | | | | | |
| 9 | Chemistry | Amplicon | | | | | | | | |
| 10 | | | | | | | | | | |
| 11 | [Reads] | | | | | | | | | |
| 12 | 100 | | | | | | | | | |
| 13 | 100 | | | | | | | | | |
| 14 | | | | | | | | | | |
| 15 | [Settings] | | | | | | | | | |
| 16 | ReverseC | 0 | | | | | | | | |
| 17 | | | | | | | | | | |
| 18 | [Data] | | | | | | | | | |
| 19 | Sample_II | Sample_N | Sample_P | Sample_W | I7_Index_ | index | I5_Index_ | index2 | Sample_P | Description |
| 20 | 1 | QXT_test_ | plate1 | A1 | P7 i1 | TAAGGCG | P5 i13 | TAGATCG | QXT_exome | |



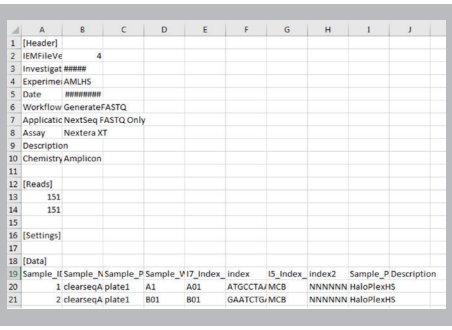
QXT without adapter trimming



| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | [Header] | | | | | | | | | |
| 2 | IEMFileVe | 4 | | | | | | | | |
| 3 | Date | ######## | | | | | | | | |
| 4 | Workflow | GenerateFASTQ | | | | | | | | |
| 5 | Applicatic | FASTQ Only | | | | | | | | |
| 6 | Assay | Nextera XT | | | | | | | | |
| 7 | Description | | | | | | | | | |
| 8 | Chemistry | Amplicon | | | | | | | | |
| 9 | | | | | | | | | | |
| 10 | [Reads] | | | | | | | | | |
| 11 | 151 | | | | | | | | | |
| 12 | 151 | | | | | | | | | |
| 13 | | | | | | | | | | |
| 14 | [Settings] | | | | | | | | | |
| 15 | CustomRe | C1 | | | | | | | | |
| 16 | CustomIn | C2 | | | | | | | | |
| 17 | CustomRe | C3 | | | | | | | | |
| 18 | ReverseC | 0 | | | | | | | | |
| 19 | Adapter | CTGTCTCTTGATCACA | | | | | | | | |
| 20 | | | | | | | | | | |
| 21 | [Data] | | | | | | | | | |
| 22 | Sample_II | Sample_N | Sample_P | Sample_W | I7_Index_ | index | I5_Index_ | index2 | Sample_P | Description |
| 23 | 1 | | | | P7 i1 | TAAGGCG | P5 i13 | TAGATCG | proj1 | |



QXT with custom primer and adapter-trimming options



| | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | [Header] | | | | | | | | | |
| 2 | IEMFileVe | 4 | | | | | | | | |
| 3 | Investigat | ##### | | | | | | | | |
| 4 | Experime | AMLHS | | | | | | | | |
| 5 | Date | ######## | | | | | | | | |
| 6 | Workflow | GenerateFASTQ | | | | | | | | |
| 7 | Applicatic | NextSeq FASTQ Only | | | | | | | | |
| 8 | Assay | Nextera XT | | | | | | | | |
| 9 | Description | | | | | | | | | |
| 10 | Chemistry | Amplicon | | | | | | | | |
| 11 | | | | | | | | | | |
| 12 | [Reads] | | | | | | | | | |
| 13 | 151 | | | | | | | | | |
| 14 | 151 | | | | | | | | | |
| 15 | | | | | | | | | | |
| 16 | [Settings] | | | | | | | | | |
| 17 | | | | | | | | | | |
| 18 | [Data] | | | | | | | | | |
| 19 | Sample_II | Sample_N | Sample_P | Sample_W | I7_Index_ | index | I5_Index_ | index2 | Sample_P | Description |
| 20 | 1 | clearseqA | plate1 | A1 | A01 | ATGCCTAA | MCB | NNNNNN | HaloPlexHS | |
| 21 | 2 | clearseqA | plate1 | B01 | B01 | GAATCTGA | MCB | NNNNNN | HaloPlexHS | |



HaloPlex$^{HS}$

B. Library Prep Kit template csv files (containing index sequences and index layout) for BaseSpace available upon request

# III. Appendices

## 3. How to add Agilent Library Prep Kits as custom Library Prep Kit types in IEM (so sample sheet can be generated without having to be edited; also refer to the "Creating Sample Prep Kit Type" section of the IEM user guide.)

**As an alternative to the method described in section I, which is to use an existing Illumina Library Prep Kit as a template and then edit the sample sheet to enter Agilent index and adapter sequences, you may add the Agilent Library Prep Kit as a custom Library Prep Kit in IEM. This way, the custom Library Prep Kit will be available in the IEM Library Prep Kit drop-down list, and the correct index and adapter sequences (if adapter-trimming option is enabled) will be entered automatically in the sample sheet. Follow these steps:**

1, Generate custom Library Prep Kit txt file(s) and add the file(s) to
   a. C:\Program Files (x86)\Illumina\Illumina Experiment Manager\SamplePrepKits
      The below files are available for Agilent Library Prep Kits:

      Haloplex_original_index_config.txt

      Haloplex$^{HS}$.txt

      SureSelect$^{XT}$-XT2-HaloPlex_revised_index_config.txt

      SureSelectQXT.txt

2. Modify the application txt file(s) in
   a. C:\Program Files (x86)\Illumina\Illumina Experiment Manager\Applications
   b. to add the custom library prep kit(s) under "Compatible Sample Prep Kits."

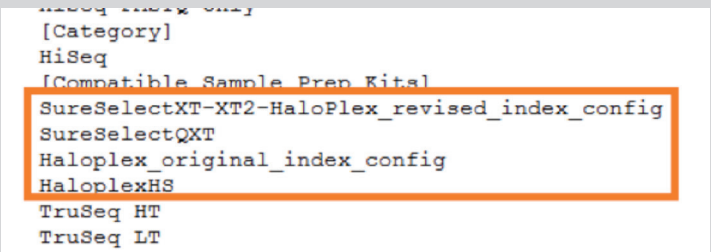
      We recommend using the Agilent Library Prep Kit under the "FASTQ Only" application, so you need to modify the following files for respective sequencers:

      NextSeqGenerateFASTQ.txt (NextSeq)

      HiSeqGenerateFASTQ.txt (HiSeq)

      GenerateFASTQ.txt (MiSeq)

      Example application txt files are available. The example application txt files have the previously listed four Agilent Library Prep Kit types added. When adding the example txt file(s) into the Applications folder, allow overwriting.




c. Re-launch IEM to enable the modifications.

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## 4. Illumina Kit Configuration Selection Guide (The ones that have been validated internally are highlighted.)

### XT/XT2/RNA-Seq

| Platform | Run Type | Read Length | SBS Kit Configration | Chemistry |
|---|---|---|---|---|
| HiSeq 2500 | Rapid | 2x100/2x75 | 200 Cycle Kit | v1/v2 |
| HiSeq 2500 | High Output | 2x100/2x75 | 4 x 50 Cycle Kits or 200+50 Cycle Kits | v3 |
| HiSeq 2500 | High Output | 2x100/2x75 | 250 Cycle Kit | v4 |
| HiSeq 2000 | All Runs | 2x100/2x75 | 200 Cycle Kit | v3 |
| MiSeq | All Runs | 2x100 | 300 Cycle Kit | v2 |
| MiSeq | All Runs | 2x75 | 150 Cycle Kit | v3 |
| NextSeq 500/550 | All Runs | 2x75 | 150 Cycle Kit | v1/v2 |
| NextSeq 500/550 | All Runs | 2x100 | 300 Cycle Kit | v1/v2 |

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## QXT

| Platform | Run Type | Read Length | SBS Kit Configration | Chemistry |
|---|---|---|---|---|
| HiSeq 2500 | Rapid | 2x100/2x75 | 200 Cycle Kit | v1/v2 |
| HiSeq 2500 | High Output | 2x75 | 200 Cycle Kit | v3 |
| HiSeq 2500 | High Output | 2x100 | 4 x 50 Cycle Kits or 200+50 Cycle Kits | v3 |
| HiSeq 2500 | High Output | 2x100/2x75 | 250 Cycle Kit | v4 |
| HiSeq 2000 | All Runs | 2x75 | 200 Cycle kit | v3 |
| HiSeq 2000 | All Runs | 2x100 | 200+50 Cycle Kit | v3 |
| MiSeq | All Runs | 2x100 | 300 Cycle Kit | v2 |
| MiSeq | All Runs | 2x75 | 150 Cycle Kit | v3 |
| NextSeq 500/550 | All Runs | 2x100/2x75 | 300 Cycle Kit | v1/v2 |

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## HaloPlex

| Platform | Run Type | Read Length | SBS Kit Configration | Chemistry |
|---|---|---|---|---|
| HiSeq 2500 | Rapid | 2x100 | 200 Cycle Kit | v1/v2 |
| HiSeq 2500 | Rapid | 2x250 | 500 Cycle Kit | v2 |
| HiSeq 2500 | High Output | 2x100 | 200 Cycle Kit | v3 |
| HiSeq 2500 | High Output | 2x100 | 250 Cycle Kit | v4 |
| HiSeq 2000 | All Runs | 2x100 | 200 Cycle kit | v3 |
| MiSeq | All Runs | 2x100/2x150 | 300 Cycle Kit | v2 |
| MiSeq | All Runs | 2x250 | 500 Cycle Kit | v2 |
| MiSeq | All Runs | 2x250 | 600 Cycle Kit | v3 |
| NextSeq 500/550 | All Runs | 2x100 | 300 Cycle Kit | v1/v2 |

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## HaloPlex$^{HS}$

| Platform | Run Type | Read Length | SBS Kit Configration | Chemistry |
|---|---|---|---|---|
| HiSeq 2500 | Rapid | 2x100 | 200 Cycle Kit | v1/v2 |
| HiSeq 2500 | Rapid | 2x250 | 500 Cycle Kit | v2 |
| HiSeq 2500 | High Output | 2x100 | 4 x 50 Cycle Kits or 200+50 Cycle Kits | v3 |
| HiSeq 2500 | High Output | 2x100 | 250 Cycle Kit | v4 |
| HiSeq 2000 | All Runs | 2x100 | 4 x 50 Cycle Kit | v3 |
| MiSeq | All Runs | 2x100/2x150 | 300 Cycle Kit | v2 |
| MiSeq | All Runs | 2x250 | 500 Cycle Kit | v2 |
| MiSeq | All Runs | 2x250 | 600 Cycle Kit | v3 |
| NextSeq 500/550 | All Runs | 2x100 | 300 Cycle Kit | v1/v2 |

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## 5. Recommended seeding concentrations based on internal experience
## Internally, we aim for 800 K/mm2 to 900 K/mm2 with the MiSeq and the HiSeq.

**MiSeq:** With the V2 sequencing kits, our range is mainly in the 9-pM to 13-pM range with XT/XT2/HaloPlex/HaloPlex$^{HS}$. Note that Methyl-Seq has not been sequenced on the MiSeq. We have not been using the V3 chemistry routinely, but the same range is applied. It is expected that the V3 chemistry can support higher cluster density, so you may be able to use higher seeding concentration.

**HiSeq 2000:** XT/XT2/HaloPlex/HaloPlex$^{HS}$, 6 pM to 8 pM. Methyl-Seq, 15 pM.

**HiSeq 2500 Rapid:** XT/XT2/HaloPlex, 8 pM to 12 pM. QXT with target enrichment, 13 pM; for whole-genome, 20 pM. This is likely because of the broader size distribution of the QXT whole-genome library. For strand-specific total RNA-Seq, we use 10 pM to 12 pM. (R&D has been using the Rapid Mode only, but this should be comparable with the high-output mode).

**NextSeq 500:** QXT/XT/XT2/HaloPlex, 1.2 pM to 1.4 pM with the V1 chemistry, and 1.8 pM with the V2 chemistry. Target cluster density, 180 K/mm2 to 200 K/mm$^2$.

**Note:**

1) The concentrations above are based on quantitation by TapeStation, with the exception of QXT whole-genome that would need to be run on a BioA because of the broader library size range;
2) The clustering outcome can vary from instrument to instrument, and libraries are not all equal even when concentration appears to be the same. We believe that these seeding concentrations should achieve a good amount of high-quality data, but please be aware that further optimization may be needed based on your own experience.

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## 6. How to modify an Agilent BED file into a manifest file

**a.** Open an Illumina manifest file in Excel for use as a template. For example: https://support.illumina.com/content/dam/illumina-support/documents/downloads/productfiles/truseq/truseq-exome-targeted-regions-manifest-v1-2-bed.zip.

**b.** Clear the contents of columns 1 to 4 in the template file.

**c.** Open the BED file in Excel. Copy columns 1 to 3 and paste into columns 2 to 4 in the above template.

**d.** If the template file has more rows than the BED file, be sure to delete the extra rows.

**e.** Now you have an empty column 1 in the manifest file. Use the CONCATENATE function to concatenate columns 2 and 3 (chr, start, and end) and create a name for each row in column 1. This way you can make sure that each row has a unique name.

**f.** Save this file as a tab delimited txt file under:

- C:\Users\useraccount\AppData\Roaming\Illumina\Illumina Experiment Manager\Manifests
- This should automatically make the manifest file available from IEM in the drop-down menu.

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## 7. Considerations for PhiX spike-in

It is recommended to always include a standard 1% to 5% PhiX spike-in. This applies to all Agilent NGS libraries, including the Methyl-Seq library.

For targeted sequencing with low complexity (e.g., <2000 amplicons), it might be helpful to use a high-concentration spike-in of up to 25%.

## 8. ION system and reagent kits that have been used for template preparation and sequencing of Agilent SureSelect$^{XT}$ PTN and HaloPlex/HaloPlex$^{HS}$ ION libraries

SureSelect$^{XT}$ PTN: Ion PI Template OT2 200 Kit v2 with the Ion OneTouch 2 system, and Ion PI Template 200 kit v2.

HaloPlex/HaloPlex$^{HS}$: Ion PGM Template OT2 400 kit with the Ion OneTouch 2 system, and Ion PGM Sequencing 400 kit..

**Note:** We have not tested the Hi-Q and Hi-Q view kits, but those also should be compatible.

## 9. Seeding concentration for ION sequencing and ION Sphere QC

Follow the dilution protocol and ION Sphere QC kit recommended for "gDNA Fragment or Amplicon Library" in the ION template-preparation kit user manuals.

## 10. How to modify an Agilent BED file for uploading to the Ion Server

a. Open the BED file in Excel.

b. Remove the first two lines, which are the browser position and the track name.

c. Remove the fourth column, which contains the annotations.