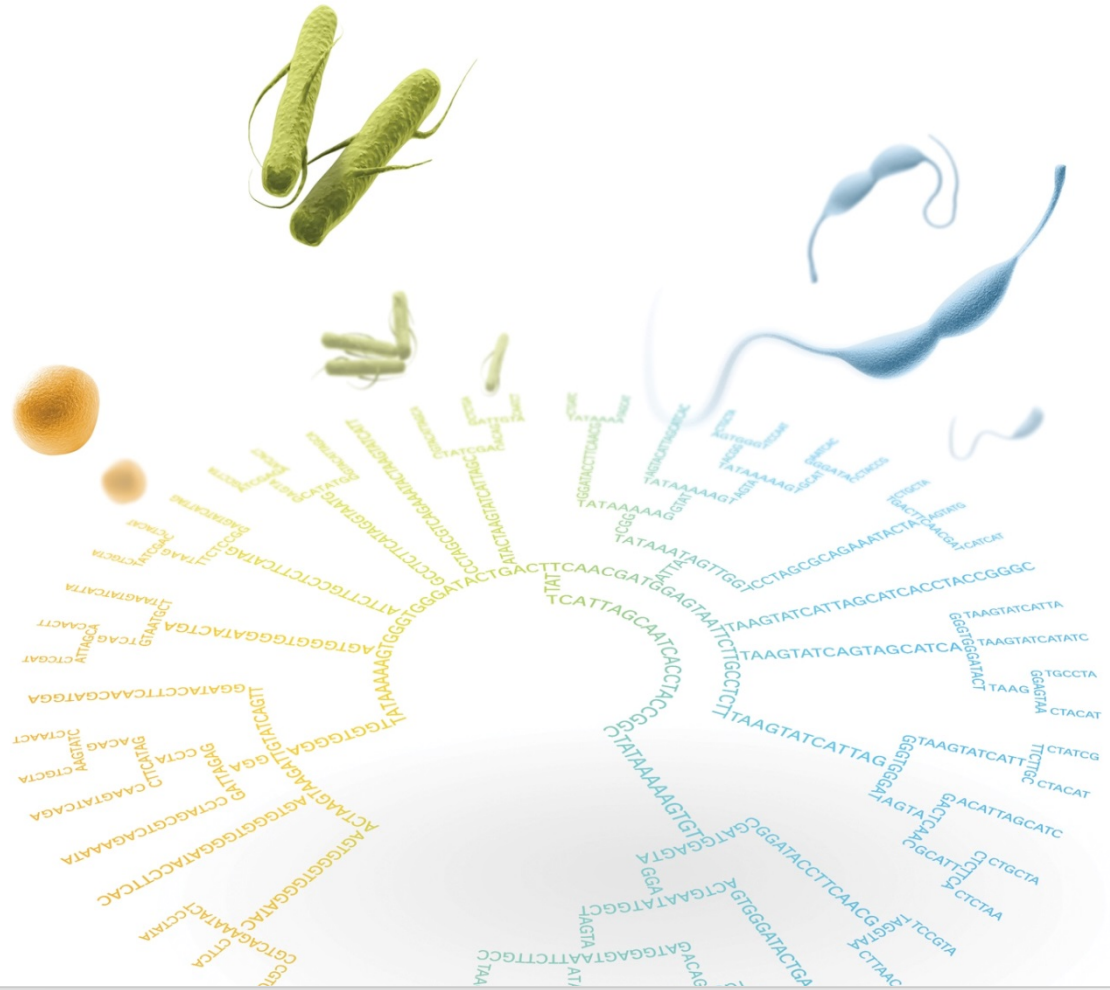


# 16S Metagenomics Sequencing Workflow

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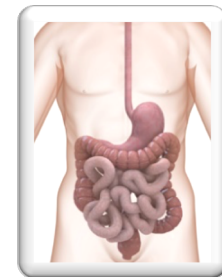


# Metagenomics and Microbial Diversity

## *Diverse Applications*

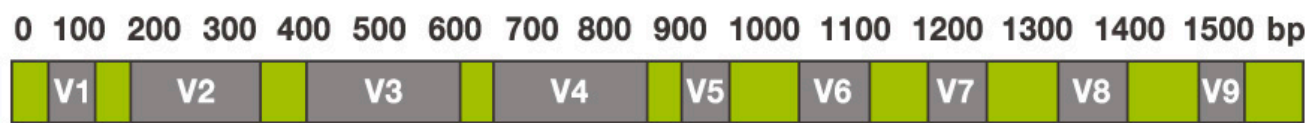
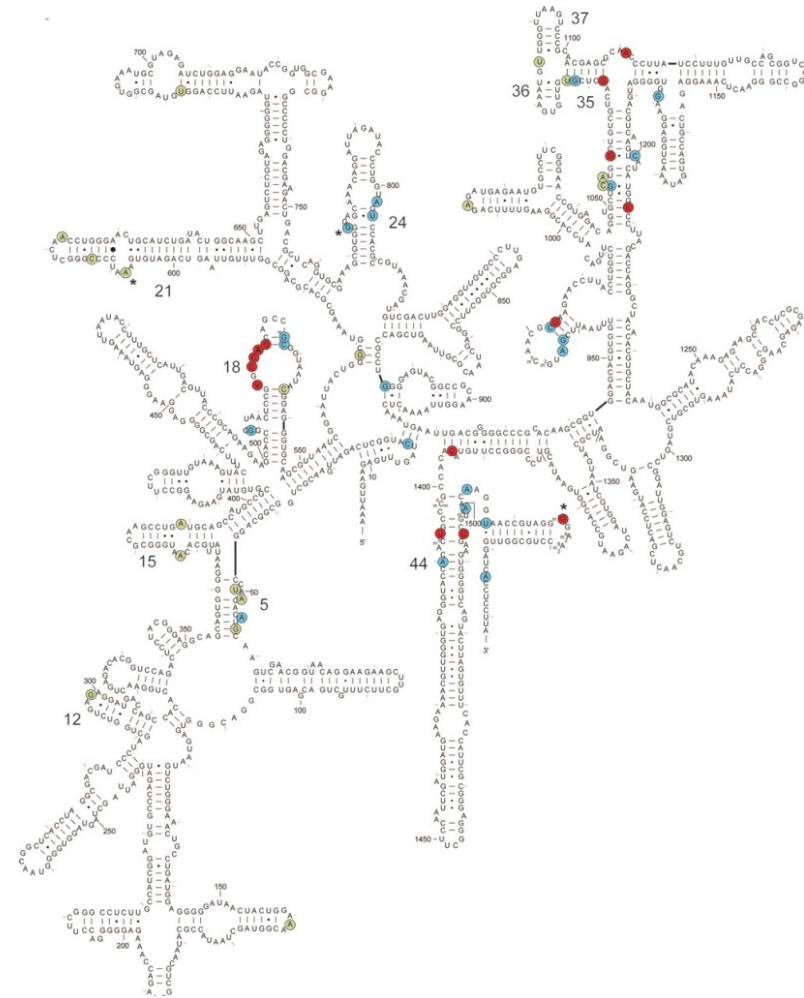
Metagenomic studies can give insight into:

- ▶ Microbial diversity in an environmental habitat
- ▶ Abundance of microbial species
- ▶ Gene content in a sample and the discovery of novel genes
- ▶ Meta-transcriptomics: analysis of the expressed genome in a sample
- ▶ Pathogen detection
- ▶ Signature profile of an environment or disease state



# 16S rRNA Gene Sequencing

- ▶ 16S rRNA is a part of the **ribosomal RNA** of prokaryotic cells which is about 1,542 nucleotides long.
- ▶ It has been observed that this molecule contains regions which are highly conserved (not altered much due to mutation) among species.
- ▶ Thus if these molecules are sequenced and the sequences of various species are compared the microbes can be phylogenetically classified.
- ▶ **This is a powerful tool used for classification and genome analysis.**

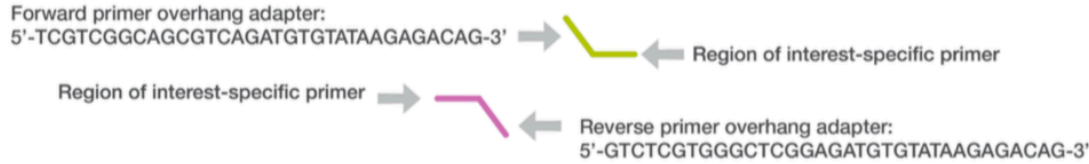


**CONSERVED REGIONS:** unspecific applications  
**VARIABLE REGIONS:** group or species-specific applications

# 16S Metagenomics Library Preparation Workflow

## *Illumina-demonstrated protocol*

PCR amplify template out of genomic DNA using region of interest-specific primers with overhang adapters



Attach indices and Illumina sequencing adapters using the Nextera<sup>®</sup> XT Index Kit



Normalize and pool libraries



Sequence

### 16S Metagenomic Sequencing Library Preparation

Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System

Introduction	2
16S Library Preparation Workflow	5
Amplicon PCR	6
PCR Clean-Up	8
Index PCR	10
PCR Clean-Up 2	13
[Optional] Validate Library	15
Library Quantification, Normalization, and Pooling	16
Library Denaturing and MiSeq Sample Loading	17
MiSeq Reporter Metagenomics Workflow	20
Supporting Information	21

**IMPORTANT NOTICE** This document provides information for an application for Illumina technology that has been demonstrated internally and may be of interest to customers. This information is provided as is and is not an Illumina product and is not accompanied by any rights or warranties. Customers using or adapting this information should obtain any licenses required and materials from authorized vendors. Illumina products mentioned herein are for research use only unless marked otherwise. While customer feedback is welcomed, this application is not supported by Illumina Technical Support and Field Application Scientists.

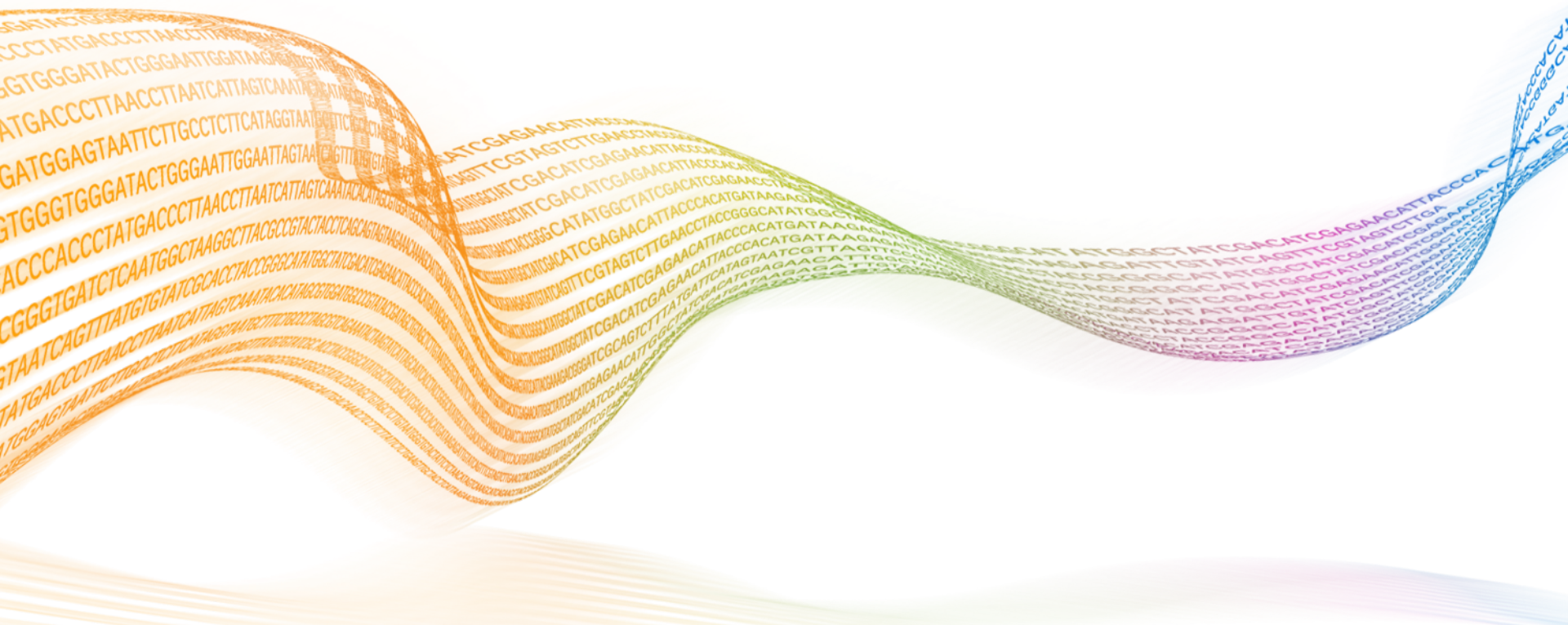
Part # 1504223 Rev. B

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# Required Reagents and Equipment



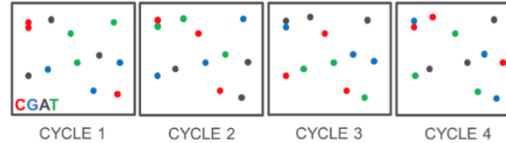
# What do I need for 16S Metagenomics workflow?

## Items to Order from Illumina



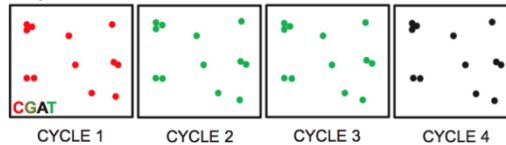
### Template Generation and Amplicons

#### PhiX



Neighboring clusters can be distinguished by their unique sequences.

#### Amplicon



Neighboring clusters can not be distinguished.



Catalog Number	Product Description
FC-131-2001~2004 20027213	<b>Nextera XT Index Kit v2 Set A~D, 384 samples (96 indices) IDT for Illumina Nextera DNA UD index Set A, 96 samples (96 indices)</b>
FC-110-3001	<b>PhiX Control v3</b>
(2019 Q2) or MS-102-3003	<b>iSeq 100 i1 Reagent (500cycle Single Kit) or MiSeq Reagent Kit v3</b>
FC-130-1005 (Optional)	<b>TruSeq Index Plate Fixture and Collar Kit (2 Each)</b>

# What do I need for 16S Metagenomics workflow?

*User-supplied equipment and consumables*

## Equipment for library prep

Plate centrifuge

Magnetic stand (96-samples)

Thermocycler

## Equipment for input & library QC

Fluorometer (Qubit preferred)

Bioanalyzer 2100

## Reagents for library prep

Forward & Reverse oligo primers

AMPure XP Beads

KAPA HiFi HotStart Ready Mix

## Reagents for input & library QC

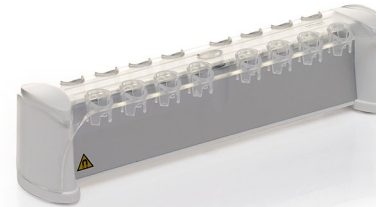
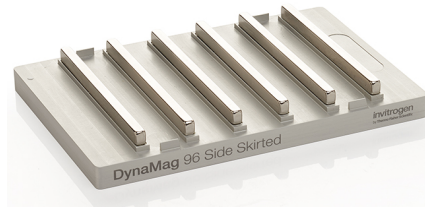
Fluorometric DNA Quant kit  
(Qubit preferred)

Bioanalyzer DNA 1000 Kit

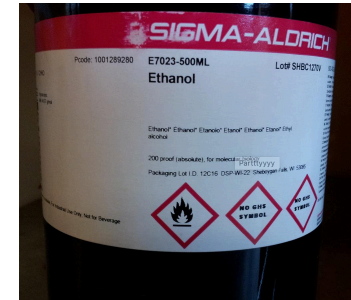
# KAPA HiFi HotStart Ready Mix



# Magnetic stand



# Ethyl alcohol



# Thermal Cycler



# Beckman Coulter AMPURE XP 60ML

encompass

Manufacturer: Beckman Coulter A63881

Agencourt AMPure XP 60 mL Kit: size 1,666/3,333 preps with kit components Agencourt AMPure XP Reagent The Agencourt AMPure XP system is a highly efficient, easily automated PCR purification system that delivers superior quality DNA with no salt carryover. Requiring no centrifugation or filtration, Agencourt AMPure XP can be easily used in manual and automated 96- or 384-well formats



# Qubit

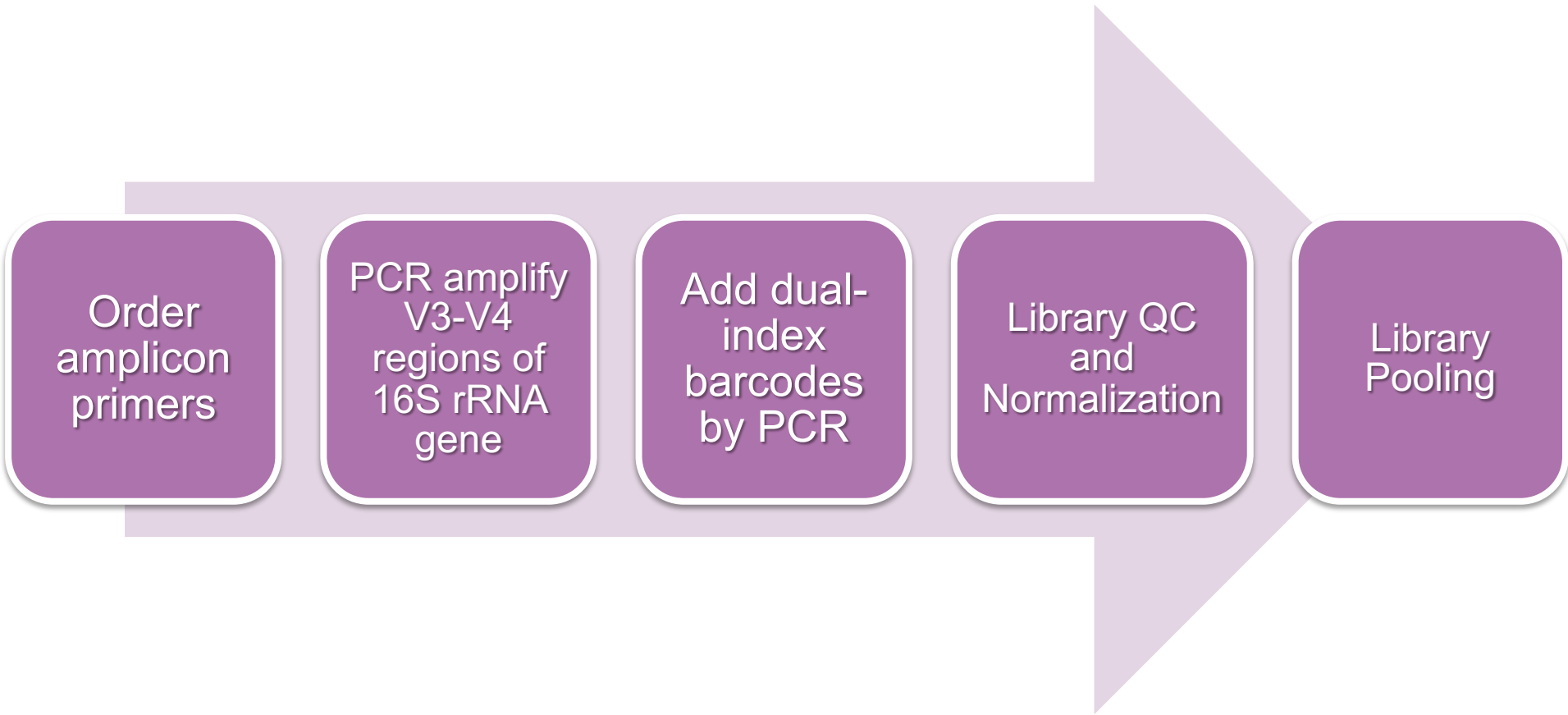


# Bioanalyzer



# 16S Metagenomics Library Preparation Workflow

*Illumina-demonstrated protocol*





# Step 1: Order Amplicon Primers

Primers target 16S rRNA gene V3-V4 region

- A single amplicon of approximately **460 bp** is created
- Primers **include overhang** adaptor sequences
- Illumina recommends using **standard desalting purification** when ordering oligo primer sets

## Forward Primer:

5' **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG**

Overhang Adapter Sequence      Locus-Specific Sequence  
16S V3-V4

## Reverse Primer:

5' **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC**

Overhang Adapter Sequence      Locus-Specific Sequence  
16S V3-V4

# DNA Isolation Kits

**Epicentre DNA isolation kits are optimized to isolate bacterial DNA obtained from various environments**

<b>Sample Type</b>	<b>Epicentre Isolation Kit</b>
Water	<b>Metagenomic DNA Isolation Kit for Water</b>
Soil	<b>SoilMaster DNA Extraction Kit</b>
Fecal matter	<b>ExtractMaster Fecal DNA Extraction Kit</b>
Difficult-to-culture species present in environmental water, soil, or compost	<b>Meta-G-Nome DNA Isolation Kit</b>

# DNA Quantitative Analysis

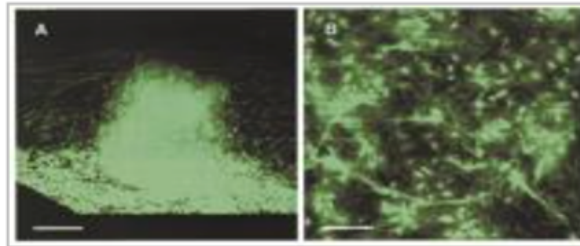
DNA input of only 12.5ng of microbial genomic DNA

- $260/280 = 1.8 - 2.0$

Quantify genomic DNA  
use fluorescence-based  
quantification method

- Qubit
- PicoGreen

## PicoGreen®



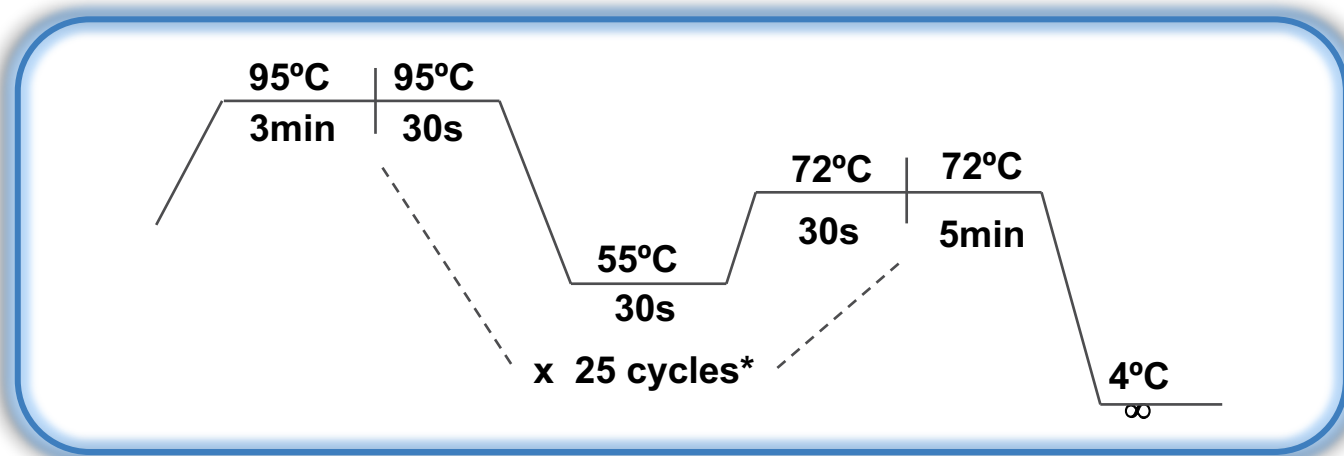
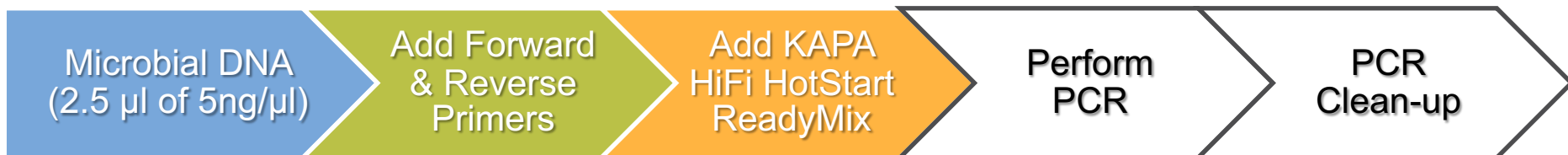
- Easier for high-throughput
- Fluorometer plate reader available

## Qubit™



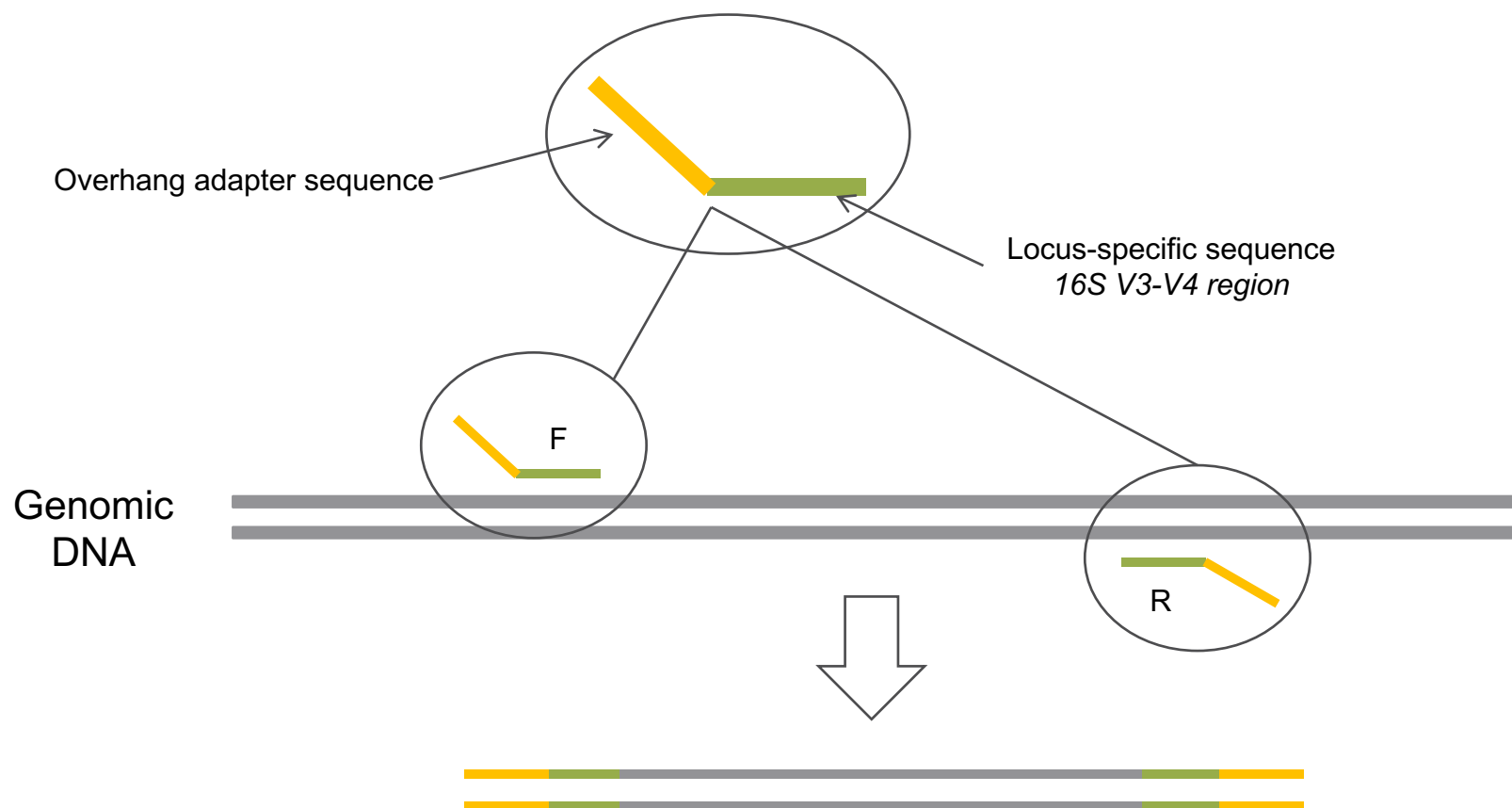
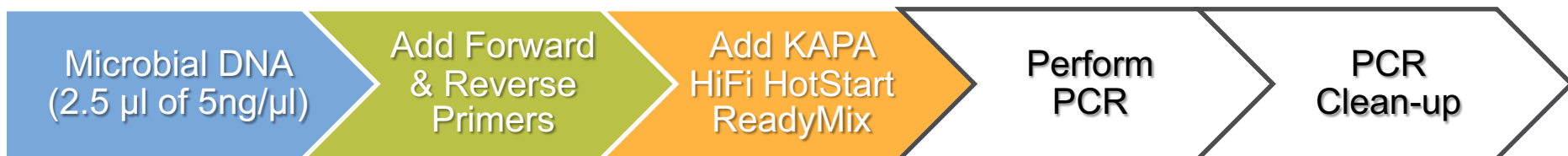
- Very easy-to-use
- Good for smaller number of samples

## Step 2: PCR Amplify V3-V4 regions of 16S rRNA gene



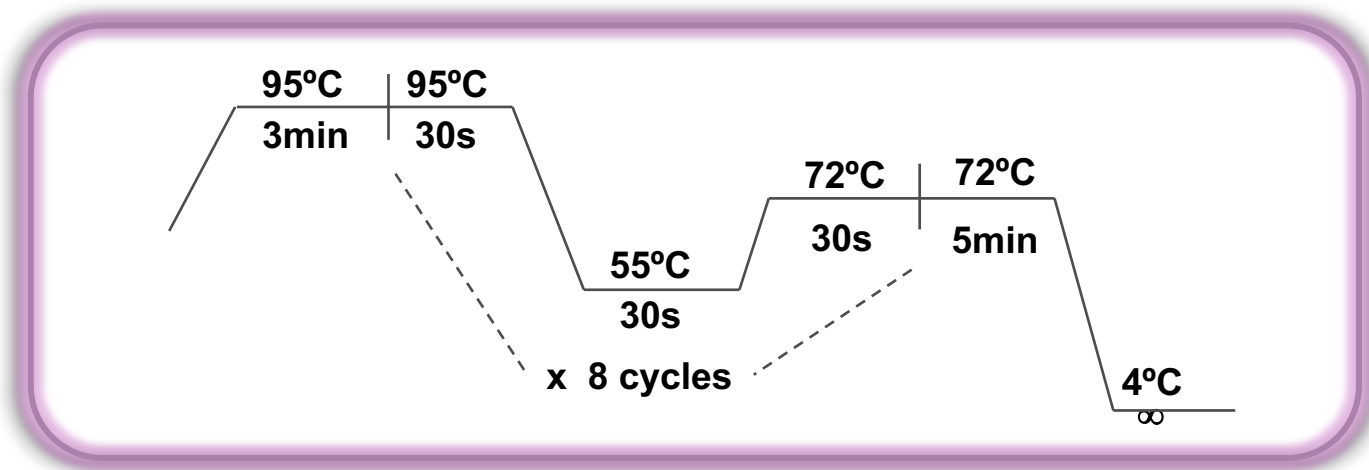
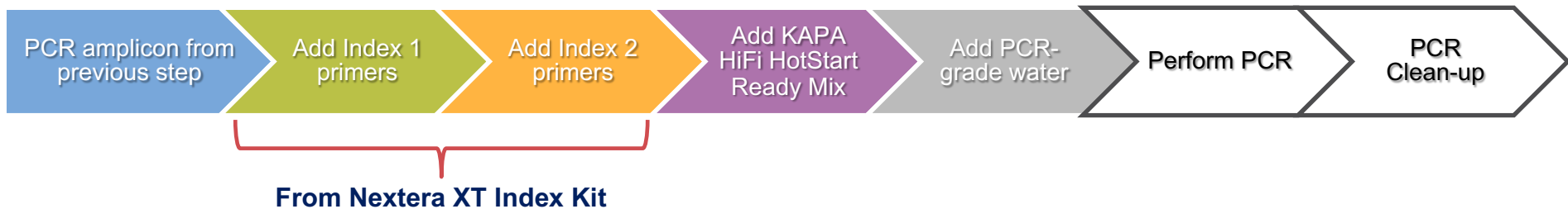
\* Number of PCR cycles may have to be optimized for different samples

## Step 2: PCR Amplify V3-V4 regions of 16S rRNA gene

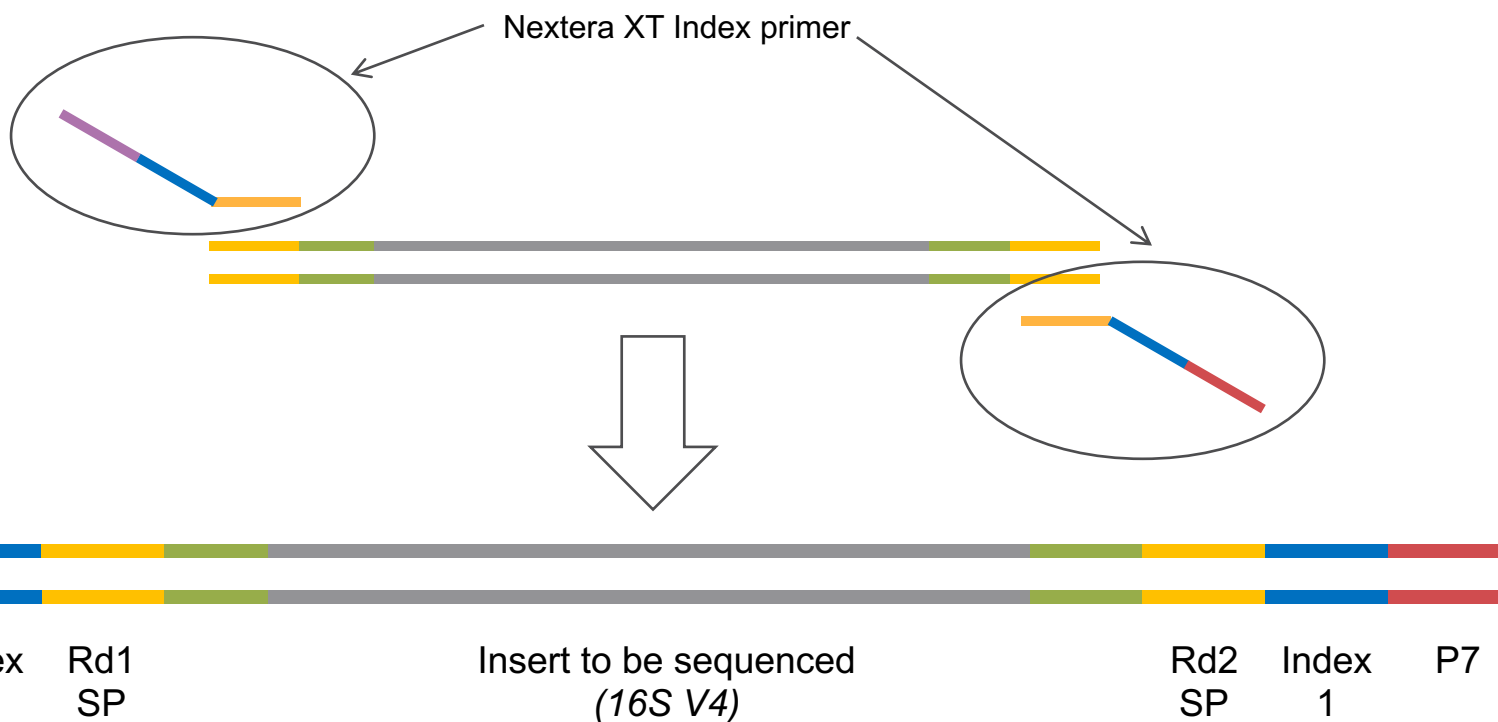
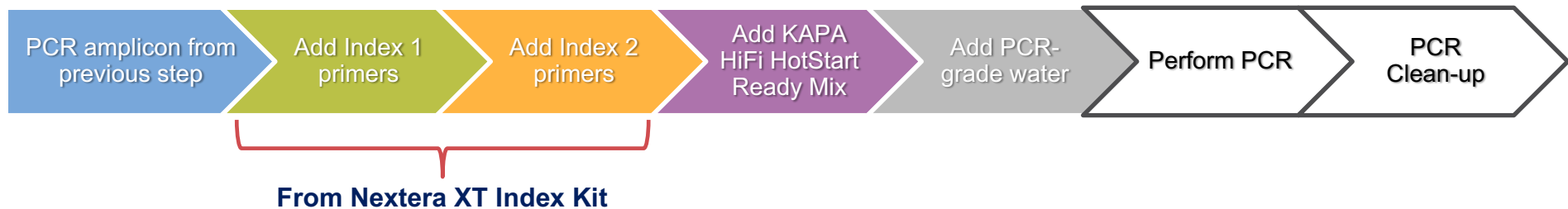




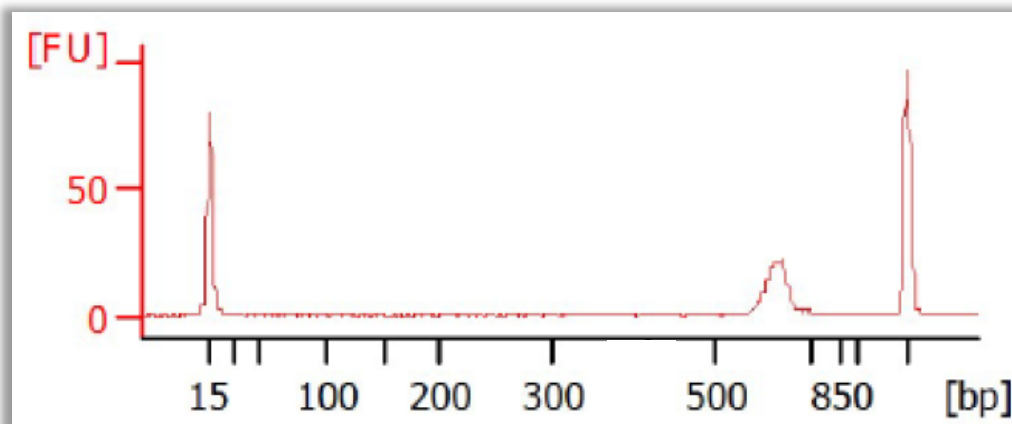
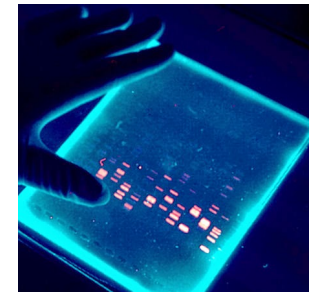
# Step 3: 2<sup>nd</sup> PCR to Add Indices and Adaptors



# Step 3: 2<sup>nd</sup> PCR to Add Indices and Adaptors



## Step 4: Library QC



Example Bioanalyzer Trace of Final Library

### Quantity

- Use dsDNA-specific fluorescent dye (Qubit or PicoGreen)

### Quality

- Run on Agilent Bioanalyzer DNA 1000 chip
- Expected library size  $\approx$  630bp

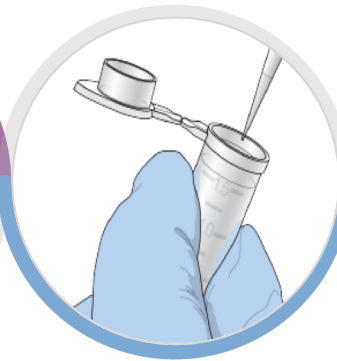
# Set Up an iSeq 100 Run

## iSeq 100 Workflow



### Thaw

Thaw cartridge IN the foil bag according to recommended methods



### Prepare

Prepare sequence-ready libraries from input DNA or RNA



### Load

Insert diluted libraries into the prepared sequencing consumables



### Start Run

Initiate a run using instrument control software

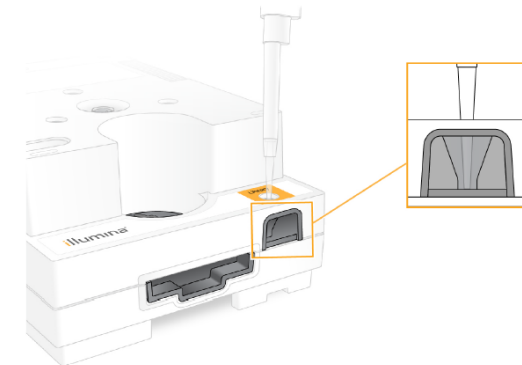
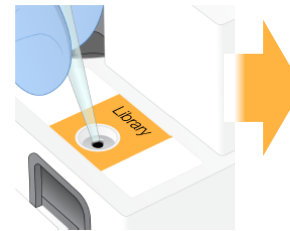
# Load Consumables Into the Cartridge

- Tear open package



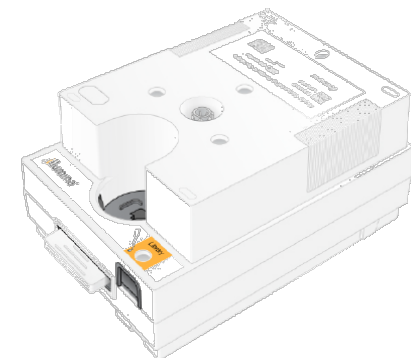
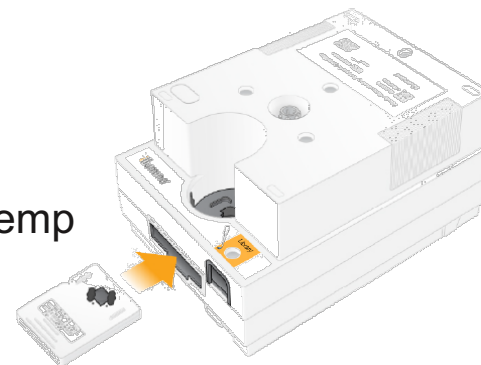
- Load library

- Use a pipette tip to pierce the foil of the Library reservoir
- Add **20  $\mu$ l** to the bottom of the reservoir



- Load flow cell

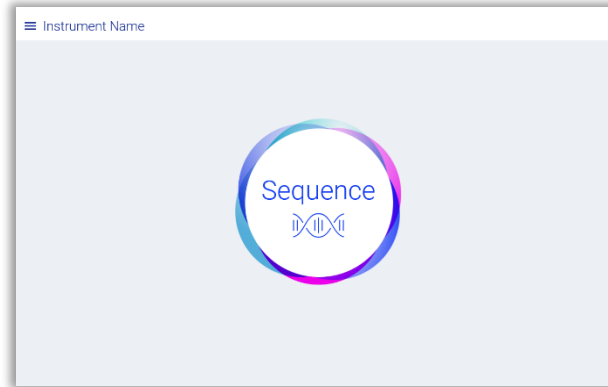
- **Tip!** Allow the flow cell to sit at room temp for 10-15 minutes to prevent forming condensation



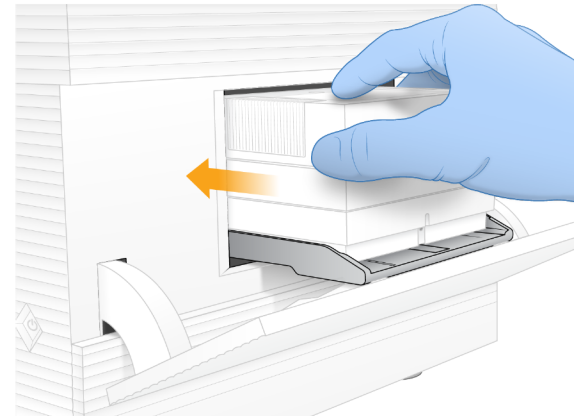
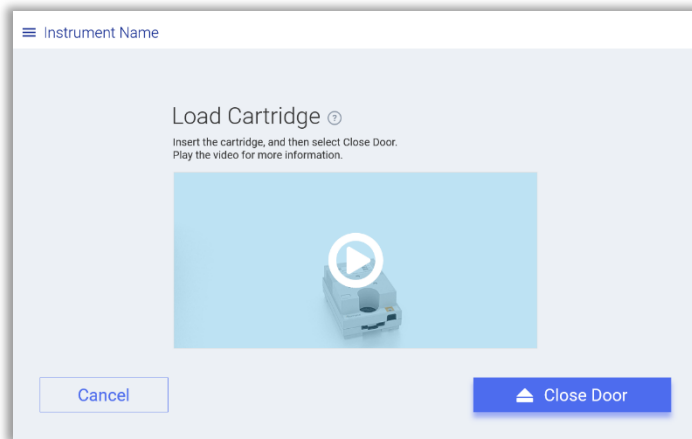


# Set Up a Run Sequencing Run

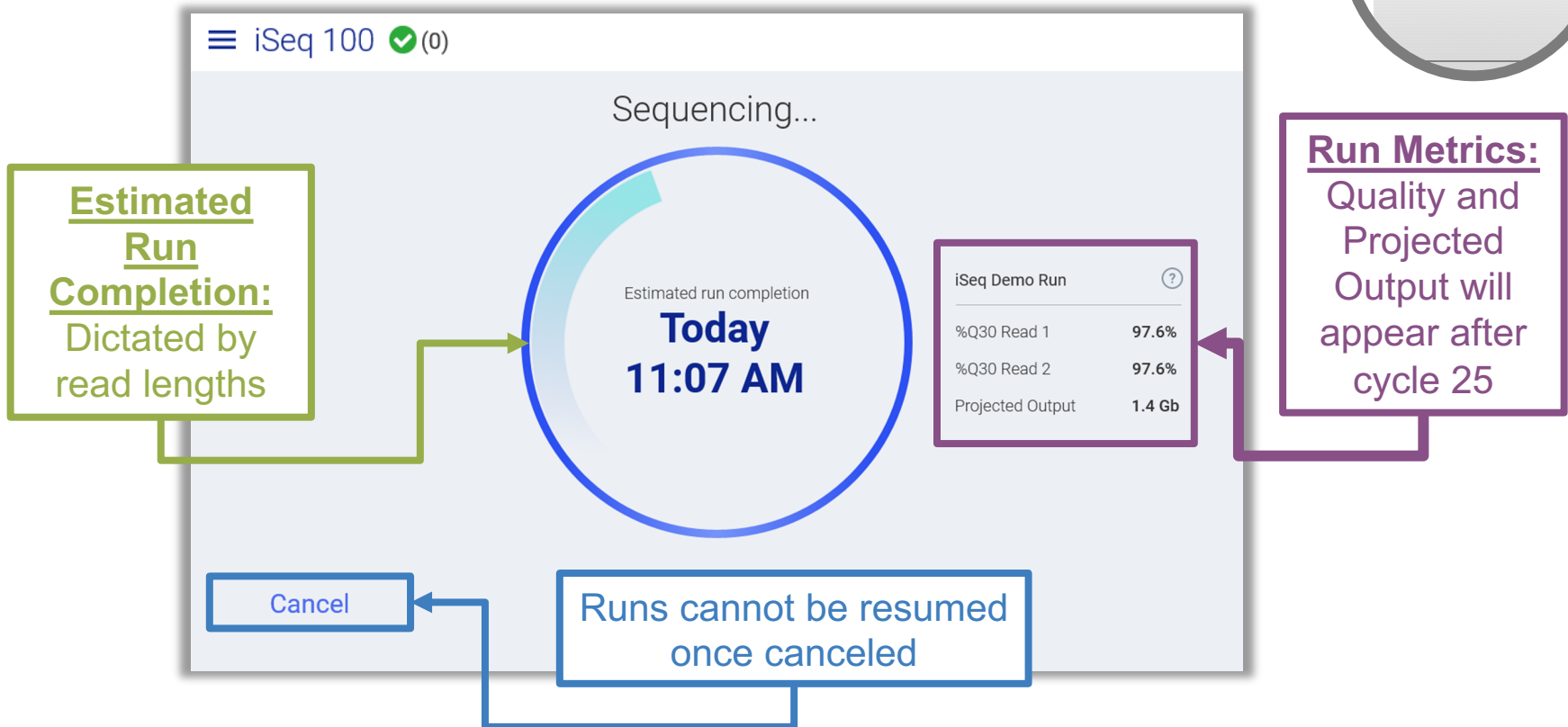
- Select *Sequence*



- Load Consumables



# Run Monitoring



The screenshot shows the iSeq 100 sequencing software interface. At the top, it says "iSeq 100" with a green checkmark and "(0)". Below that, it says "Sequencing...". In the center, there is a circular progress indicator with a teal segment. Text inside the circle reads "Estimated run completion Today 11:07 AM". To the right of the circle is a table titled "iSeq Demo Run" with a help icon (?). The table contains the following data:

iSeq Demo Run	
%Q30 Read 1	97.6%
%Q30 Read 2	97.6%
Projected Output	1.4 Gb

Annotations include:

- A green box on the left: **Estimated Run Completion:** Dictated by read lengths. An arrow points from this box to the circular progress indicator.
- A purple box on the right: **Run Metrics:** Quality and Projected Output will appear after cycle 25. An arrow points from this box to the table.
- A blue box at the bottom: **Runs cannot be resumed once canceled**. An arrow points from this box to a "Cancel" button.

- Paired-end 2X151 plus dual index 8+8 run time is approximately 18.5 hours

# Questions?

