

Praxis[®] Extended RAS Panel Reference Guide

FOR *IN VITRO* DIAGNOSTIC USE

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Introduction

Intended Use

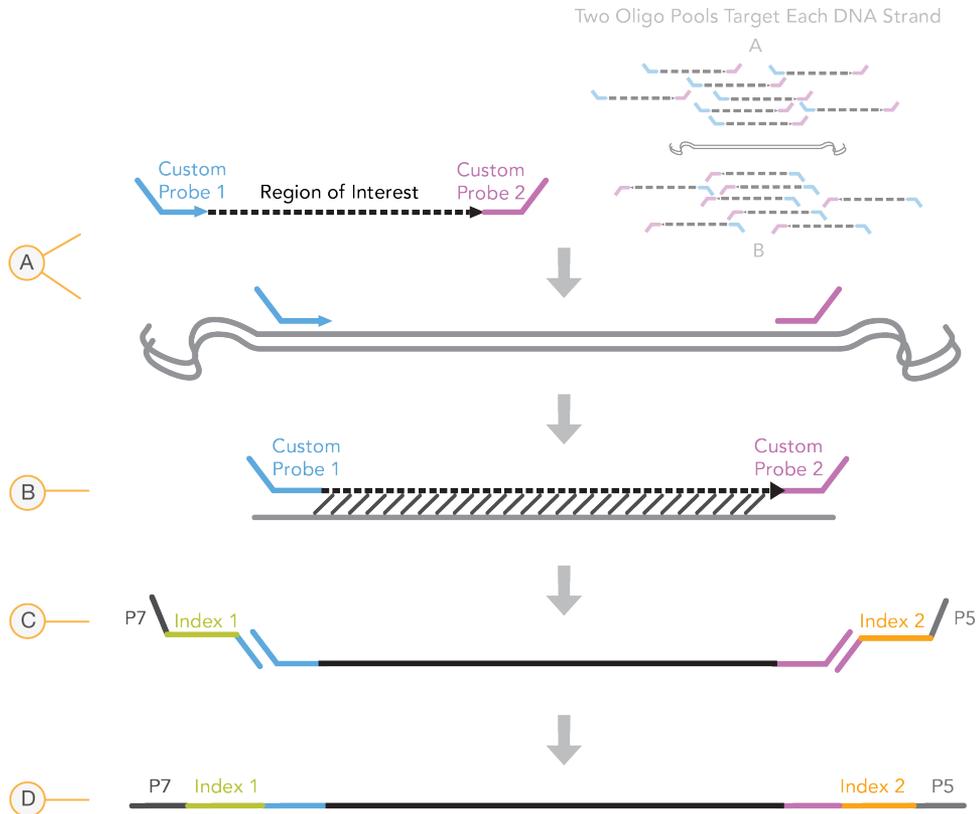
The Praxis™ Extended RAS Panel is a qualitative *in vitro* diagnostic test using targeted high throughput parallel sequencing for the detection of 56 specific mutations in RAS genes [*KRAS* (exons 2, 3, and 4) and *NRAS* (exons 2, 3, and 4)] in DNA extracted from formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) tissue samples. The Praxis™ Extended RAS Panel is indicated to aid in the identification of patients with colorectal cancer for treatment with Vectibix® (panitumumab) based on a no mutation detected test result. The test is intended to be used on the Illumina MiSeqDx® instrument.

About this Guide

This reference guide provides more detailed instructions, technique tips, and helpful hints to newly trained users to help guide proper execution of the Praxis Extended RAS Panel protocol. This is intended as a supplement and is not meant to replace the package insert.

How Does the Assay Work?

Pairs of oligonucleotides targeting specific mutations in RAS genes are hybridized to genomic DNA. Each genomic DNA sample is tested with two oligonucleotide pools that independently target each strand of DNA followed by extension and ligation to form DNA templates consisting of the regions of interest flanked by universal primer sequences. Using indexed primers supplied with the kit, DNA templates are then amplified by PCR, pooled into a single tube, and sequenced on the MiSeqDx instrument.



- A** Hybridization of oligonucleotide probes targeting specific RAS mutations (each DNA strand is independently targeted by two oligonucleotide probe pools)
- B** Extension and ligation
- C** Addition of indices and sequencing adapters by PCR
- D** Final amplicon ready for sequencing with MiSeqDx

Process Overview

The Illumina Praxis Extended RAS Panel process can be summarized into the following steps:

Create a Run

First, create a run. The MiSeqDx uses the run information to identify each sample and its corresponding indexes.

To create a run, use the Local Run Manager and the Praxis Extended RAS Panel analysis module. Enter the sample name and indexes for the 96-well plate in the module. You can print out the plate layout from the module and use the information for setting up the plate during the assay workflow.

Prepare Libraries

Prepare the libraries using the protocol detailed in this user guide.

Sequence Samples on the MiSeqDx

The Praxis Extended RAS Panel must be sequenced on a MiSeqDx instrument using a paired-end 141 cycle run with dual indexing. For instructions for performing a sequencing run on the MiSeqDx, see the *MiSeqDx Instrument Reference Guide* (document # 1000000021961).

Automated Sequencing and Data Analysis

The Praxis Extended RAS Panel analysis module processes the base calls generated by the MiSeqDx instrument. It is an on-instrument software, which is built in to the instrument's processes. The analysis module produces details indicating whether each sample contains any of the Praxis Extended RAS Panel mutations. For more information about this software, see the *Local Run Manager Software Reference Guide for MiSeqDx* (document # 1000000011880) and the *Local Run Manager Extended RAS Panel Analysis Module Guide* (document # 1000000011875).

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- ▶ The **Lab Tracking Form** can be used to record information such as operator name, sample and index information, start and stop times, reagent lot numbers, and barcodes.
- ▶ The **Praxis Extended RAS Panel** analysis module is used to create a run. The analysis module provides a feature for recording parameters for the sample plate, such as sample name and dual indexes applicable to the run.



NOTE

You can download the above Illumina Praxis Extended RAS Panel documents from the Illumina website. Go to the Illumina Praxis Extended RAS Panel support page and click the **Documentation & Literature** tab.

Getting Started

This section describes the Illumina Praxis Extended RAS Panel kit contents, consumables and equipment used, DNA input recommendations, and best practices to apply during the protocol.

Praxis Extended RAS Panel Kit Contents

The Illumina Praxis Extended RAS Panel Kit (Catalog # 20012431) contains the following components. Store the kit components at the specified temperature and in designated pre- and post-amplification areas.

Because the pre- and post-amp reagents are shipped together, it is important to unpack the reagents in the pre-amp lab area, and then move the post-amp reagents to the proper post-amp storage area.

Praxis Extended RAS Panel, Box 1

Table 1 Box 1A Pre-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|--------------------------------|-------------------|-------------|--|----------------|
| Hybridization Buffer | 1 tube | 4.32 ml | Buffered aqueous solution containing salts and formamide | -25°C to -15°C |
| Extension-Ligation Mix | 1 tube | 4.8 ml | Buffered aqueous solution containing proprietary blend of DNA polymerases, DNA ligase, and dNTPs | -25°C to -15°C |
| Index Primers A-H (A501-A508) | 1 tube per primer | 192 µl | PCR primers with index sequences and sequencing adapters | -25°C to -15°C |
| Index Primers 1-12 (A701-A712) | 1 tube per primer | 128 µl | PCR primers with index sequences and sequencing adapters | -25°C to -15°C |
| PCR Polymerase | 1 tube | 56 µl | Proprietary DNA polymerase | -25°C to -15°C |
| PCR Master Mix | 1 tube | 2.8 ml | Buffered aqueous solution containing salts and dNTPs | -25°C to -15°C |

Table 2 Box 1B Post-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|-------------------------------|----------|-------------|--|----------------|
| Library Normalization Diluent | 1 tube | 4.6 ml | Buffered aqueous solution containing salts, 2-Mercaptoethanol, and formamide | -25°C to -15°C |
| Library Dilution Buffer | 2 tubes | 4.5 ml | Buffered aqueous solution | -25°C to -15°C |
| PhiX Control | 1 tube | 10 µl | Buffered aqueous solution containing PhiX genomic DNA | -25°C to -15°C |

Praxis Extended RAS Panel, Box 2

Table 3 Box 2 Post-Amp Reagents

| Component | Quantity | Contents | Storage |
|--------------------------------------|--------------|---|----------------|
| MiSeqDx Cartridge–Extended RAS Panel | 2 cartridges | Single-use cartridge that contains cluster generation and sequencing reagents for use with the MiSeqDx, including formamide, 2-Mercaptoethanol, and < 2% DMSO | -25°C to -15°C |

Praxis Extended RAS Panel, Box 3

Table 4 Box 3A Pre-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|-----------------------|----------|-------------|---|------------|
| Stringent Wash Buffer | 1 bottle | 24 ml | Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide | 2°C to 8°C |
| Universal Wash Buffer | 1 tube | 4.8 ml | Buffered aqueous solution containing salts | 2°C to 8°C |

Table 5 Box 3B Post-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|----------------------------|--------------|-------------|---|------------|
| PCR Clean-Up Beads | 1 tube | 5 ml | Buffered aqueous solution containing solid phase paramagnetic beads and polyethylene glycol | 2°C to 8°C |
| Library Normalization Wash | 2 tubes | 4.8 ml | Buffered aqueous solution containing salts, 2-Mercaptoethanol and formamide | 2°C to 8°C |
| Library Beads | 1 tube | 1.2 ml | Buffered aqueous solution containing solid phase paramagnetic beads | 2°C to 8°C |
| MiSeqDx Flow Cell | 2 containers | 1 flow cell | Glass substrate with covalently bound oligonucleotides | 2°C to 8°C |

Praxis Extended RAS Panel, Box 4

Table 6 Box 4 Post-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|---|-----------|-------------|---------------------------|------------|
| MiSeqDx SBS Solution (PR2)–Extended RAS Panel | 2 bottles | 500 ml | Buffered aqueous solution | 2°C to 8°C |

Praxis Extended RAS Panel, Box 5

Table 7 Box 5 Pre-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|--------------|----------|-------------|--|--------------|
| Filter Plate | 2 plates | N/A | Polypropylene microtiter plate with a modified polyethersulfone membrane | 15°C to 30°C |

Table 8 Box 5 Post-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|------------------------|----------|-------------|---------------------------|--------------|
| Elution Buffer | 1 tube | 4.8 ml | Buffered aqueous solution | 15°C to 30°C |
| Library Storage Buffer | 1 tube | 3.5 ml | Buffered aqueous solution | 15°C to 30°C |

Praxis Extended RAS Panel, Box 6

Table 9 Box 6 Pre-Amp Reagents

| Component | Quantity | Fill Volume | Active Ingredients | Storage |
|-------------------------------|----------|-------------|--|----------------|
| Oligo Pool A–Extended RAS | 1 tube | 150 µl | Buffered aqueous solution containing oligonucleotides targeting the RAS genes | -25°C to -15°C |
| Oligo Pool B–Extended RAS | 1 tube | 150 µl | Buffered aqueous solution containing oligonucleotides targeting the RAS genes | -25°C to -15°C |
| Quality Control (QC) Primers | 4 tubes | 75 µl | Buffered aqueous solution containing oligonucleotides for DNA sample qualification | -25°C to -15°C |
| Quality Control (QC) Template | 1 tube | 220 µl | Buffered aqueous solution containing genomic DNA for sample qualification | -25°C to -15°C |
| qPCR Master Mix | 2 tubes | 1 ml | Buffered aqueous solution containing qPCR reaction components | -25°C to -15°C |
| Panel Control–Extended RAS | 1 tube | 8 µl | Buffered aqueous solution containing genomic DNA for assay positive control | -25°C to -15°C |

Reagents Required, Not Provided

Pre-Amp Reagents

- ▶ 10 N NaOH (prepare from tablets or use a standard solution)
- ▶ TE Buffer
- ▶ RNase/DNase-free water

Post-Amp Reagents

- ▶ 10 N NaOH (prepare from tablets or use a standard solution)
- ▶ Ethanol, 200 proof for molecular biology
- ▶ TE Buffer
- ▶ RNase/DNase-free water

Equipment and Materials

Equipment and Materials Required, Sold Separately

- 1 **MiSeqDx Instrument**, Catalog # DX-410-1001
- 2 **TruSeq[®] Index Plate Fixture Kit**, Catalog # FC-130-1005
- 3 **TruSeq Index Plate Fixture & Collar Kit**, Catalog # FC-130-1007

Equipment and Materials Required, Not Provided

Pre-Amp Equipment and Materials

- 1 **DNA Extraction and Purification Reagents**—See the package insert for more information about DNA Extraction.
- 2 **Heat Block**—One heat block for a 96-well PCR plate is required. The heat block must meet the following performance specifications. Heat blocks with heated lids are required for use.
 - ▶ Temperature Range: Ambient +5°C to 99°C
 - ▶ Temperature Regulation: ±0.1°C at 37°C; ±0.4°C at 60°C
- 3 **Sample Incubator**—One incubator (hybridization oven) is required. The incubator must meet the following performance specifications.
 - ▶ Temperature Range: 10°C to 100°C
 - ▶ Temperature Regulation: ±0.2°C
- 4 **Tabletop Centrifuge**—One tabletop centrifuge is required. (A separate centrifuge is required in the post-amp area.) Any plate centrifuge that attains the designated speeds of the protocol (280 to 2400 × g) is acceptable.
- 5 **Microcentrifuge**—One microcentrifuge for microcentrifuge tubes.
- 6 **Precision Pipettes**—One set of precision pipettes is required. (A separate set is required in the post-amp area.) The use of precision pipettes is required to ensure accurate reagent and sample delivery. If they are calibrated regularly and are accurate within 5% of stated volume, single-channel or multi-channel pipettes can be used.
- 7 **Sealing Rollers or Sealing Wedge**—A sealing roller or sealing wedge are required for sealing microplates.
- 8 **Consumables**—The following consumables are required.
 - ▶ 96-well or 384-well optical PCR plates and seals
 - ▶ 96-well skirted PCR plates, 0.2 ml, polypropylene, or equivalent
 - ▶ 96-well storage plates, 0.8 ml (MIDI plates)



NOTE

Make sure that the 96-well plate is fit compatible with the magnetic stand.

- ▶ PCR eight-tube strips
- ▶ Solution basin, PVC, RNase/DNase-free (trough)
- ▶ Thermal microplate sealer



NOTE

If a thermal microplate sealer is not available, adhesive aluminum foil seal can be used.

- ▶ Adhesive aluminum foil seal
- ▶ Appropriate PCR plate seals
- ▶ Aerosol resistant pipette tips

Post-Amp Equipment and Materials

- 1 **96-Well or 384-Well Real-Time PCR Detection System**—One Real-Time PCR Detection system is required. The system must meet the following performance specifications. In addition, the system must have a heated lid and support a 96-well or 384-well format.
 - ▶ Minimum Temperature Range: 37°C to 95°C
 - ▶ Accuracy/Uniformity: $\pm 0.5^{\circ}\text{C}$
 - ▶ Dye: FAM
 - 2 **Thermal Cycler**—One thermal cycler is required. The thermal cycler must have a heated lid and meet the following performance specifications:
 - ▶ Minimum Temperature Control Range: 4°C to 99°C
 - ▶ Control Accuracy: $\pm 0.3^{\circ}\text{C}$
 - 3 **Optical Compression Pad**—An optical compression pad is recommended to provide a proper thermal seal between the PCR thermal cycler and reaction plates when using adhesive plate seals.
 - 4 **Microplate Shaker**—One microplate shaker is required in the post-amp lab area. The plate shaker must meet the following performance specifications:
 - ▶ Maximum Mixing Speed: 3000 rpm
 - ▶ Mixing Speed Range: 200 to 3000 rpm
 - 5 **Tabletop Centrifuge**—One tabletop centrifuge is required. (A separate centrifuge is required in the pre-amp area.) Any plate centrifuge that attains the designated speeds of the protocol (280 to 2400 \times g) is acceptable.
 - 6 **Heat Block**—One heat block for tubes is required. The heat block must meet the following performance specifications.
 - ▶ Temperature Range: Ambient +5°C to 99°C
 - ▶ Temperature Regulation: $\pm 0.1^{\circ}\text{C}$ at 37°C; $\pm 0.4^{\circ}\text{C}$ at 60°C
 - 7 **Magnetic Stand**—One magnetic stand for a 96-well plate is required. Better performance is seen when the magnets are on the side of the stand and not on the bottom.
 - 8 **Precision Pipettes**—One set of precision pipettes is required. (A separate set is required in the pre-amp area.) The use of precision pipettes is required to ensure accurate reagent and sample delivery. If they are calibrated regularly and are accurate within 5% of stated volume, single-channel or multi-channel pipettes can be used.
 - 9 **Gel Electrophoresis Supplies**—Gel electrophoresis supplies and apparatus are required along with an appropriate staining method to visualize PCR products in the gel.
 - 10 **Consumables**—The following consumables are required.
 - ▶ 96-well skirted PCR plates, 0.2 ml, polypropylene, or equivalent
 - ▶ 96-well storage plates, 0.8 ml (MIDI plates)
-  **NOTE**
Make sure that the 96-well plate is fit compatible with the magnetic stand.
- ▶ 2-4% TBE Agarose gel

- ▶ 100 bp DNA molecular weight marker
- ▶ DNA loading dye
- ▶ Conical tubes, 15 ml
- ▶ Microcentrifuge tubes
- ▶ PCR eight-tube strips
- ▶ Solution basins, PVC, RNase/DNase-free (trough)
- ▶ Adhesive plate seals
- ▶ Aerosol resistant pipette tips

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

- ▶ **Physically Separate Pre- and Post-Amp Areas**
 - ▶ Physically separate laboratory space where pre-amp processes are performed (DNA extraction, quantification, and normalization) from the laboratory space where post-amp processes are performed.
 - ▶ Never use the same sink to wash pre-amp and post-amp troughs.
 - ▶ Never share the same water purification system for pre-amp and post-amp processes.
 - ▶ Store all supplies used in the protocols in the pre-amp area, and transfer to the post-amp area as needed.
- ▶ **Use Dedicated Equipment and Supplies**
 - ▶ Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-amp and post-amp lab processes, and never share between processes.
 - ▶ Dedicate separate storage areas (freezers and refrigerators) to pre-amp and post-amp consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-amp lab area, and then move the post-amp reagents to the proper post-amp storage area.

Pre- and Post-Amp Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach).



CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-Amp Area

A daily cleaning of the pre-amp area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-amp area.

Identify pre-amp areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-amp processes. High-risk areas might include, but are not limited to, the following items:

- ▶ Bench tops
- ▶ Door handles
- ▶ Refrigerator/freezer door handles
- ▶ Computer mouse
- ▶ Keyboards

Daily Cleaning of Post-Amp Area

Reducing the amount of PCR product in the post-amp area helps reduce the risk of contamination in the pre-amp area. Daily cleaning of the post-amp area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps achieve this.

Identify post-amp areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:

- ▶ Thermal cyclers
- ▶ Bench space used to process amplified DNA
- ▶ Door handles
- ▶ Refrigerator/freezer door handles
- ▶ Computer mouse
- ▶ Keyboards

Weekly Cleaning of All Lab Areas

- ▶ Once a week, perform a thorough cleaning of the pre-amp and post-amp areas using 0.5% Sodium Hypochlorite (10% Bleach).
- ▶ Clean all bench tops and laboratory surfaces.
- ▶ Clean all instruments that are not cleaned daily.
- ▶ Thoroughly mop lab floors.
- ▶ Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-amp area; therefore, anything falling to the floor must be treated as contaminated.

- ▶ Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.
- ▶ Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned with a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
- ▶ Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

Precautions

Adhere to the following recommendations when preparing libraries for sequencing using this protocol.

Ensuring Consistency

- ▶ **Use multi-channel pipettes**—To ensure consistency across samples, use a multi-channel pipette where possible. Calibrate pipettes regularly.
- ▶ **Consistency for smaller sample preparations**—Each reagent tube supplied with the kit contains sufficient volume to generate results using manual pipettes and reagent troughs following standard laboratory techniques. To ensure accurate reagent volume delivery, single pipette reagent into each well or multi-channel pipette from reagents aliquoted into a PCR 8-tube strip.

Handling Magnetic Beads

- ▶ **Use at room temperature**—Allow the beads to reach room temperature prior to use.
- ▶ **Vortex until well-suspended**—Immediately prior to use, vortex the beads until they are well-suspended and the color appears homogeneous.
- ▶ **Mix samples thoroughly**—After adding the beads to the samples, mix thoroughly by pipetting up and down ten times. Illumina also recommends using a shaker to thoroughly mix samples.
- ▶ **Allow maximum binding**—For best results, incubate the bead/sample mixtures at room temperature for the entire duration indicated in the protocol.
- ▶ **Slowly aspirate cleared solution**—After placing the plate on the magnetic stand, wait for the solution to clear before proceeding. Keep the plate on the magnetic stand when slowly aspirating cleared solution, taking care not to disturb the separated beads.

Avoiding Cross-Contamination

- ▶ **Change tips between dispensing reagents and samples**—Always use fresh pipette tips between dispensing reagents and samples.
- ▶ **Mix plates as directed**—Mix samples with a multi-channel pipette and centrifuge the plate when indicated. Do not vortex the plates.
- ▶ **Use aerosol-resistant tips**—Using aerosol-resistant pipette tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.

Washing with 80% Ethanol During the PCR Clean-Up Step

- ▶ **Prepare fresh 80% ethanol**—Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air and impact results.
- ▶ **Remove all ethanol from wells**—Make sure that all ethanol is removed from the bottom of the wells, as it may contain residual contaminants. Use a P20 multi-channel pipette to remove residual ethanol and accelerate drying.
- ▶ **Allow complete evaporation**—Allow at least five minutes of drying time off the magnetic stand at room temperature for complete evaporation. Residual ethanol can impact the performance of subsequent reactions.

DNA Input Requirements

- ▶ The Illumina Praxis Extended RAS Panel is designed to be used with genomic DNA extracted from formalin-fixed paraffin embedded (FFPE) colorectal cancer (CRC) tissue samples.
- ▶ **Assessing DNA Quality**—The Praxis Extended RAS Panel workflow starts with a FFPE DNA qualification step to determine if the DNA is of sufficient quality for use with the

Praxis Extended RAS Panel, by measuring the amplifiable amounts of DNA in the sample relative to a control template. The qualification test determines a difference in quantification cycle (delta Cq value) for each sample relative to the control template.

Genomic DNA (gDNA) samples giving a delta Cq value less than or equal to 5.00 can be used for the Praxis Extended RAS Panel. Illumina does not support the use of gDNA samples giving a delta Cq value of greater than 5.00 with the Praxis Extended RAS Panel.

Quality Controls

- ▶ Good laboratory practices mandate that the Panel Control sample and a negative (no-template) control sample be included in every run.

Acronyms

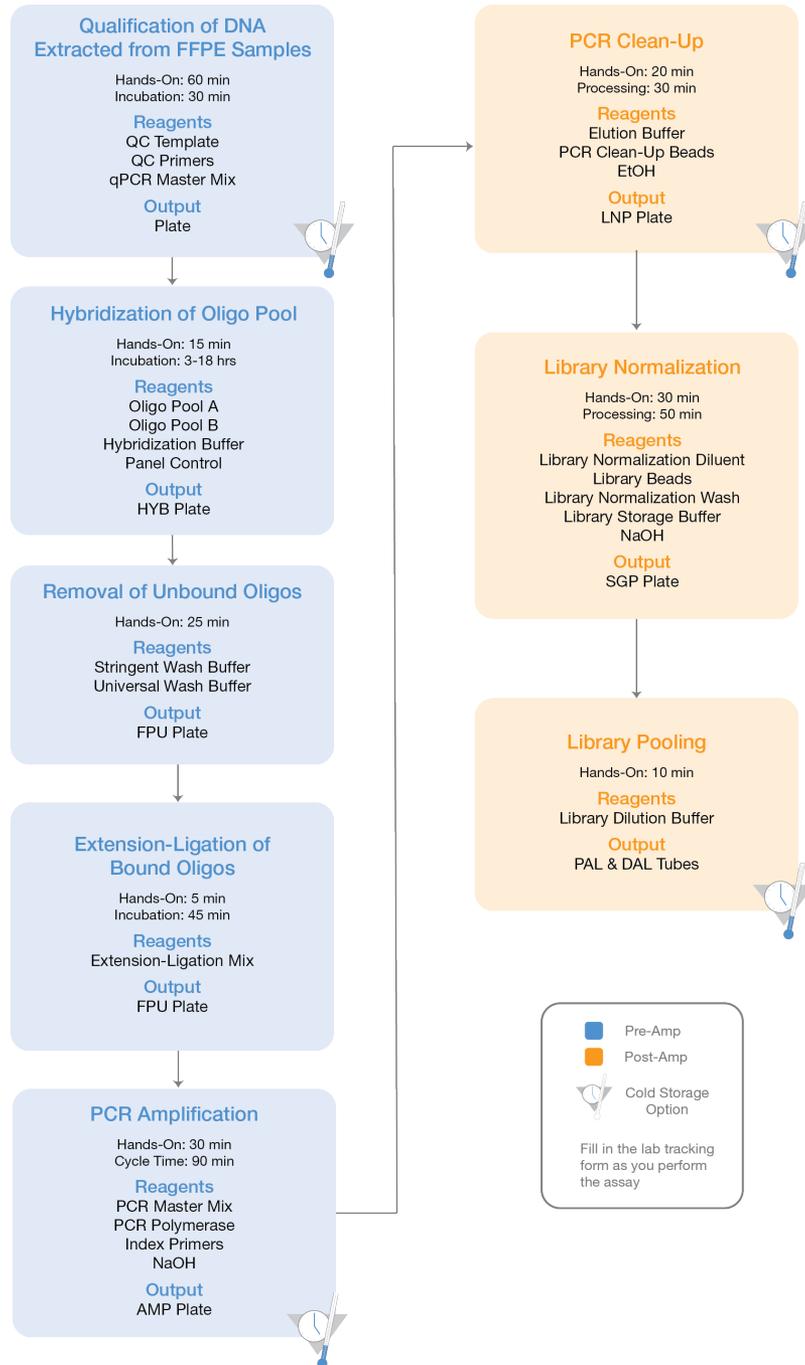
Table 10 Illumina Praxis Extended RAS Panel Acronyms

| Acronym | Definition |
|---------|------------------------------|
| AMP | AMplification Plate |
| CLP | CLean-up Plate |
| DAL | Diluted Amplicon Library |
| FPU | Filter Plate Unit |
| HYB | HYBridization Plate |
| LNP | Library Normalization Plate |
| NTC | Negative/No Template Control |
| PAL | Pooled Amplicon Library |
| SGP | StoraGe Plate |

Assay Workflow

The following diagram illustrates the Illumina Praxis Extended RAS Panel workflow. Safe stopping points are marked between steps.

Figure 1 Illumina Praxis Extended RAS Panel Workflow



Qualification of FFPE DNA Samples

During this step, a qPCR reaction will be performed to determine the amplifiability of your FFPE-extracted gDNA samples. By comparing the amplifiability of FFPE DNA relative to that of the Quality Control Template non-FFPE reference gDNA, a ΔCq value can be calculated for each sample and used to predict its performance in the Praxis Extended RAS Panel assay. The exact amount of FFPE DNA input will vary according to the quality of the extracted DNA.

Estimated Time

- ▶ Total duration: 3 hours
- ▶ Hands-on: 60 minutes

Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|---|-----------|----------------|-------------|---------|
| QC Primers | 4 tubes | -25°C to -15°C | Illumina | 6 |
| QC Template | 1 tube | -25°C to -15°C | Illumina | 6 |
| qPCR Master Mix | 2 tubes | -25°C to -15°C | Illumina | 6 |
| Genomic DNA | As needed | -25°C to -15°C | User | — |
| RNase/DNase-free Water | As needed | 15°C to 30°C | User | — |
| 96 or 384-well Optical PCR Plate and Seal | 1 plate | 15°C to 30°C | User | — |
| PCR Eight-tube Strips | As needed | 15°C to 30°C | User | — |
| Microcentrifuge Tubes | As needed | 15°C to 30°C | User | — |

Preparation

- 1 Remove the QC Template, QC Primers, qPCR Master Mix, and genomic DNA from -25°C to -15°C storage and thaw at room temperature for up to 30 minutes.
- 2 Place thawed tubes on ice.



NOTE

If using QC Template for the first time, aliquot 7 μ l of QC Template into different PCR strip tubes for long-term storage to avoid freeze-thawing.

Procedure

- 1 Determine the plate layout of the qPCR reaction.
For 10 samples, Illumina recommends the layout in Figure 2 for a 384-well format. A similar layout can be adapted for a 96-well format. Test QC Template, NTC, and each sample in **triplicate**.

Figure 2 Suggested qPCR Plate Layout for 10 Samples

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|---|---|----------|----------|----------|---|-----------|-----------|-----------|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| A | | | | | | | | | | | | | | | | | | | | | | | | |
| B | | | | | | | | | | | | | | | | | | | | | | | | |
| C | | Sample 1 | Sample 1 | Sample 1 | | Sample 7 | Sample 7 | Sample 7 | | | | | | | | | | | | | | | | |
| D | | | | | | | | | | | | | | | | | | | | | | | | |
| E | | Sample 2 | Sample 2 | Sample 2 | | Sample 8 | Sample 8 | Sample 8 | | | | | | | | | | | | | | | | |
| F | | | | | | | | | | | | | | | | | | | | | | | | |
| G | | Sample 3 | Sample 3 | Sample 3 | | Sample 9 | Sample 9 | Sample 9 | | | | | | | | | | | | | | | | |
| H | | | | | | | | | | | | | | | | | | | | | | | | |
| I | | Sample 4 | Sample 4 | Sample 4 | | Sample 10 | Sample 10 | Sample 10 | | | | | | | | | | | | | | | | |
| J | | | | | | | | | | | | | | | | | | | | | | | | |
| K | | Sample 5 | Sample 5 | Sample 5 | | QCT | QCT | QCT | | | | | | | | | | | | | | | | |
| L | | | | | | | | | | | | | | | | | | | | | | | | |
| M | | Sample 6 | Sample 6 | Sample 6 | | NTC | NTC | NTC | | | | | | | | | | | | | | | | |
| N | | | | | | | | | | | | | | | | | | | | | | | | |
| O | | | | | | | | | | | | | | | | | | | | | | | | |
| P | | | | | | | | | | | | | | | | | | | | | | | | |

QCT = QC Template

NTC = No Template Control

- 2 Add 5 µl of QC Template to 495 µl of RNase/DNase-free water in a microcentrifuge tube.
- 3 Vortex the dilution to mix the sample.
- 4 Add 1.5 µl of extracted genomic DNA to 148.5 µl of RNase/DNase-free water in a PCR eight-tube strip to make a 100-fold dilution.



NOTE

Illumina recommends performing sample dilutions in a PCR eight-tube strip to facilitate multi-channel pipetting.

- 5 Using a P200, pipette up and down 10 times to mix the dilutions.
- 6 Add 150 µl of the diluted QC Template to an unused well in the PCR eight-tube strip consistent with the plate layout.
- 7 To another unused well, add 150 µl of nuclease-free water for use as NTC sample.
- 8 Prepare the qPCR Master Mix reaction per Table 11 (384-well format) or Table 12 (96-well format). The master mix contains extra volume.

Table 11 qPCR Master Mix Reactions (384-well Format)

| Consumable | µl per Well | µl for 36 Reactions |
|------------------------|-------------|---------------------|
| qPCR Master Mix | 5.0 µl | 205 µl |
| QC Primers | 0.8 µl | 33 µl |
| RNase/DNase-free Water | 2.2 µl | 90 µl |

Table 12 qPCR Master Mix Reactions (96-well Format)

| Consumable | µl per Well | µl for 36 Reactions |
|------------------------|-------------|---------------------|
| qPCR Master Mix | 10.0 µl | 410 µl |
| QC Primers | 1.6 µl | 66 µl |
| RNase/DNase-free Water | 4.4 µl | 180 µl |

- 9 Mix reaction mix gently but thoroughly.
- 10 Place the reaction mix on ice and protect it from light until use.
- 11 Aliquot master mix into a trough or eight-tube strip to aid in dispensing with multi-channel pipettor.
- 12 Add 8 µl (384-well format) or 16 µl (96-well format) of the master mix to each well of the qPCR plate. Take care to pipette accurately into the wells as small variations will affect the assay.
- 13 Add 2 µl (384-well format) or 4 µl (96-well format) of the QC Template dilution, the sample dilutions, or nuclease-free water to each well of the plate. Take care to pipette accurately into the wells as small variations will affect the assay.
- 14 Seal the plate with the appropriate seal for your instrument, taking care to avoid cross-contamination and to avoid smudging the surface of the lids. Verify that the seal is oriented correctly and uniformly sealed.
- 15 Place the plate on an adapter (if needed) and centrifuge the plate to 1000 × g for 1 minute.
- 16 Ensure that the seal is free of any liquid or dust, place the plate on the qPCR machine in the correct orientation, then close the lid and run the following qPCR thermal profile:
 - ▶ Hot Start
 - ▶ 50°C for 2 minutes
 - ▶ 95°C for 10 minutes
 - ▶ x40
 - ▶ 95°C for 30 seconds
 - ▶ 57°C for 30 seconds
 - ▶ 72°C for 30 seconds
 - ▶ Confirm that the instrument captures images after the 72°C step.

**NOTE**

The C_q threshold should be set to a value appropriate for your instrument that avoids inaccurate measurements due to background.

- 17 After the final step, the thermal cycler analyzes the samples. Ensure that the QC Template produces expected amplification curves (see green amplification curves in Figure 3). On most systems, the QC Template should amplify at a C_q of approximately 15–22 cycles. Exclude replicates from a triplicate group that are > 0.5 C_q different from the rest of the group. At least two of the three replicates less than or equal to 0.5 C_q apart must be included in the final calculation. If these criteria are not met, the qualification process should be repeated for those samples.

**NOTE**

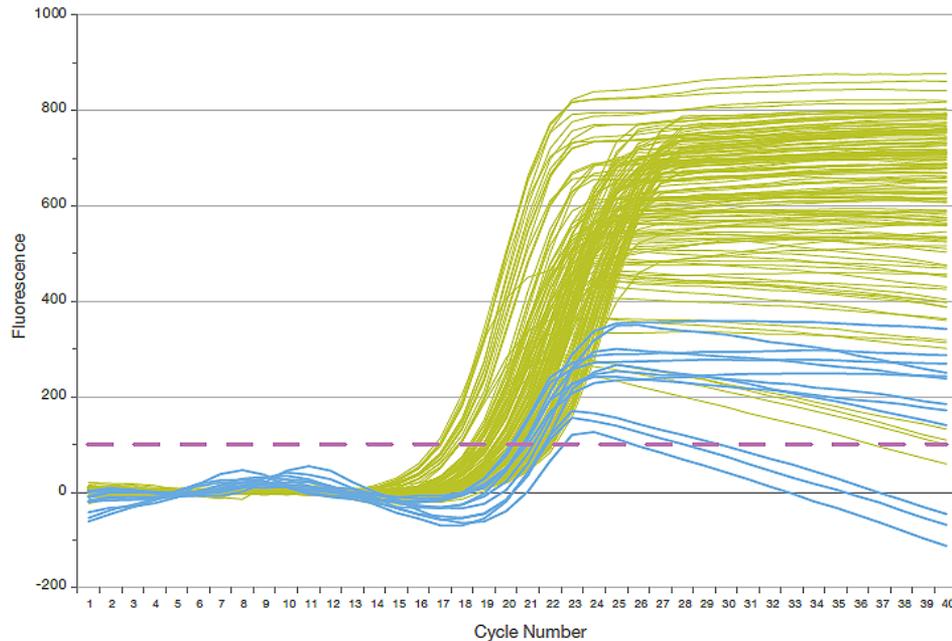
Four or more samples per 10-sample run having replicates removed indicate technical errors and the qualification process for all samples should be repeated.

**NOTE**

If little to no measurable amplification occurs for two or more replicates, the qualification process should be repeated for those samples.

- 18 Replicates exhibiting abnormal amplification curves should be excluded (see blue amplification curves in Figure 3).

Figure 3 Qualification of Samples* by qPCR



* DNA samples shown in Figure 3 were qualified for use in creating libraries for next-generation sequencing using real-time PCR following parameters stated in the text. The graph shows qPCR amplification curves generated by these samples.

Purple Line—Cq Threshold

Blue Curves—DNA replicates with abnormal amplification profiles that should be excluded from dCq calculations

Green Curves—DNA replicates with expected amplification curves that can be used for delta Cq calculations

- 19 Subtract the average Cq for the QC Template from the average Cq for each sample (Sample Average Cq – QC Template Average Cq) to yield the delta Cq values for each sample. Record the delta Cq values and the sample dilution factors. The sample dilution factors are based on the recommendations in Table 16.
- 20 Make sure that amplification of the NTC occurs at least 10 cycles after QC Template amplification.

**NOTE**

Repeat entire qualification process for all samples if NTC fails.

Table 13 dCq Calculation Examples

| Example # | Replicate 1 | Replicate 2 | Replicate 3 | Final values included in Cq calculations | Average Cq | dCq* |
|--------------------------------|--|-------------|-------------|--|--------------|------|
| 1 | 24.80 | 25.11 | 25.06 | All | 24.99 | 6.87 |
| Actions: | Does not meet dCq requirement (≤ 5.00). Sample does not qualify for the assay. | | | | | |
| 2 | 17.22 | 18.58 | 17.91 | None | N/A | N/A |
| Actions: | Remove replicates >0.5 Cq different from the rest of triplicates (all three). Sample qualification process must be repeated as a minimum of two valid replicates are required. | | | | | |
| 3 | 22.45 | 22.63 | 23.52 | 22.45 & 22.63 | 22.54 | 4.42 |
| Actions: | Remove replicate which is >0.5 Cq different from the rest of triplicates. Calculate dCq using two valid replicate values. dCq requirement is met (≤ 5.00). | | | | | |
| 4 | 21.56 | 21.73 | 21.48 | All | 21.59 | 3.46 |
| Actions: | Sample passed with three replicate values that are not >0.5 Cq apart. dCq requirement is met (≤ 5.00). | | | | | |
| 5 | 18.21 | 18.50 | 19.04 | All | 18.70 | 0.58 |
| Actions: | Sample passed with three replicate values that are not >0.5 Cq apart. dCq requirement is met (≤ 5.00). | | | | | |
| QC Template (Example 1) | 17.86 | 18.11 | 18.40 | All | 18.12 | 0 |
| Actions: | QC Template passes with three valid replicate values that are not >0.5 Cq apart. QC Template passes QC (average Cq ≥ 15 and ≤ 22). | | | | | |
| Control considerations | | | | | | |
| NTC (negative control) | 26.00 | 25.64 | 25.83 | All | 25.82 | 7.7 |
| Actions: | Entire sample qualification fails. Amplification of the NTC must occur ≥ 10 cycles after QC Template amplification. Repeat qualification process for all samples, QC Template, and NTC. | | | | | |
| QC Template (Example 2) | 9.85 | 9.73 | 9.87 | All | 9.82 | 0 |
| Actions: | QC Template fails. QC Template average Cq must be ≥ 15 and ≤ 22 . Repeat sample qualification process for all samples, QC Template, and NTC. | | | | | |

*dCq is calculated by [sample Average Cq - QC Template (Example 1) Average Cq]

SAFE STOPPING POINT

Illumina recommends storing extracted genomic DNA between 2°C to 8°C for a maximum of 28 days or storing frozen between -15°C to -25°C for a maximum of 161 days. Genomic DNA stored frozen is stable for a maximum of 2 freeze/thaw cycles.

Set Parameters

- 1 Log in to Local Run Manager.
- 2 Click **Create Run**, and select **Extended RAS**.
- 3 Make sure that "Create Run Praxis Extended RAS" is visible in the top left corner of the screen.
- 4 Enter a run name that identifies the run from sequencing through analysis.
Use alphanumeric characters, spaces, underscores, or dashes.
- 5 [Optional] Enter a run description to help identify the run.
Use alphanumeric characters.

Specify Samples for the Run

Specify samples for the run using the following options:

- ▶ **Enter samples manually**—Use the table on the Create Run screen.
- ▶ **Import samples**—Navigate to an external file in a comma-separated values (*.csv) format.

After you have populated the samples table, you can export the sample information to an external file, and import the file for another run.

Enter Samples Manually

- 1 Click the plate region A1 or E7 to enter sample information in the samples table.
Region A1 is equivalent to the upper left quadrant of wells in the Hyb plate layout, and includes samples for Oligo Pools A and B.
Region E7 is equivalent to the lower right quadrant of wells in the Hyb plate layout, and includes samples for Oligo Pools A and B.
- 2 In the Pool A table, enter a unique sample name in the Sample Name field for each well you use.
Use alphanumeric characters, dashes, or underscores.
The sample name automatically populates the corresponding well in the Pool B table.
Dual index adapters for each Index Read, specific to the well, display as you enter a sample name.
- 3 [Optional] Although the default layout is recommended, to change the position of control samples, use the Positive Control or Negative Control drop down menu.
- 4 Click the print  icon to display the plate layout.
- 5 Select **Print** to print the plate layout as a reference for preparing libraries.
- 6 Click **Close**.
- 7 Click **Save Run**.

Import Samples

- 1 Click the plate region A1 or E7.
- 2 Click the **Export**  icon for an example template.

- Enter a unique sample name in the template for up to 10 samples and save the file. Use alphanumeric characters, dashes, or underscores.

| [Data] | Sample Names as a 4 * 3 matrix | |
|---------|--------------------------------|------------------|
| Sample1 | Sample5 | Sample9 |
| Sample2 | Sample6 | Sample10 |
| Sample3 | Sample7 | Positive_Control |
| Sample4 | Sample8 | Negative_Control |

- Click **Import Samples**.
- Browse to the location of the sample information file in *.csv file format.
- When finished, click the print  icon to display the plate layout.
- Select **Print** to print the plate layout as a reference for preparing libraries.
- Click **Close**.
- Click **Save Run**.

Index Primer Sequences

Table 14 Sequences for Index Primers A-H (A501-A508)

| Index Primer | Sequence |
|-----------------------|----------|
| Index Primer A (A501) | TGAACCTT |
| Index Primer B (A502) | TGCTAAGT |
| Index Primer C (A503) | TGTTCTCT |
| Index Primer D (A504) | TAAGACAC |
| Index Primer E (A505) | CTAATCGA |
| Index Primer F (A506) | CTAGAACA |
| Index Primer G (A507) | TAAGTTCC |
| Index Primer H (A508) | TAGACCTA |

Table 15 Sequences for Index Primers 1-12 (A701-A712)

| Index Primer | Sequence |
|------------------------|----------|
| Index Primer 1 (A701) | ATCACGAC |
| Index Primer 2 (A702) | ACAGTGGT |
| Index Primer 3 (A703) | CAGATCCA |
| Index Primer 4 (A704) | ACAAACGG |
| Index Primer 5 (A705) | ACCCAGCA |
| Index Primer 6 (A706) | AACCCCTC |
| Index Primer 7 (A707) | CCCAACCT |
| Index Primer 8 (A708) | CACCACAC |
| Index Primer 9 (A709) | GAAACCCA |
| Index Primer 10 (A710) | TGTGACCA |
| Index Primer 11 (A711) | AGGGTCAA |
| Index Primer 12 (A712) | AGGAGTGG |

Hybridization of Oligonucleotide Pool

During this step, the Extended RAS oligonucleotide pools containing upstream and downstream oligonucleotides specific to the RAS genes are hybridized to genomic DNA samples.



WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.



NOTE

Illumina does not support the use of gDNA samples giving a delta Cq value of greater than 5.00.

Estimated Time

- ▶ Total duration: 3–18 hours (overnight)
- ▶ Hands-on: 15 minutes

Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|---|-----------|----------------|-------------|---------|
| Oligo Pool A | 1 tube | -25°C to -15°C | Illumina | 6 |
| Oligo Pool B | 1 tube | -25°C to -15°C | Illumina | 6 |
| Hybridization Buffer | 1 tube | -25°C to -15°C | Illumina | 1A |
| Genomic DNA | 20 µl | -25°C to -15°C | User | — |
| Panel Control | 1 tube | -25°C to -15°C | Illumina | 6 |
| TE Buffer | As needed | 15°C to 30°C | User | — |
| 96-well skirted PCR plate | 1 plate | 15°C to 30°C | User | — |
| Thermal Microplate Sealer or Adhesive Aluminum Foil | 2 seals | 15°C to 30°C | User | — |
| Sterile Troughs | As needed | 15°C to 30°C | User | — |

Preparation

- 1 Determine genomic DNA samples to be used in the assay. Samples giving a delta Cq value less than or equal to 5.00 can be used for the Praxis Extended RAS Panel.
- 2 [Optional] If extracted DNA samples do not meet the delta Cq threshold, additional tissue sections can be used for DNA extraction if available.
- 3 Remove the Oligo Pool A, Oligo Pool B, Hybridization Buffer, genomic DNA samples, and Panel Control from -25°C to -15°C storage and thaw at room temperature.
- 4 Vortex the Oligo Pool A, Oligo Pool B, and Hybridization Buffer vigorously to make sure that all precipitates have dissolved, then briefly centrifuge the tubes to collect liquid.



NOTE

Before using Hybridization Buffer, hold the tube in front of a light and visually inspect to make sure that all precipitates have completely dissolved.

- 5 Set a 96-well heat block to 95°C.
- 6 Pre-heat an incubator to 37°C to prepare for the extension-ligation step.
- 7 Create the run and enter sample plate information in the Praxis Extended RAS Panel analysis module. Verify the location of positive and negative controls and then print sample plate layout. Illumina recommends processing samples in batches no larger than 12 samples/24 libraries.



NOTE

Use of controls enables Illumina Technical Support to provide effective troubleshooting assistance. Illumina Technical Support will not provide assistance unless these control reactions were included in the run.

Procedure

- 1 Label a new 96-well PCR plate "HYB_Plate_ID".
See Figure 4 for the suggested **HYB** plate layout for the following steps.

Figure 4 **HYB** Plate Layout

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|----------|----------|------------------|----------|----------|------------------|-----------|-----------|------------------|-----------|-----------|------------------|
| A | Sample 1 | Sample 5 | Sample 9 | Sample 1 | Sample 5 | Sample 9 | | | | | | |
| B | Sample 2 | Sample 6 | Sample 10 | Sample 2 | Sample 6 | Sample 10 | | | | | | |
| C | Sample 3 | Sample 7 | Positive Control | Sample 3 | Sample 7 | Positive Control | | | | | | |
| D | Sample 4 | Sample 8 | Negative Control | Sample 4 | Sample 8 | Negative Control | | | | | | |
| E | | | | | | | Sample 11 | Sample 15 | Sample 19 | Sample 11 | Sample 15 | Sample 19 |
| F | | | | | | | Sample 12 | Sample 16 | Sample 20 | Sample 12 | Sample 16 | Sample 20 |
| G | | | | | | | Sample 13 | Sample 17 | Positive Control | Sample 13 | Sample 17 | Positive Control |
| H | | | | | | | Sample 14 | Sample 18 | Negative Control | Sample 14 | Sample 18 | Negative Control |

Extended RAS-Oligo Pool A

Extended RAS-Oligo Pool B

- 2 Using the plate layout in Figure 4, add 10 µl of TE Buffer to the wells indicated as Negative Control (No Template Control) for Oligo Pool A and Oligo Pool B.
- 3 For each 12 sample/ 24 library preparation, add 2.5 µl of Panel Control to 22.5 µl of TE Buffer in a microcentrifuge tube. Mix the diluted Panel Control thoroughly.
- 4 Using the plate layout in Figure 4, add 10 µl of diluted Panel Control prepared in the previous step to each of the two indicated Positive Control wells for Oligo Pool A and Oligo Pool B.
- 5 Determine if sample dilution is required based on Table 16. If dilution is necessary, take 15 µl of genomic DNA extracted from FFPE samples and dilute in TE buffer according to Table

16. For example, for a sample with delta C_q of -1.50, add 15 µl sample to 15 µl of TE Buffer. For a sample with delta C_q of -2.50, add 15 µl sample to 45 µl of TE Buffer.

Table 16 Delta C_q Dilution Table

| Delta C _q | < -1.51 | -1.50 to -0.51 | -0.50 to 0.50 | 0.51 to 1.50 | 1.51 to 5.00 | > 5.00 |
|----------------------|---------|----------------|---------------|--------------|--------------|---------------|
| Dilution | 4x | 2x | No dilution | No dilution | No dilution | Not supported |

- 6 Using the plate layout in Figure 4, add 10 µl of each genomic DNA sample as prepared in step 5 to the Oligo Pool A wells of the **HYB** plate, starting with columns 1 or 7. Repeat this process for the Oligo Pool B wells of the **HYB** plate, starting with columns 4 or 10.
-  **NOTE**
Verify that DNA sample layout and the positions of positive and negative controls match the plate layout in Figure 4.
- 7 Using a multi-channel pipette, add 5 µl of Oligo Pool A to all wells containing genomic DNA, Panel Control, and Negative Control indicated for Oligo Pool A in columns 1-3 or 7-9 in the **HYB** plate. Then add 5 µl of Oligo Pool B to all wells containing genomic DNA, Panel Control, and Negative Control indicated for Oligo Pool B in columns 4-6 or 10-12 in the **HYB** plate. Change tips after each column to avoid cross-contamination.
 - 8 Using a multi-channel pipette, add 40 µl of Hybridization Buffer to each sample in the **HYB** plate. Gently pipette up and down 3–5 times to mix. Change tips after each column to avoid cross-contamination.
-  **NOTE**
Ensure any crystals or precipitate in Hybridization Buffer have dissolved.
-  **NOTE**
Do not mix the Oligo Pools and Hybridization Buffer for storage. If combined, the Oligo Pools become unstable, even when stored frozen.
- 9 Seal the **HYB** plate with a thermal microplate sealer. If a thermal sealer is not available, use an adhesive aluminum foil seal and secure the seal with a sealing roller or wedge, especially on the perimeter of the plate.
 - 10 Centrifuge at 1000 × g at room temperature for 1 minute.
 - 11 Place the **HYB** plate in the pre-heated block at 95°C and incubate for 1 minute.
 - 12 Change the temperature of the same heat block to 40°C and incubate for 3–18 hours.

 **NOTE**
Moving the plate from the 95°C heat block to another pre-heated block set to 40°C will adversely affect hybridization.

Removal of Unbound Oligonucleotides

This process removes unbound oligonucleotides from genomic DNA using a filter capable of size selection. Two wash steps using Stringent Wash Buffer ensure complete removal of unbound oligonucleotides. A third wash step using Universal Wash Buffer removes residual Stringent Wash Buffer and prepares samples for the extension-ligation step.



WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Estimated Time

- ▶ Total duration: 25 minutes
- ▶ Hands-on: 25 minutes

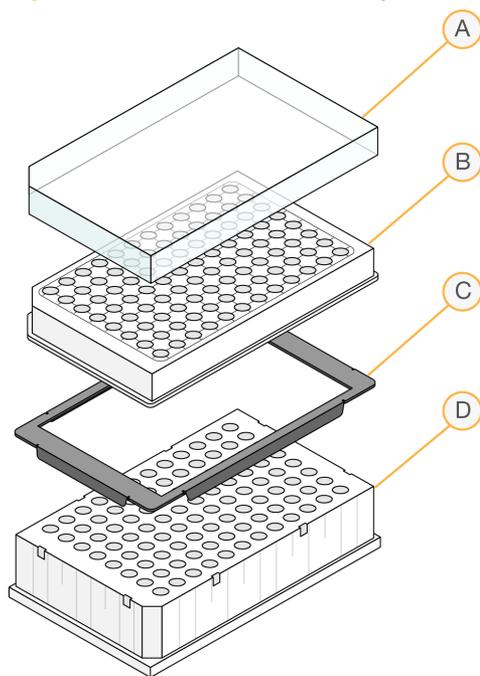
Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|------------------------|-----------|----------------|-------------|---------|
| Extension-Ligation Mix | 1 tube | -25°C to -15°C | Illumina | 1A |
| Stringent Wash Buffer | 1 bottle | 2°C to 8°C | Illumina | 3A |
| Universal Wash Buffer | 1 tube | 2°C to 8°C | Illumina | 3A |
| Filter Plate | 1 plate | 15°C to 30°C | Illumina | 5 |
| Adapter collar | 1 plate | 15°C to 30°C | User | — |
| MIDI plate | 1 plate | 15°C to 30°C | User | — |
| Troughs | As needed | 15°C to 30°C | User | — |

Preparation

- 1 Remove Extension-Ligation Mix from -25°C to -15°C storage and thaw at room temperature.
- 2 Extension-Ligation Mix is used in the Extension-Ligation step and takes approximately 20 minutes to thaw.
- 3 Remove Stringent Wash Buffer and Universal Wash Buffer from 2°C to 8°C storage and set aside at room temperature.
- 4 Assemble the filter plate assembly unit (FPU) in the following order (from bottom to top):

Figure 5 Filter Plate Unit Assembly



- A Lid
- B Filter plate
- C Adapter collar
- D MIDI plate

- 5 Label the filter plate **FPU_Plate_ID**.
- 6 Pre-wash the filter plate membrane as follows:
 - a Using a multi-channel pipette, add 50 μ l of Stringent Wash Buffer to each well.
 - b Cover the **FPU** plate with the filter plate lid and keep it covered during each centrifugation step.
 - c Centrifuge the **FPU** at $2400 \times g$ at room temperature for 5 minutes.
 - d If the wash buffer does not drain completely, centrifuge the filter plate again at $2400 \times g$ at room temperature for 5 minutes.

**NOTE**

Illumina strongly recommends keeping spare filter plates (FC-130-1006) on hand as general lab supplies.

Procedure

- 1 After the hybridization incubation, confirm the heat block has cooled to 40°C.



NOTE
If the heat block fails to cool to 40°C overnight, library preparation will need to be repeated.
- 2 Remove the **HYB** plate from the heat block and centrifuge at $1000 \times g$ at room temperature for 1 minute to collect condensation.

- 3 Using a multi-channel pipette set to 65 μl , transfer the entire volume of each sample onto the center of the corresponding pre-washed wells of the filter plate. Change tips after each column to avoid cross-contamination.
- 4 Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.

**NOTE**

Make sure that liquid has drained after centrifugation. Repeat centrifugation if necessary.

- 5 Wash the filter plate as follows:
 - a Using a multi-channel pipette, add 50 μl of Stringent Wash Buffer to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.
 - c If the wash buffer does not drain completely, centrifuge the filter plate again at $2400 \times g$ at room temperature for 5 minutes.
- 6 Repeat the wash as follows:
 - a Using a multi-channel pipette, add 50 μl of Stringent Wash Buffer to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.
 - a Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.
 - b If the wash buffer does not drain completely, centrifuge the filter plate again at $2400 \times g$ at room temperature for 5 minutes.
- 7 Discard all the flow-through (containing formamide) collected up to this point in an appropriate hazardous waste container, then reassemble the **FPU**. The same MIDI plate can be re-used for the rest of the pre-amplification process.
- 8 Using a multi-channel pipette, add 45 μl of Universal Wash Buffer to each sample well. Changing tips between columns is not required if you use care to avoid cross-contamination.
- 9 Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.

**NOTE**

Make sure that all liquid has drained after centrifugation. Repeat centrifugation if necessary. Residual wash buffer may inhibit subsequent enzymatic reactions.

Extension-Ligation of Bound Oligonucleotides

This process connects the hybridized upstream and downstream oligonucleotides. A DNA polymerase extends from the upstream oligonucleotide through the targeted region, followed by ligation to the 5' end of the downstream oligonucleotide using a DNA ligase. This results in the formation of products containing the targeted regions of interest flanked by sequences required for amplification.

Estimated Time

- ▶ Total duration: 50 minutes
- ▶ Hands-on: 5 minutes

Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|-----------------------------|-----------|----------------|-------------|---------|
| Extension-Ligation Mix | 1 tube | -25°C to -15°C | Illumina | 1A |
| Adhesive aluminum foil seal | 1 seal | 15°C to 30°C | User | — |
| Troughs | As needed | 15°C to 30°C | User | — |

Procedure

- 1 Using a multi-channel pipette, add 45 µl of Extension-Ligation Mix to each sample well of the filter plate. The Extension-Ligation reaction takes place on the filter plate membrane. Changing tips between columns is not required if you use care to avoid cross-contamination.
- 2 Seal the filter plate with adhesive aluminum foil, and then cover with the lid to secure the foil during incubation.
- 3 Incubate the entire **FPU** assembly in the pre-heated 37°C incubator for 45 minutes.
- 4 While the **FPU** plate is incubating, prepare the AMP (Amplification Plate) as described in the following section.

PCR Amplification

In this step, the extension-ligation products are amplified using primers that add index sequences for sample multiplexing, as well as common adapters required for cluster generation.

Estimated Time

- ▶ Total duration: ~105 minutes
- ▶ Hands-on: 30 minutes

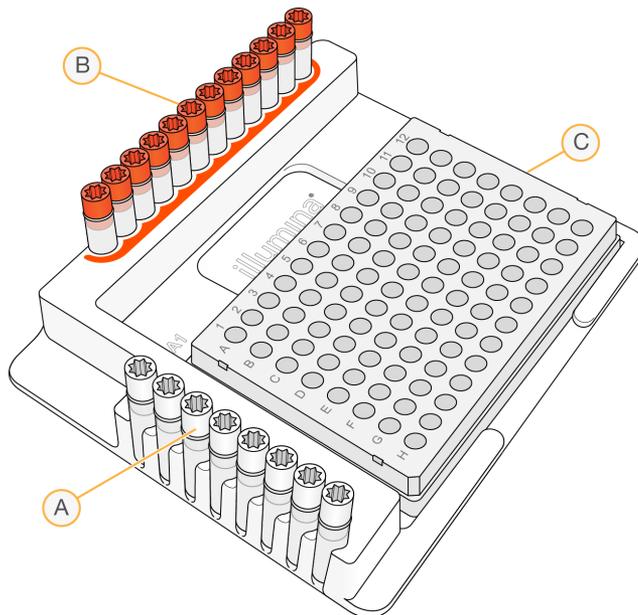
Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|--------------------------------|-------------------|----------------|-------------|---------|
| PCR Master Mix | 1 tube | -25°C to -15°C | Illumina | 1A |
| Index Primers A-H (A501-A508) | 1 tube per primer | -25°C to -15°C | Illumina | 1A |
| Index Primers 1-12 (A701-A712) | 1 tube per primer | -25°C to -15°C | Illumina | 1A |
| PCR Polymerase | 1 tube | -25°C to -15°C | Illumina | 1A |
| Appropriate PCR plate seal | 1 | 15°C to 30°C | User | — |
| 0.05 N NaOH, freshly prepared | 5 mL | 15°C to 30°C | User | — |
| 96-well skirted PCR plate | 1 plate | 15°C to 30°C | User | — |
| Troughs | As needed | 15°C to 30°C | User | — |

Preparation

- 1 Prepare fresh 0.05 N NaOH by adding 25 µl of 10 N NaOH to 4975 µl of sterile water.
- 2 Determine the index primers to be used according to the plate graphic printout from Praxis Extended RAS Panel analysis module.
- 3 Remove PCR Master Mix and the appropriate index primers from -25°C to -15°C storage and thaw on a bench at room temperature. Allow approximately 20 minutes to thaw the reagents.
- 4 After the index primers are completely thawed, vortex each tube to mix and briefly centrifuge the tubes in a microcentrifuge. Use 1.7 ml microcentrifuge tubes as adapters for the microcentrifuge.
- 5 Arrange the primers in a rack using the following arrangements:
 - a Arrange Index Primers A-H (A501-A508) primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
 - b Arrange Index Primers 1-12 (A701-A712) primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

Figure 6 Index Plate Fixture



- A Index Primers A-H (A501-A508) (white caps)
- B Index Primers 1-12 (A701-A712) (orange caps)
- C AMP plate

- 6 Label a new 96-well PCR plate "AMP" (Amplification Plate).
- 7 Add index primers to the AMP plate as follows:
 - a Using a multi-channel pipette, add 9 μ l of the selected index primers [A–H (A501–A508)] (clear solution) to the appropriate well in a column of the AMP plate.
 - b Using a multi-channel pipette, add 9 μ l of the selected index primers [1–12 (A701–A712)] (yellow solution) to the appropriate row of the AMP plate. *Tips must be changed after each row to avoid index cross-contamination.*
 - c Remove all the index primer tubes from the working area.



NOTE

Index primers are single use only and should be discarded immediately after use.

- 8 Prepare the PCR Master Mix/PCR Polymerase PCR working solution as follows:
 - a For 12 samples/24 libraries, add 21 μ l of PCR Polymerase to 1050 μ l of PCR Master Mix.
 - b Invert the prepared PCR working solution 20 times to mix.

You will add this working solution to the AMP plate in the next section. The PCR working solution is stable at room temperature for 10 minutes and can also be stored on ice if needed during preparation.



NOTE

Always add PCR Polymerase to PCR Master Mix just prior to use. Never store the combined PCR working solution.

Procedure

- 1 When the 45-minute extension-ligation reaction is complete, remove the **FPU** from the incubator. Remove the aluminum foil seal and replace with the filter plate lid. Removing the aluminum foil seal before centrifugation ensures the reaction supernatant will drain into the waste plate effectively.
- 2 Centrifuge the **FPU** at $2400 \times g$ at room temperature for 2 minutes.
- 3 Using a multi-channel pipette, add $25 \mu\text{l}$ of 0.05 N NaOH to each sample well on the filter plate. Ensuring that pipette tips come in contact with the membrane, pipette the NaOH up and down 5–6 times. Tips must be changed after each column.
- 4 Cover and incubate the filter plate at room temperature for 5 minutes.
- 5 While the filter plate is incubating, use a multi-channel pipette to transfer $22 \mu\text{l}$ of the PCR working solution to each well of the AMP plate containing index primers. Change tips between samples.
- 6 Transfer samples eluted from the filter to the AMP plate as follows:
 - a Set a multi-channel P20 pipette to $20 \mu\text{l}$.
 - b Pipette the samples in the first column of the filter plate up and down 5–6 times.

NOTE
Slightly tilt the **FPU** plate to ensure complete aspiration and to avoid air bubbles.
 - c Transfer $20 \mu\text{l}$ from the filter plate to the corresponding column of the **AMP** plate.
 - d Gently pipette up and down 5–6 times to thoroughly combine the DNA with the PCR working solution.
 - e Transfer the remaining columns from the filter plate to the AMP plate in a similar manner. *Tips must be changed after each column to avoid index and sample cross-contamination.*
 - f After all the samples have been transferred, the waste collection MIDI plate of the **FPU** can be discarded. The metal adapter collar should be cleaned and put away for future use.
- 7 Cover the **AMP** plate with the appropriate plate seal and secure with a rubber roller.
- 8 Centrifuge at $1000 \times g$ at room temperature for 1 minute.
- 9 Transfer the AMP plate to the post-amplification area.
- 10 Perform PCR using the following program on a thermal cycler:
 - ▶ 95°C for 3 minutes
 - ▶ 32 cycles of:
 - ▶ 95°C for 30 seconds
 - ▶ 66°C for 30 seconds
 - ▶ 72°C for 60 seconds
 - ▶ 72°C for 5 minutes
 - ▶ Hold at 10°C



NOTE
Illumina strongly recommends using an Optical Compression Pad to provide a proper thermal seal between the PCR thermal cycler and reaction plates and prevent sample evaporation.



NOTE

On the PCR machine, set the reaction volume to 60 μ l and the temperature ramp speed to maximum.

SAFE STOPPING POINT

If not proceeding immediately to PCR Clean-Up, the **AMP** plate can remain on the thermal cycler overnight, or can be stored at 2°C to 8°C up to 48 hours.

Verify Library Preparation

After PCR:

- 1 Combine 5 μ l of amplified product with 15 μ l of RNase/DNase-free water and DNA loading dye if necessary.
- 2 Run on a 2-4% TBE agarose gel with 100 bp ladder to confirm the presence and brightness of the 250-280 bp library product.
 - ▶ Samples that show amplification in both Oligo Pool A and B are considered valid and can be processed through the remainder of the workflow (Figure 7).
 - ▶ Samples showing little to no amplification in one Oligo Pool (Figure 8, left) or both Oligo Pools (Figure 8, right) are considered invalid and should not be processed through the remainder of the workflow. If an invalid gel result is observed, library preparation for that sample or samples will need to be repeated.
 - ▶ The Negative Control sample should show an Invalid gel result. If the Negative Control sample shows amplification in both Oligo Pool A and B, this indicates potential processing errors, i.e. contamination, pipetting errors, or sample mix-ups. The operator should evaluate the root cause and repeat library preparation as appropriate. If contamination is suspected, library preparation for all samples should be repeated. If pipetting errors or mix-ups are suspected, repeat library preparation for the affected samples.
 - ▶ The presence of a 500 bp band is normal and does not affect assay performance.

Figure 7 Valid Gel Results, Example

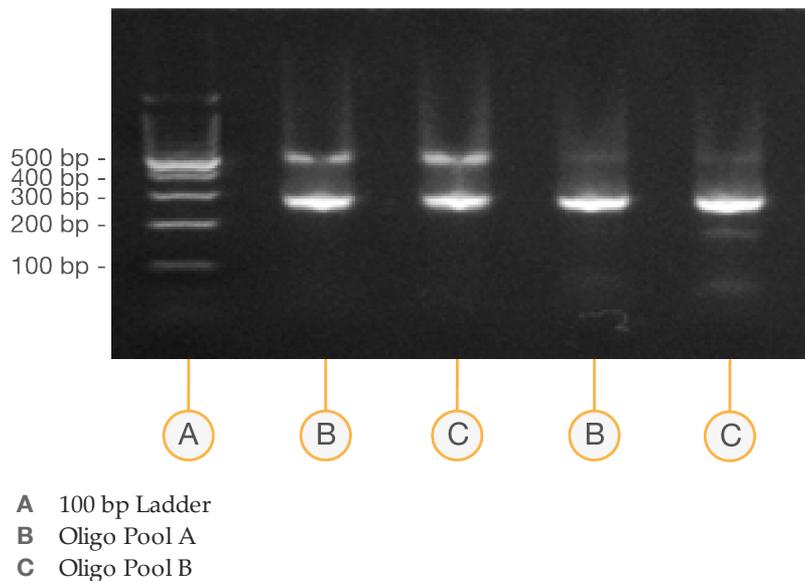
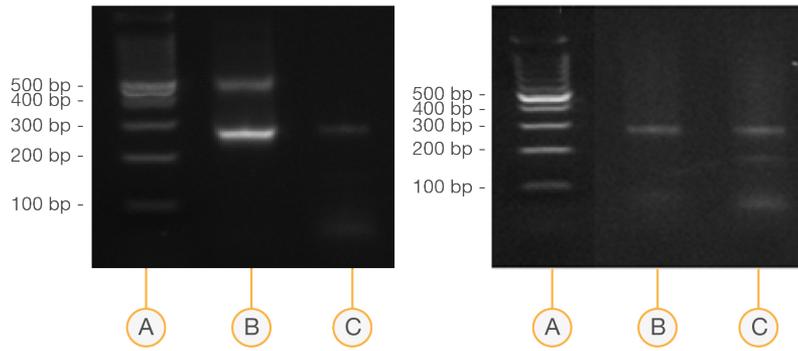
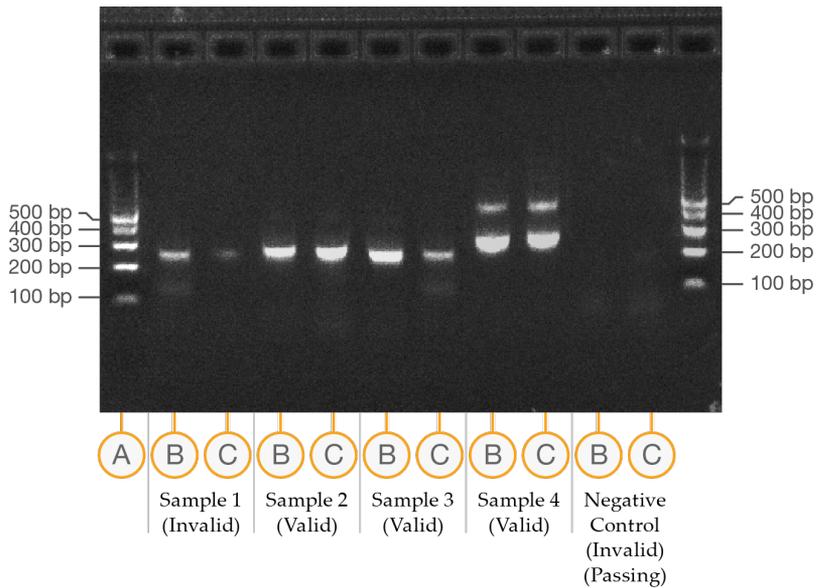


Figure 8 Invalid Gel Results, Examples



- A** 100 bp Ladder
- B** Oligo Pool A
- C** Oligo Pool B

Figure 9 Sample Outcomes, Example



- A** 100 bp Ladder
- B** Oligo Pool A
- C** Oligo Pool B

PCR Clean-Up

This process uses PCR Clean-Up Beads to purify the PCR products from the other reaction components.

Estimated Time

- ▶ Total duration: 50 minutes
- ▶ Hands-on: 20 minutes

Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|-------------------------------|------------------------------------|--------------|-------------|---------|
| Elution Buffer | 1 tube | 15°C to 30°C | Illumina | 5 |
| PCR Clean-Up Beads | 1.8 ml per 12 samples/24 libraries | 2°C to 8°C | Illumina | 3B |
| 80% Ethanol, freshly-prepared | 15 ml per 12 samples/24 libraries | 15°C to 30°C | User | — |
| 96-well MIDI plates | 2 | 15°C to 30°C | User | — |
| Adhesive plate seal | As needed | 15°C to 30°C | User | — |
| Troughs | As needed | 15°C to 30°C | User | — |

Preparation



NOTE

Please review the **Precautions** section at the beginning of this protocol regarding the handling of magnetic beads and washing with 80% ethanol during the PCR clean-up.

- 1 Bring the PCR Clean-Up Beads to room temperature.
- 2 Prepare fresh 80% ethanol from absolute ethanol. For 12 samples/24 libraries, prepare 80% ethanol using 12 mL of absolute ethanol and 3 mL of DNase/RNase-free water. Mix thoroughly.



NOTE

Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air impacting the results.

Procedure

- 1 Centrifuge the **AMP** plate at 1000 × g at room temperature for 1 minute to collect condensation.
- 2 Label a new MIDI plate "**CLP_Plate_ID**" (Clean-up Plate).
- 3 Invert PCR Clean-Up Beads 10 times. Vortex vigorously and then invert again 10 times.
- 4 Visually inspect solution to ensure that beads are resuspended well.
- 5 Using a multi-channel pipette, add 55 µl of PCR Clean-Up Beads to each well of the **CLP** plate.

- 6 Using a multi-channel pipette set to 60 μ l, transfer the entire PCR product from the **AMP** plate to the **CLP** plate. Change tips between samples.
- 7 Seal the **CLP** plate with an adhesive plate seal.
- 8 Shake the **CLP** plate on a microplate shaker at 1800 rpm for 2 minutes.
- 9 Incubate at room temperature (15°C to 30°C) without shaking for 10 minutes.
- 10 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 11 With the **CLP** plate on the magnetic stand and a multi-channel pipette set to 100 μ l, carefully remove and discard the supernatant. Change tips between samples.

**NOTE**

If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes and confirm that the supernatant has cleared.

**NOTE**

Delays during this step may lead to bead clumping following removal of the supernatant. Proceed immediately to the next step as soon as all supernatant is removed.

- 12 With the **CLP** plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
 - a Using a multi-channel pipette, add 200 μ l of freshly prepared 80% ethanol to each sample well. Changing tips is not required if you use care to avoid cross-contamination. You do not need to resuspend the beads at this time.
 - b Incubate the plate on the magnetic stand for a minimum of 30 seconds or until the supernatant is clear.
 - c Carefully remove and discard the supernatant.
- 13 With the **CLP** plate on the magnetic stand, perform a second ethanol wash as follows:
 - a Using a multi-channel pipette, add 200 μ l of freshly prepared 80% ethanol to each sample well.
 - b Incubate the plate on the magnetic stand for a minimum of 30 seconds or until the supernatant appears clear.
 - c Carefully remove and discard the supernatant.
- 14 Use a P20 multi-channel pipette set to 20 μ l to remove excess ethanol. Do not disturb or touch the beads.
- 15 Remove the **CLP** plate from the magnetic stand and allow the beads to air-dry for 5 minutes.
- 16 Using a multi-channel pipette, add 25 μ l of Elution Buffer to each sample. Changing tips is not required if you use care to avoid cross-contamination.
- 17 Seal the plate with an adhesive plate seal.
- 18 Shake the **CLP** plate on a microplate shaker at 1800 rpm for 5 minutes.
 - NOTE
Ensure all samples are completely resuspended. If there are samples in which the beads are not completely resuspended, gently pipette up and down to resuspend the beads and repeat the previous two steps.
- 19 Place the **CLP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.

- 20 Label a new MIDI plate "**LNP_Plate_ID**" (Library Normalization Plate).
- 21 Using a P20 multi-channel pipette and fine tips, carefully transfer 20 μ l of the supernatant from the **CLP** plate to the **LNP** plate. Change tips between samples to avoid cross-contamination.



NOTE

If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest on the magnet for 2 minutes and confirm that the supernatant has cleared.

- 22 [Optional] Transfer the remaining 5 μ l of supernatant from the **CLP** plate to a new plate and label the plate with a run name and date. Store this plate at -25°C to -15°C until completion of the sequencing run and data analysis. The cleaned up PCR products can be used for troubleshooting efforts in the event of sample failures.
- 23 Seal the **LNP** plate with an adhesive plate seal, and then centrifuge at 1000 \times g at room temperature for 1 minute to ensure all the supernatant is at the bottom of the well.

SAFE STOPPING POINT

After PCR Clean-Up, the plate is stable for up to 3 hours at 2°C to 8°C.

Library Normalization

This process normalizes the quantity of each library to ensure equal library representation in the pooled sample.

Estimated Time

- ▶ Total duration: 1 hour 20 minutes
- ▶ Hands-on: 30 minutes

Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|-------------------------------|-----------|----------------|-------------|---------|
| Library Normalization Diluent | 1 tube | -25°C to -15°C | Illumina | 1B |
| Library Beads | 1 tube | 2°C to 8°C | Illumina | 3B |
| Library Normalization Wash | 2 tubes | 2°C to 8°C | Illumina | 3B |
| Library Storage Buffer | 1 tube | 15°C to 30°C | Illumina | 5 |
| 0.1 N NaOH, freshly prepared | 3 ml | 15°C to 30°C | User | — |
| 96-well skirted PCR plate | 1 plate | 15°C to 30°C | User | — |
| 15 ml conical tube | 1 tube | 15°C to 30°C | User | — |
| Adhesive plate seal | As needed | 15°C to 30°C | User | — |



WARNING

This set of reagents contains potentially hazardous chemicals. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and laboratory coat appropriate for risk of exposure. Handle used reagents as chemical waste and discard in accordance with applicable regional, national, and local laws and regulations. For additional environmental, health, and safety information, see the SDS at support.illumina.com/sds.html.

Preparation

- 1 Prepare fresh 0.1 N NaOH by adding 30 µl of 10 N NaOH to 2970 µl of RNase/DNase-free water.
- 2 Remove Library Normalization Diluent from -25°C to -15°C storage and bring to room temperature. Use a 20°C to 25°C water bath as needed.



NOTE

Library Normalization Diluent might form visible precipitates or crystals. Before use, vortex vigorously, and then hold the tube in front of a light and visually inspect to make sure that all precipitate has completely dissolved.

- 3 Remove Library Beads and Library Normalization Wash from 2°C to 8°C storage and bring to room temperature.
Use a 20°C to 25°C water bath as needed.

- 4 Vortex Library Beads vigorously for 1 minute with intermittent inversion until the beads are resuspended and no pellet is found at the bottom of the tube when the tube is inverted.

Procedure

- 1 For 12 samples/24 libraries, add 1575 μl of Library Normalization Diluent to a fresh 15 ml conical tube.
- 2 To homogenize beads, use a P1000 pipette set to 1000 μl to resuspend Library Beads thoroughly by pipetting up and down 10 times.



NOTE

It is critical to completely resuspend the library bead pellet at the bottom of the tube. The use of a P1000 ensures that the beads are homogeneously resuspended and that there is no bead mass at the bottom of the tube. This is essential for achieving consistent cluster density on the flow cell.

- 3 For 12 samples/24 libraries, pipette 300 μl of Library Beads to the tube containing Library Normalization Diluent. Mix well by inverting the tube 15–20 times.



NOTE

A P1000 set to 1000 μl is required to resuspend the beads completely in step 2. Mix only the specified amounts of Library Normalization Diluent and Library Beads. You must store the remaining Library Normalization Diluent and Library Beads separately at their respective recommended temperatures. To preserve stability, Library Beads beads should never be frozen or mixed with Library Normalization Diluent if not used immediately.

- 4 Using a multi-channel pipette, add 45 μl of the combined Library Normalization Diluent/Library Beads working solution to each well of the LNP plate containing libraries. Changing tips between columns is not required if you use care to avoid cross-contamination.
- 5 Seal the LNP plate with an adhesive plate seal.
- 6 Shake the LNP plate on a microplate shaker at 1,800 rpm for 30 minutes.



NOTE

This 30-minute incubation is critical for proper library normalization. Incubations of greater or less than 30 minutes may affect library representation and cluster density.

- 7 Place the LNP plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 8 With the LNP plate on the magnetic stand, using a multi-channel pipette set to 80 μl , carefully remove and discard the supernatant in an appropriate hazardous waste container.



NOTE

If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest for 2 minutes or until the supernatant has cleared.

- 9 Remove the LNP plate from the magnetic stand and wash the beads with Library Normalization Wash as follows:
 - a Using a multi-channel pipette, add 45 μl of Library Normalization Wash to each sample well.
Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Seal the LNP plate with an adhesive plate seal.
 - c Shake the LNP plate on a microplate shaker at 1,800 rpm for 5 minutes.

- d Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
 - e Carefully remove and discard the supernatant in an appropriate hazardous waste container.
 - 10 Remove the **LNP** plate from the magnetic stand and repeat the wash with Library Normalization Wash as follows:
 - a Using a multi-channel pipette, add 45 μ l of Library Normalization Wash to each well. Changing tips between columns is not required if you use care to avoid cross-contamination.
 - b Seal the **LNP** plate with an adhesive plate seal.
 - c Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
 - d Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes.
 - e Carefully remove and discard the supernatant in an appropriate hazardous waste container.
 - f Seal the **LNP** plate with an adhesive plate seal.
 - 11 Centrifuge the **LNP** plate at $1000 \times g$ at room temperature for 30 seconds to collect residual wash buffer.
 - 12 Place the **LNP** plate on the magnetic stand.
 - 13 Use a P20 multi-channel pipette set to 20 μ l to remove excess Library Normalization Wash.
 - 14 Remove the **LNP** plate from the magnetic stand and add 30 μ l of 0.1 N NaOH to each well to elute the sample.
 - 15 Seal the **LNP** plate with an adhesive plate seal.
 - 16 Shake the **LNP** plate on a microplate shaker at 1,800 rpm for 5 minutes.
 - 17 During the 5-minute shake, label a new 96-well PCR plate **SGP_Plate_ID**.
 - 18 Add 30 μ l Library Storage Buffer to each well to be used in the **SGP** plate.
 - 19 After the 5-minute shake, ensure that all samples in the **LNP** plate are resuspended. If the samples are not resuspended, gently pipette those samples up and down or lightly tap the plate on the bench to resuspend the beads, then shake for another 5 minutes.
 - 20 [Optional] Centrifuge the **LNP** plate at $1000 \times g$ at room temperature for 30 seconds to collect contents to the bottom of the wells.
 - 21 Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes.
 - 22 Using a multi-channel pipette set to 30 μ l, transfer the supernatant from the **LNP** plate to the **SGP** plate. Gently pipette up and down 5 times to mix.
-  **NOTE**
If any beads are inadvertently aspirated into the tips, dispense the beads back to the plate and let the plate rest for 2 minutes or until the supernatant has cleared.
- 23 Seal the **SGP** plate with an adhesive plate seal and then centrifuge at $1,000 \times g$ at room temperature for 1 minute. Proceed immediately to Library Pooling.

Library Pooling

In preparation for cluster generation and sequencing, equal volumes of normalized library are combined, diluted in hybridization buffer, and heat denatured prior to sequencing on the MiSeqDx. PhiX is used as an internal control for sequencing.

Estimated Time

- ▶ Total duration: 10 minutes
- ▶ Hands-on: 10 minutes

Consumables

| Item | Quantity | Storage | Supplied By | Kit Box |
|------------------------------|-----------|----------------|-------------|---------|
| Library Dilution Buffer | 2 tubes | -25°C to -15°C | Illumina | 1B |
| PhiX Control | 2 µl | -25°C to -15°C | Illumina | 1B |
| 0.1 N NaOH, freshly prepared | 2.5 ml | 15°C to 30°C | User | — |
| 1X TE buffer | 8 µl | 15°C to 30°C | User | — |
| Microcentrifuge tubes | As needed | 15°C to 30°C | User | — |
| PCR eight-tube strip | 1 | 15°C to 30°C | User | — |
| Ice bucket | 1 | 15°C to 30°C | User | — |

Prepare for Library Pooling

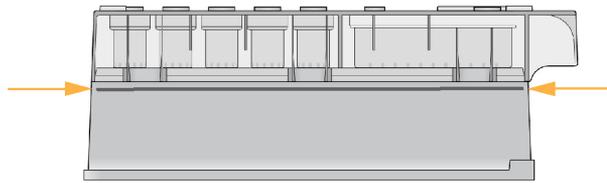
- 1 Set a heat block suitable for 1.5 ml centrifuge tubes to 96°C.
- 2 In an ice bucket, prepare an ice-water bath. Chill the Library Dilution Buffer in the ice-water bath.
- 3 Begin thawing the MiSeqDx reagent cartridge.

Prepare the Reagent Cartridge

The following instructions describe how to thaw the reagent cartridge using a room temperature water bath. This method requires approximately one hour.

- 1 Remove the reagent cartridge from -25°C to -15°C storage.
- 2 Place the reagent cartridge in a water bath containing enough room temperature deionized water to submerge the base of the reagent cartridge up to the water line printed on the reagent cartridge. Do not allow the water to exceed the maximum water line.

Figure 10 Maximum Water Line



- 3 Allow the reagent cartridge to thaw in the room temperature water bath for approximately 60-90 minutes or until completely thawed.
- 4 Remove the cartridge from the water bath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge. Make sure that no water has splashed on the top of the reagent cartridge.

Denature and Dilute PhiX Control

- 1 Prepare 0.1 N NaOH by combining the following volumes in a conical tube:
 - ▶ DNase, RNase-free water (2475 μ l)
 - ▶ Stock 10 N NaOH (25 μ l)
 - 2 Invert the tube several times to mix.
-  **CAUTION**
Using freshly diluted NaOH is essential in order to denature PhiX for cluster generation on the MiSeqDx.
- 3 Combine the following volumes to make Diluted PhiX Control:
 - ▶ PhiX Control (2 μ l)
 - ▶ 1X TE Buffer (8 μ l)
 - 4 Combine the following volumes of Diluted PhiX Control and 0.1 N NaOH in a microcentrifuge tube to make Denatured Diluted PhiX Control:
 - ▶ Diluted PhiX Control (10 μ l)
 - ▶ 0.1 N NaOH (10 μ l)
 - 5 Vortex briefly to mix the Denatured Diluted PhiX Control.
 - 6 Briefly centrifuge the Denatured Diluted PhiX Control to collect contents.
 - 7 Incubate for 5 minutes at room temperature to denature the PhiX Control into single strands.
 - 8 Combine the following volume of pre-chilled Library Dilution Buffer and Denatured Diluted PhiX Control to make PhiX/Library Dilution Buffer solution.
 - ▶ Denatured Diluted PhiX Control (20 μ l)
 - ▶ Pre-chilled Library Dilution Buffer (980 μ l)



NOTE

You can store the the PhiX/Library Dilution Buffer solution up to 3 weeks at -25°C to -15°C as single-use aliquots. After 3 weeks, cluster numbers tend to decrease.

Inspect the Reagent Cartridge

- 1 Invert the reagent cartridge ten times to mix the thawed reagents, and then inspect that all positions are thawed.

**NOTE**

It is critical that the reagents in the cartridge are thoroughly thawed and mixed to ensure proper sequencing.

- 2 Inspect reagents in positions 1, 2, and 4 to make sure that they are fully mixed and free of precipitates.
- 3 Gently tap the cartridge on the bench to reduce air bubbles in the reagents.

**NOTE**

The MiSeqDx sipper tubes go to the bottom of each reservoir to aspirate the reagents, so it is important that the reservoirs are free of air bubbles.

- 4 Place the reagent cartridge on ice or set aside at 2°C to 8°C (up to 6 hours) until ready to set up the run. For best results, proceed directly to loading the sample and setting up the run.

Prepare Samples for Sequencing

- 1 Bring Library Dilution Buffer to room temperature. Vortex Library Dilution Buffer and ensure that all precipitates have dissolved completely.
- 2 Centrifuge the **SGP** plate at 1000 × g at room temperature for 1 minute to collect condensation.
- 3 Label a fresh microcentrifuge tube "**PAL_Plate_ID**" (Pooled Amplicon Library).
- 4 Determine the samples to be pooled for sequencing. A maximum of 12 samples/24 libraries can be pooled for each sequencing run.
- 5 Transfer 10 µl of each library to be sequenced from the **SGP** plate, column by column, to a PCR eight-tube strip. Seal **SGP** with an adhesive plate seal and set aside.
- 6 Combine and transfer the contents of the PCR eight-tube strip into the **PAL** tube. Mix the **PAL** tube thoroughly.
- 7 Label 3–6 fresh microcentrifuge tubes "**DAL_Plate_ID**" (Diluted Amplicon Library).

**NOTE**

Illumina recommends creating 3 **DAL** tubes per **PAL** for future use.

- 8 Add 784 µl of Library Dilution Buffer to each **DAL** tube.
- 9 Add 8 µl of PhiX/Library Dilution Buffer solution to each **DAL** tube. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
- 10 Transfer 8 µl of **PAL** to each **DAL** tube containing Library Dilution Buffer. Using the same tip, pipette up and down 3–5 times to rinse the tip and ensure complete transfer.
- 11 Mix the **DAL** tubes by vortexing the tube at top speed.

**NOTE**

The diluted library **DAL** can be stored at -25°C to -15°C for up to 30 days.

- 12 Centrifuge the one **DAL** tube at room temperature for 1 minute to collect contents.
- 13 Using a heat block, incubate the **DAL** tube at 96°C for 2 minutes.

**WARNING**

Use caution when removing the tube. The tube and the heat block may be hot.

- 14 After the incubation, invert the **DAL** tube 1–2 times to mix, then immediately place in the ice-water bath.

- 15 Keep the **DAL** tube in the ice-water bath for 5 minutes.



NOTE

You must perform the heat denaturation step immediately before loading **DAL** into the MiSeqDx reagent cartridge to ensure efficient template loading on the MiSeqDx flow cell. Replicate **DAL** tubes must be heat denatured immediately before MiSeqDx loading.

Library Sequencing

The flow cell is washed, dried, and loaded into MiSeqDx, samples are loaded into the reagent cartridge, the reagent cartridge is loaded into MiSeqDx, and the sequencing run is started. The MiSeqDx performs cluster generation, sequencing by synthesis, and data analysis.

Estimated Time

- ▶ Total sequencing duration: ~28 hours
- ▶ Hands-on: ~5 minutes

Consumables

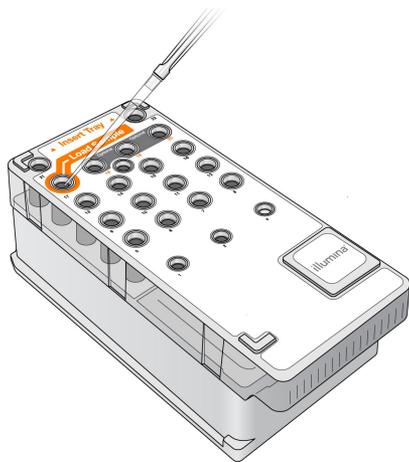
| Item | Quantity | Storage | Supplied By | Kit Box |
|----------------------------|------------------------|----------------|-------------|---------|
| MiSeqDx Cartridge | 1 pre-filled cartridge | -25°C to -15°C | Illumina | 2 |
| MiSeqDx SBS Solution (PR2) | 1 bottle | 2°C to 8°C | Illumina | 4 |
| MiSeqDx Flow Cell | 1 flow cell | 2°C to 8°C | Illumina | 3B |

Procedure

For details on the steps outlined here, see the *MiSeqDx Instrument Reference Guide* (document # 1000000021961).

- 1 Use a separate, clean, and empty 1 ml pipette tip to pierce the foil seal over the reservoir on the MiSeqDx Cartridge labeled **Load Samples**.
- 2 Pipette 600 µl of the sample DAL libraries into the **Load Samples** reservoir. Take care to avoid touching the foil seal while dispensing the sample.
- 3 Check for air bubbles in the reservoir after loading sample. If air bubbles are present, gently tap the cartridge on the bench to release the bubbles.

Figure 11 Load Libraries



- 4 Log in to the MiSeq Operating Software (MOS) using your Local Run Manager user name and password.



NOTE

If a previous user is logged in, select **Log Off** before logging in. Confirm that the proper username or login credentials are displayed.

- 5 From the Home screen, select **Sequence**.
- 6 Select a run from the list.
- 7 Confirm the run settings.
- 8 Clean the flow cell.
- 9 Load the flow cell.
- 10 Empty the waste bottle and load the MiSeqDx SBS Solution (PR2) bottle.
- 11 Load the reagent cartridge.
- 12 The run automatically starts after passing pre-run checks.
- 13 Perform a post-run wash.

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Website: www.illumina.com
Email: techsupport@illumina.com

Illumina Customer Support Telephone Numbers

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|-----------------|-----------------|----------------|
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| Austria | +43 800006249 | +43 19286540 |
| Belgium | +32 80077160 | +32 34002973 |
| China | 400.635.9898 | |
| Denmark | +45 80820183 | +45 89871156 |
| Finland | +358 800918363 | +358 974790110 |
| France | +33 805102193 | +33 170770446 |
| Germany | +49 8001014940 | +49 8938035677 |
| Hong Kong | 800960230 | |
| Ireland | +353 1800936608 | +353 016950506 |
| Italy | +39 800985513 | +39 236003759 |
| Japan | 0800.111.5011 | |
| Netherlands | +31 8000222493 | +31 207132960 |
| New Zealand | 0800.451.650 | |
| Norway | +47 800 16836 | +47 21939693 |
| Singapore | +1.800.579.2745 | |
| Spain | +34 911899417 | +34 800300143 |
| Sweden | +46 850619671 | +46 200883979 |
| Switzerland | +41 565800000 | +41 800200442 |
| Taiwan | 00806651752 | |
| United Kingdom | +44 8000126019 | +44 2073057197 |
| Other countries | +44.1799.534000 | |

Safety data sheets (SDSs)—Available on the Illumina website at support.illumina.com/sds.html.

Product documentation—Available for download in PDF from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.



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