

# Praxis™ Extended RASPanel

FOR *IN VITRO* DIAGNOSTIC USE

Catalog # 20012431: 2 Runs, up to 10 samples plus 2 controls per run/20 samples per kit

## 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.

## Summary and Explanation of the Assay

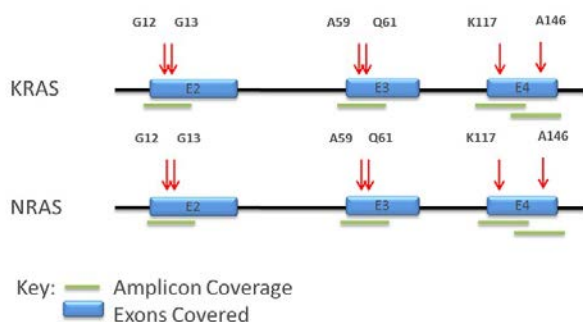
### Clinical Description

Mutations in the RAS oncogenes are frequently found in human cancers. These mutations activate the mitogen-activated protein kinase pathway contributing to the malignant phenotype. Historically, *KRAS* exon 2 (codon 12 and 13) mutations were an established predictive biomarker of resistance to anti-epidermal growth factor receptor (EGFR) therapy for metastatic colorectal cancer (mCRC). Multiple clinical trials have since established the value of extended RAS testing to include exon 2 (codons 12 and 13), exon 3 (codons 59 and 61), and exon 4 (codons 117 and 146) of either *KRAS*, or *NRAS*.<sup>1</sup> Overall, extended RAS mutations (*KRAS* exons 3, 4 and *NRAS* exons 2, 3, 4) account for 11% of patients with mCRC.<sup>1</sup> Taken together, mutations in *NRAS* and *KRAS* may affect up to approximately 53% of patients.<sup>1,2,3</sup> The clinical benefit of Vectibix (panitumumab), a fully human anti-EGFR antibody, is restricted to patients who have no tumor mutations in extended RAS.

### RAS Mutations in Panel

The Praxis Extended RAS Panel is an *in vitro* diagnostic test used in the detection of specific mutations in exons 2, 3, and 4 of *KRAS* and *NRAS*. [Figure 1](#) depicts the general locations of the mutations within the exons of the genes. Codon changes with red arrows show location of mutations targeted in the assay.

**Figure 1** RAS Genes (*KRAS* and *NRAS*) and Targeted Sequencing



The assay is designed specifically for the detection of the mutations from the *KRAS* and *NRAS* genes listed in [Table 1](#). The assay simultaneously determines the presence or absence of each of the 56 mutations in every sequenced sample.

**Table 1** Praxis Extended RAS Panel Summary of Mutations\*

| Chromosome | Gene | Exon | Amino Acid  | Wild Type > Mutation |
|------------|------|------|-------------|----------------------|
| chr12      | KRAS | 2    | p.Gly13Val  | c.38_39GC>TT         |
| chr12      | KRAS | 2    | p.Gly13Glu  | c.38_39GC>AA         |
| chr12      | KRAS | 2    | p.Gly13Asp  | c.38_39GC>AT         |
| chr12      | KRAS | 2    | p.Gly13Asp  | c.38G>A              |
| chr12      | KRAS | 2    | p.Gly13Cys  | c.37G>T              |
| chr12      | KRAS | 2    | p.Gly13Arg  | c.37G>C              |
| chr12      | KRAS | 2    | p.Gly12Trp  | c.34_36GGT>TGG       |
| chr12      | KRAS | 2    | p.Gly12Ala  | c.35G>C              |
| chr12      | KRAS | 2    | p.Gly12Asp  | c.35G>A              |
| chr12      | KRAS | 2    | p.Gly12Phe  | c.34_35GG>TT         |
| chr12      | KRAS | 2    | p.Gly12Asn  | c.34_35GG>AA         |
| chr12      | KRAS | 2    | p.Gly12Val  | c.35G>T              |
| chr12      | KRAS | 2    | p.Gly12Ser  | c.34G>A              |
| chr12      | KRAS | 2    | p.Gly12Cys  | c.34G>T              |
| chr12      | KRAS | 2    | p.Gly12Arg  | c.34G>C              |
| chr12      | KRAS | 3    | p.Gln61His  | c.183A>C             |
| chr12      | KRAS | 3    | p.Gln61His  | c.183A>T             |
| chr12      | KRAS | 3    | p.Gln61Leu  | c.182A>T             |
| chr12      | KRAS | 3    | p.Gln61Arg  | c.182A>G             |
| chr12      | KRAS | 3    | p.Gln61Lys  | c.181C>A             |
| chr12      | KRAS | 3    | p.Gln61Glu  | c.181C>G             |
| chr12      | KRAS | 3    | p.Ala59Gly  | c.176C>G             |
| chr12      | KRAS | 3    | p.Ala59Thr  | c.175G>A             |
| chr12      | KRAS | 4    | p.Ala146Val | c.437C>T             |
| chr12      | KRAS | 4    | p.Ala146Thr | c.436G>A             |
| chr12      | KRAS | 4    | p.Ala146Pro | c.436G>C             |
| chr12      | KRAS | 4    | p.Lys117Asn | c.351A>T             |
| chr12      | KRAS | 4    | p.Lys117Asn | c.351A>C             |
| chr1       | NRAS | 2    | p.Gly13Glu  | c.38_39GT>AG         |
| chr1       | NRAS | 2    | p.Gly13Glu  | c.38_39GT>AA         |
| chr1       | NRAS | 2    | p.Gly13Asp  | c.38G>A              |
| chr1       | NRAS | 2    | p.Gly13Val  | c.38G>T              |
| chr1       | NRAS | 2    | p.Gly13Cys  | c.37G>T              |
| chr1       | NRAS | 2    | p.Gly13Arg  | c.37G>C              |
| chr1       | NRAS | 2    | p.Gly12Trp  | c.34_36GGT>TGG       |
| chr1       | NRAS | 2    | p.Gly12Ala  | c.35G>C              |
| chr1       | NRAS | 2    | p.Gly12Val  | c.35G>T              |
| chr1       | NRAS | 2    | p.Gly12Asp  | c.35G>A              |
| chr1       | NRAS | 2    | p.Gly12Phe  | c.34_35GG>TT         |

| Chromosome | Gene        | Exon | Amino Acid  | Wild Type > Mutation |
|------------|-------------|------|-------------|----------------------|
| chr1       | <i>NRAS</i> | 2    | p.Gly12Asn  | c.34_35GG>AA         |
| chr1       | <i>NRAS</i> | 2    | p.Gly12Ser  | c.34G>A              |
| chr1       | <i>NRAS</i> | 2    | p.Gly12Cys  | c.34G>T              |
| chr1       | <i>NRAS</i> | 2    | p.Gly12Arg  | c.34G>C              |
| chr1       | <i>NRAS</i> | 3    | p.Gln61His  | c.183A>T             |
| chr1       | <i>NRAS</i> | 3    | p.Gln61His  | c.183A>C             |
| chr1       | <i>NRAS</i> | 3    | p.Gln61Arg  | c.182A>G             |
| chr1       | <i>NRAS</i> | 3    | p.Gln61Leu  | c.182A>T             |
| chr1       | <i>NRAS</i> | 3    | p.Gln61Lys  | c.181C>A             |
| chr1       | <i>NRAS</i> | 3    | p.Gln61Glu  | c.181C>G             |
| chr1       | <i>NRAS</i> | 3    | p.Ala59Gly  | c.176C>G             |
| chr1       | <i>NRAS</i> | 3    | p.Ala59Thr  | c.175G>A             |
| chr1       | <i>NRAS</i> | 4    | p.Ala146Val | c.437C>T             |
| chr1       | <i>NRAS</i> | 4    | p.Ala146Thr | c.436G>A             |
| chr1       | <i>NRAS</i> | 4    | p.Ala146Pro | c.436G>C             |
| chr1       | <i>NRAS</i> | 4    | p.Lys117Asn | c.351G>C             |
| chr1       | <i>NRAS</i> | 4    | p.Lys117Asn | c.351G>T             |

\* *KRAS* proto-oncogene, GTPase [ Homo sapiens (human) ] (Gene ID: 3845) - NM\_004985.4, NP\_004976.2.

\* *NRAS* neuroblastoma RAS viral oncogene homolog [ Homo sapiens (human) ] (Gene ID: 4893) - NM\_002524.4, NP\_002515.1.

Testing for *KRAS* and *NRAS* mutations is recommended by the NCCN Guidelines® for all patients with mCRC. Only patients who have wild type RAS are eligible for anti-EGFR therapy<sup>4</sup>.

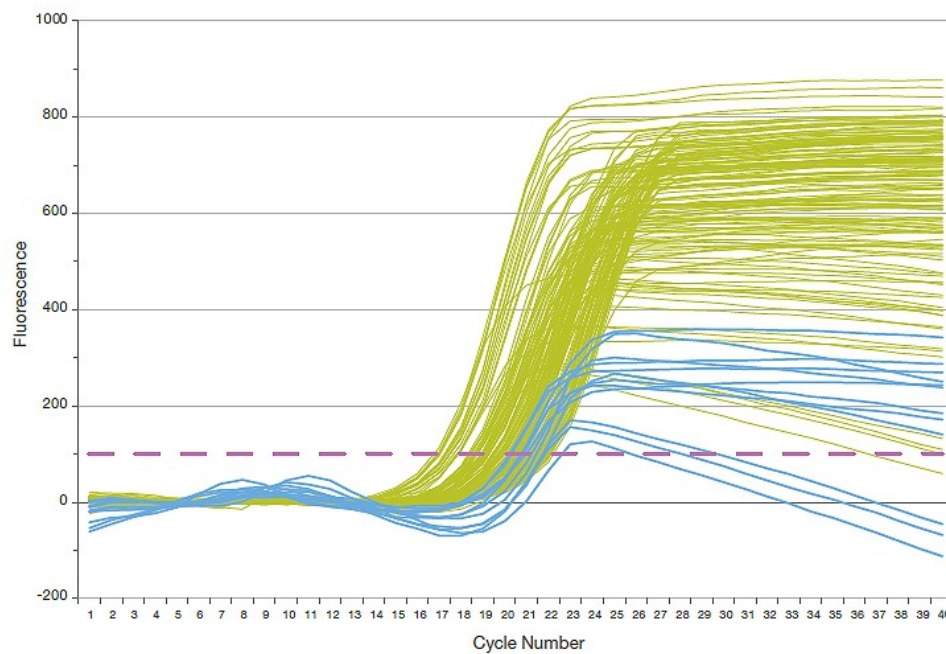
## Principles of Procedure

The Illumina Praxis Extended RAS Panel involves three main procedures. The first is to qualify the DNA sample that can be used for the test (Figure 2). The second is to manually prepare the samples for sequencing, which is called library preparation. Library preparation consists of four key steps: Hybridization, Extension-Ligation, PCR Amplification, and Library Normalization (Figure 3). The third procedure is to sequence the prepared sample using SBS (sequencing by synthesis) chemistry on the MiSeqDx.

### Qualification of FFPE Samples

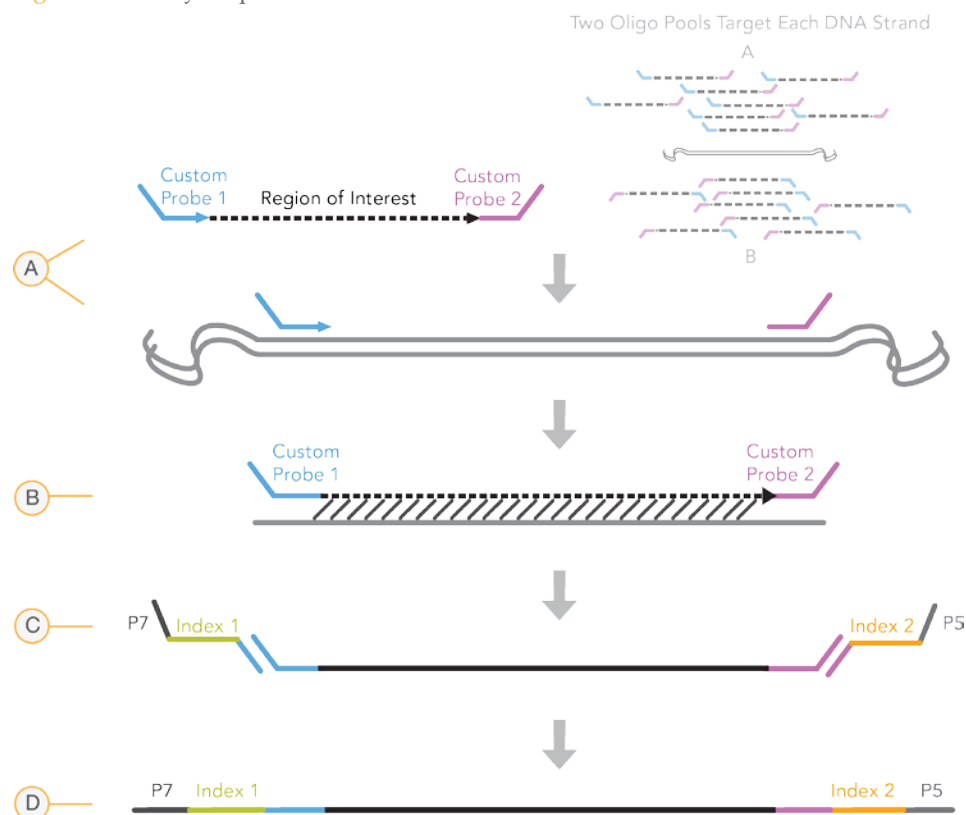
The Praxis Extended RAS Panel workflow starts with DNA qualification step which is performed to determine if the DNA is of sufficient quality for use with the Praxis Extended RAS Panel. Genomic DNA samples are qualified by measuring amplifiability relative to a control DNA template.

Figure 2 Amplification Graph



## Library Preparation

Figure 3 Library Preparation Process



**A Hybridization**—A pool of upstream and downstream oligonucleotides specific to the targeted regions in the Praxis Extended RAS Panel is hybridized to the sample genomic DNA. Two oligonucleotide pools (A and B) independently test each strand of DNA. At the end of this process, a three-step wash procedure with a filter

capable of size selection removes unbound oligonucleotides from the genomic DNA.

- B Extension-Ligation**—The second step, Extension-Ligation, connects the hybridized upstream and downstream oligonucleotides. A DNA polymerase extends from the upstream oligonucleotides through the targeted region, followed by ligation to the 5' end of the downstream oligonucleotide using a DNA ligase. The result is the formation of products that contain the RAS-specific oligonucleotides flanked by sequences required for amplification.
- C PCR Amplification**—The third step, PCR Amplification, amplifies the extension-ligation products using primers that add index sequences for sample multiplexing, as well as common adapters required for cluster generation on the MiSeqDx. At the end of this process, a PCR clean-up procedure purifies the PCR products (referred to as a library).
- D Library Normalization**—The final step, Library Normalization, normalizes the quantity of each library to ensure more equal library representation in the final pooled library. At the end of this process, the pooled library is loaded onto the MiSeqDx for sequencing using SBS chemistry.

## Sequencing

SBS chemistry uses a reversible-terminator method to detect single nucleotide bases as they are incorporated into growing DNA strands. During each sequencing cycle, a single fluorescently-labeled deoxynucleotide triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide. Because all four-reversible terminator-bound dNTPs (A, G, T, C) are present as single, separate molecules, natural competition minimizes incorporation bias. Base calls are made directly from signal intensity measurements during each sequencing cycle. The result is base-by-base sequencing.

## Data Analysis

The Praxis Extended RAS Panel module of the Local Run Manager software processes base calls generated during primary analysis and produces information about each sample, called secondary analysis. Secondary analysis includes demultiplexing, FASTQ file generation, alignment, variant calling, and report generation.

- } **Demultiplexing**— This is the first step in secondary analysis. Demultiplexing separates data from pooled samples based on the unique sequence indexes that were added during the PCR amplification step.
- } **FASTQ File Generation**—After demultiplexing, the Praxis Extended RAS Panel module generates intermediate files in the FASTQ format, which is a text format used to represent sequences. FASTQ files contain the reads for each sample and the quality scores, excluding reads from any clusters that did not pass filter.
- } **Alignment**—Alignment compares sequences against the reference to identify a relationship between the sequences and assigns a score based on regions of similarity. Aligned reads are written to files in BAM format. Local Run Manager uses a banded Smith-Waterman algorithm that performs local sequence alignments to determine similar regions between two sequences.
- } **Variant Calling**—Developed by Illumina, the somatic variant caller identifies mutations present at low frequency in the DNA sample. In order to minimize false positives due to FFPE artifacts, mutations are first called for each pool separately. Then, mutations from the two pools are compared and combined into a single output file. If found, only mutations listed in [Table 1](#) are reported. For more information, see the *Praxis Extended RAS Panel Reference Guide (document # 15054440)*.
- } **Report Generation**—Results are accessible through Local Run Manager in the Samples and Results tab for the run, and the following simple-text output file is generated:
  - } **Extended RAS Panel Report**—Details whether each sample contains any of the mutations listed in the reference panel.

## Limitations of the Procedure

- 1 For *in vitro* diagnostic use.
- 2 In this procedure, the **Extended RAS Panel Report** only determines the presence or absence of the *NRAS* and *KRAS* mutations listed in [Table 1](#).
- 3 In a study assessing 685 banked specimens, 25% failed to meet the QC requirement of dCq≤5.0.

- 4 Neoplastic content is required to be  $\geq 50\%$  in order for a FFPE tissue to be processed for sequencing.

## Product Components

The Illumina MiSeqDx Platform consists of the following:

- } Praxis Extended RAS Panel (Catalog # 20012431)
- } MiSeqDx Instrument (Catalog # DX-410-1001)

## Reagents

### Reagents Provided

Illumina provides the following reagents for the Illumina Praxis Extended RAS Panel ([Table 2–Table 10](#)). Catalog # 20012431 has been configured for 2 runs with a maximum of 10 samples plus 2 controls per run.

#### Praxis Extended RAS Panel, Box 1

**Table 2** 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 $\mu$ l | PCR primers with index sequences and sequencing adapters   | -25°C to -15°C |
| Index Primers 1-12 (A701-A712) | 1 tube per primer | 128 $\mu$ l | PCR primers with index sequences and sequencing adapters   | -25°C to -15°C |
| PCR Polymerase                 | 1 tube            | 56 $\mu$ l  | 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 3** 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 $\mu$ l  | Buffered aqueous solution containing PhiX genomic DNA                        | -25°C to -15°C |

#### Praxis Extended RAS Panel, Box 2

**Table 4** 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 5 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 6 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 7 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 8 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 9 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 10** 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

## Storage and Handling

- 1 Room temperature is defined as 15°C to 30°C.
- 2 Reagents are provided in sufficient quantity for 2 separate uses of a maximum of 12 samples/24 libraries each, including controls. Between uses, reagents should be recapped and returned to their original storage temperatures, unless otherwise indicated as single use only.
- 3 The following reagents are shipped frozen and are stable when stored at -25°C to -15°C until the specified expiration date.
  - } Oligo Pool A—Extended RAS
  - } Oligo Pool B—Extended RAS
  - } Hybridization Buffer
  - } Extension-Ligation Mix
  - } Index Primers A-H (A501-A508)
  - } Index Primers 1-12 (A701-A712)
  - } PCR Polymerase
  - } PCR Master Mix
  - } Library Normalization Diluent
  - } Library Dilution Buffer
  - } PhiX Control
  - } MiSeqDx Cartridge
  - } QC Primers
  - } QC Template



- } Panel Control—Extended RAS

- } qPCR Master Mix

Except for the reagent cartridge, the reagents above are stable for a maximum of 5 freeze/thaw cycles that occur before the specified expiration date.

Index primers and the MiSeqDx Cartridge are single-use only.



**CAUTION**

Do not store reagents in a frost-free storage unit or store in door compartments.

Do not refreeze the reagent cartridge after it has been thawed. It can be stored up to 6 hours at 2°C to 8°C.

- 4 The following reagents are shipped refrigerated and are stable when stored at 2°C to 8°C until the specified expiration date.
  - } Stringent Wash Buffer
  - } Universal Wash Buffer
  - } PCR Clean-Up Beads
  - } Library Beads
  - } Library Normalization Wash
  - } MiSeqDx SBS Solution (PR2)
  - } MiSeqDx Flow Cell

SBS Solution and Flow Cell are single-use only.
- 5 The following reagents are shipped ambient and are stable when stored at room temperature until the specified expiration date:
  - } Elution Buffer
  - } Filter Plate
  - } Library Storage Buffer

Filter plate is single-use only.
- 6 Changes in the physical appearance of the reagents provided can indicate deterioration of the materials. If changes in the physical appearance occur (e.g., obvious changes in reagent color or cloudiness apparent with microbial contamination), do not use the reagents.
- 7 The Hybridization Buffer, Stringent Wash Buffer, and Library Normalization Diluent reagents might form visible precipitates or crystals. Before use, vortex vigorously, and then visually inspect to ensure that no precipitates are present.
- 8 Adhere to the following best practices when handling PCR Clean-Up Beads and Library Beads:
  - } The beads should never be frozen.
  - } Allow the beads to reach room temperature.
  - } Immediately before use, vortex the beads until well-suspended and color appears homogeneous.
  - } Thoroughly mix the sample after the beads are added by pipetting up and down 10 times. Alternatively, use a shaker to thoroughly mix samples.
  - } Incubate the bead/sample mixture at room temperature for the entire duration indicated.
  - } Follow instructions when using the magnetic stand. Wait for the solution to clear before aspirating. Keep the plate on the magnetic stand when slowly aspirating the supernatant, taking care not to disturb the separated beads.
- 9 The PCR amplification plate can remain on the thermal cycler overnight, or it can be stored at 2°C to 8°C for up to 2 days. Before storing the plate at 2°C to 8°C, seal the plate well.



**CAUTION**

Do not freeze the Library Beads or mix with the Library Normalization Diluent reagent if not used immediately.

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

## 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 [DNA Extraction on page 12](#).
- 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: ±0.5°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: ±0.3°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 × 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: ±0.1°C at 37°C; ±0.4°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

## Specimen Collection, Transport, and Storage

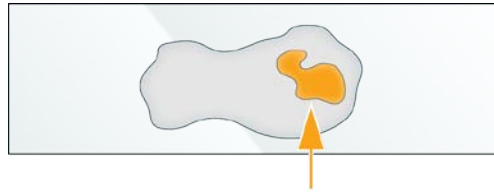
### Tissue Assessment

The Praxis Extended RAS Panel utilizes DNA samples extracted from formalin-fixed paraffin-embedded (FFPE) colorectal cancer (CRC) tissue samples.

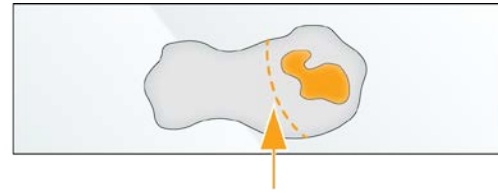
To prepare FFPE CRC tissue samples for DNA extraction:

- 1 Scrape excess paraffin away from the tissue using a fresh, sterile scalpel. Use serial sections for DNA extraction.
- 2 Use sections with ≥ 50% neoplastic content by area for processing without macrodissection.
- 3 For sections that are less than 50% neoplastic content by area, enrich neoplastic content by macrodissecting the sections to obtain ≥ 50% neoplastic content by area. In order to maximize total tissue area captured per slide, macrodissecting to obtain 50% neoplastic content by area is recommended.

**Figure 4** Maximizing Total Tissue Area Captured per Slide During Macrodissection



20% neoplastic content by area.



Macrodissect to obtain  $\geq 50\%$  neoplastic content by area, while maximizing total tissue area captured per slide.

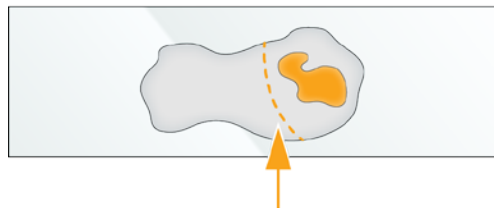
- 4 Cumulative tissue area is the total tissue area per section times the total number of 5  $\mu\text{m}$  sections. For optimal results, recommended cumulative tissue area using 5  $\mu\text{m}$  sections is 240  $\text{mm}^2$  or greater with  $\geq 50\%$  neoplastic content by area. For optimal yield of amplifiable DNA, use at least  $8 \times 5 \mu\text{m}$  sections or equivalent alternate thickness serial sections.



**NOTE**

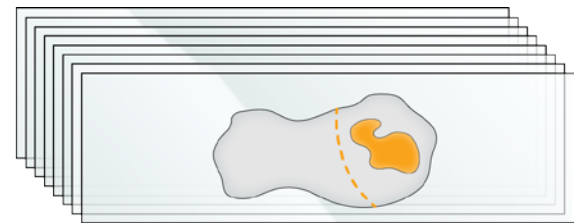
If macrodissection was performed, the macrodissected tissue area should be used in the determination of cumulative tissue area.

**Figure 5** Recommended Cumulative Tissue Area is  $\geq 240 \text{ mm}^2$



**Per Slide as Required:**

Macrodissect to obtain  $\geq 50\%$  neoplastic content by area, while maximizing total tissue area captured per slide.

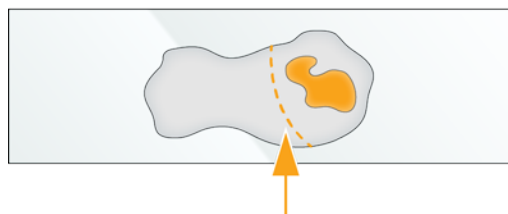


**Minimum Cumulative Tissue Area:**

For 5  $\mu\text{m}$  sections,  $\geq 240 \text{ mm}^2$  cumulative tissue area with  $\geq 50\%$  neoplastic content by area. For optimal DNA yield, use at least eight slides.

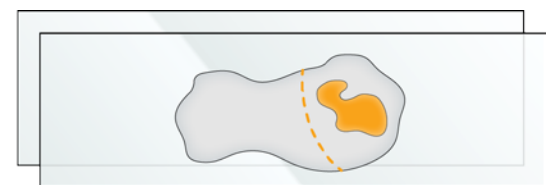
- 5 Minimum cumulative tissue area using 5  $\mu\text{m}$  sections is 80  $\text{mm}^2$  with  $\geq 50\%$  neoplastic content by area. Equivalent cumulative tissue area for alternate thickness serial sections can also be used.

**Figure 6** Minimum Cumulative Tissue Area is 80  $\text{mm}^2$



**Per Slide as Required:**

Macrodissect to obtain  $\geq 50\%$  neoplastic content by area, while maximizing total tissue area captured per slide.



**Recommended Cumulative Tissue Area:**

For 5  $\mu\text{m}$  sections, 80  $\text{mm}^2$  cumulative tissue area with  $\geq 50\%$  neoplastic content by area.

- 6 Extracted DNA samples giving a delta Cq value  $\leq 5.00$  can be used for the Praxis Extended RAS Panel.
- 7 [Optional] If extracted DNA samples do not meet the delta Cq threshold, additional sections can be used for extraction if available.

## DNA Extraction

- } Illumina recommends column-based DNA extraction kits, using double the amount of Proteinase K, overnight Proteinase K incubations with agitation, and final elutions in at least 30  $\mu\text{l}$  volume.
- } Bead-based extraction methods and methods using only lysis of crude cell extracts are not recommended for use with this assay.

- } Illumina recommends storing extracted genomic DNA between 2°C and 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.

## Warnings and Precautions



### CAUTION

Federal law restricts this device to sale by or on the order of a physician or other practitioner licensed by the law of the State in which he/she practices, to use or order the use of the device.

- 1 **Some components of this assay contain 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](http://support.illumina.com/sds.html). (See [Reagents on page 6](#) for more information.)
- 2 Handle all specimens as if they are potentially infectious agents.
- 3 Failure to follow the procedures as outlined may result in erroneous results or significant reduction in sample quality.
- 4 Use routine laboratory precautions. Do not pipette by mouth. Do not eat, drink, or smoke in designated work areas. Wear disposable gloves and laboratory coats when handling specimens and assay reagents. Wash hands thoroughly after handling specimens and assay reagents.
- 5 Do not use any assay components beyond their stated expiration date on the assay carton label. Do not interchange assay components from different assay lots. Assay lots are identified on the assay carton label.
- 6 Store the assay components at the specified temperature in designated pre-amplification and post-amplification areas.
- 7 Repeated freeze-thaw cycles (up to 5) of the Box 1 and Box 6 components will not compromise the integrity of the assay.
- 8 Index primers are single use only and should be discarded after use.
- 9 To prevent sample or reagent degradation, ensure that all sodium hypochlorite vapors have fully dissipated before starting the protocol.
- 10 Proper laboratory practices and good laboratory hygiene is required to prevent PCR products from contaminating reagents, instrumentation, and genomic DNA samples. PCR contamination may cause inaccurate and unreliable results.
- 11 To prevent contamination, ensure that pre-amplification and post-amplification areas have dedicated equipment (e.g., pipettes, pipette tips, vortexer, and centrifuge).
- 12 Avoid cross contamination. Use fresh pipette tips between samples and between dispensing reagents. Mix samples with a pipette and centrifuge the plate when indicated. Do not vortex the plates. Using aerosol-resistant tips reduces the risk of amplicon carry-over and sample-to-sample cross-contamination.
- 13 Index-sample pairing must match the printed plate layout exactly. Mismatches between the printed plate layout and the sample will result in loss of positive sample identification and incorrect result reporting.
- 14 Always prepare fresh 80% ethanol for wash steps. Ethanol can absorb water from the air, impacting results.
- 15 Ensure that all ethanol is removed from the bottom of the wells during wash steps. Residual ethanol may impact results.
- 16 Adhere to the specified drying time following the magnetic stand step to ensure complete evaporation. Residual ethanol can impact the performance of subsequent reactions.
- 17 Do not mix the Oligo Pool A and Oligo Pool B and Hybridization Buffer for storage. When combined, the Oligo Pools become unstable, even when stored frozen.
- 18 The use of thermal cyclers with active cooling (e.g., Peltier, thermoelectric cooled) is not recommended for the hybridization step. The passive cooling step is critical for proper hybridization.
- 19 Always add PCR Polymerase to the PCR Master Mix immediately before use. Never store the combined working solution.
- 20 During the library normalization step, it is critical to resuspend the library bead pellet. This is essential for achieving consistent cluster density on the MiSeqDx flow cell.

- 21 Adhere to the specified incubation times in the library normalization step. Improper incubation can affect library representation and cluster density.
- 22 Due to the number of plate transfers and subsequent potential for contamination, extreme care should be taken to ensure that well contents remain fully in the well. Do not splash contents.
- 23 DNA 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.

## Index Primer Sequences

**Table 11** 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 12** 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 |

## Instructions for Use

### Qualification of DNA Extracted from FFPE Samples

#### Preparation

- 1 Bring the QC Template, QC Primers, qPCR Master Mix, and genomic DNA to room temperature.
- 2 Place thawed tubes on ice.



#### NOTE

If using QC Template for the first time, aliquot 7 µl 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.



**NOTE**

Samples, QC Template, and No Template Control (NTC) all must be tested in triplicate. See the *Praxis Extended RAS Panel Reference Guide* (document # 15054440) for more information on plate layouts.

- 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.
- 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 as follows for a 384-well or 96-well format. The master mix contains extra volume.
  - } 384-Well Format
    - } qPCR Master Mix: 5.0 µl/well (205 µl for 36 reactions)
    - } QC Primers: 0.8 µl/well (33 µl for 36 reactions)
    - } RNase/DNase-free Water: 2.2 µl/well (90 µl for 36 reactions)
  - } 96-Well Format
    - } qPCR Master Mix: 10.0 µl/well (410 µl for 36 reactions)
    - } QC Primers: 1.6 µl/well (66 µl for 36 reactions)
    - } RNase/DNase-free Water: 4.4 µl/well (180 µl for 36 reactions)

**NOTE**

See the *Praxis Extended RAS Panel Reference Guide* (document # 15054440) for more information on preparing the qPCR Master Mix reaction.

- 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<sub>q</sub> 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. On most systems, the QC Template should amplify at a C<sub>q</sub> of approximately 15–22 cycles. Exclude replicates from a triplicate group that are > 0.5 C<sub>q</sub> different from the rest of the group. At least two of the three replicates less than or equal to 0.5 C<sub>q</sub> 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.

**NOTE**

See the *Praxis Extended RAS Panel Reference Guide* (document # 15054440) for more information on appropriate amplification curves.

- 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 the *Praxis Extended RAS Panel Reference Guide* (document # 15054440).
- 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.

## 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

For more information on the Local Run Manager software, see the *Local Run Manager Software Reference Guide for MiSeqDx* (document # 1000000011880) and the *Local Run Manager Praxis Extended RAS Panel Analysis Module Workflow Guide* (document # 1000000011875).

- 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.

### 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.
- 3 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 |

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

## Hybridization of Oligonucleotide Pool

### 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 Bring the Oligo Pool A, Oligo Pool B, Hybridization Buffer, genomic DNA samples, and Panel Control sample to 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.
- 5 Set a 96-well heat block to 95°C.
- 6 Pre-heat an incubator to 37°C.
- 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.

### Procedure

- 1 Set out a new 96-well PCR plate (hereafter referred to as **HYB** plate).



#### NOTE

See the *Praxis Extended RAS Panel Reference Guide* (document # 15054440) for more information on suggested **HYB** plate layout.

- 2 Add 10 µl of TE Buffer to each of the Negative Control (No Template Control) wells 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 Add 10 µl of diluted Panel Control prepared in the previous step to each of the two Positive Control wells for Oligo Pool A and Oligo Pool B.
- 5 Dilute genomic DNA samples (if necessary) according to the recommendations in [Table 13](#).

Table 13 Delta C<sub>q</sub> Dilution Table

| Delta C <sub>q</sub> | < -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 |

- Add 10 µl of each genomic DNA sample (diluted as necessary) to the appropriate wells for Oligo Pool A and Oligo Pool B.

**NOTE**

For information on diluting your samples and the suggested plate layout for correct Oligo Pool A and Oligo Pool B well selection, see the *Praxis Extended RAS Panel Reference Guide* (document # 15054440).

- Add 5 µl of the Oligo Pool A to all Oligo Pool A wells containing genomic DNA, Panel Control, and Negative Control. Then, add 5 µl of the Oligo Pool B to all Oligo Pool B wells containing genomic DNA, Panel Control, and Negative Control.
- Add 40 µl of Hybridization Buffer to each sample in the **HYB** plate. Gently pipette up and down 3–5 times to mix.

**NOTE**

Ensure any crystals or precipitate in Hybridization Buffer have dissolved.

- Seal the **HYB** plate and centrifuge 1000 × g at room temperature for 1 minute.
- Place the **HYB** plate in the pre-heated block at 95°C and incubate for 1 minute.
- 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

### Preparation

- Bring Extension-Ligation Mix, Stringent Wash Buffer, and Universal Wash Buffer to room temperature, and then vortex briefly.
- Assemble the filter plate assembly (hereafter referred to as the **FPU**) in order from bottom to top: MIDI plate, adapter collar, filter plate, and lid.
- Pre-wash the filter plate membrane as follows:
  - Add 50 µl of Stringent Wash Buffer to each well.
  - Cover the filter plate with the lid and centrifuge at 2400 × g at room temperature for 5 minutes.
  - If the wash buffer does not drain completely, centrifuge the filter plate again at 2400 × g at room temperature for 5 minutes.

### Procedure

- Remove the **HYB** plate from the heat block and centrifuge at 1000 × g at room temperature for 1 minute.
- Transfer the entire volume (approximately 65 µl) of each sample to the corresponding wells of the filter plate.
- Cover the filter plate with the lid and centrifuge at 2400 × g at room temperature for 5 minutes.

**NOTE**

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

- Wash the filter plate as follows:
  - Add 50 µl of Stringent Wash Buffer to each sample well.
  - Cover the filter plate with the lid and centrifuge at 2400 × g at room temperature for 5 minutes.
  - If the wash buffer does not drain completely, centrifuge the filter plate again at 2400 × g at room temperature for 5 minutes.
- Repeat the wash as described in the previous step.

**NOTE**

If the wash buffer does not drain completely, centrifuge again at  $2400 \times g$  at room temperature until all liquid has gone through (an additional 5–10 minutes).

- 6 Discard all the flow-through (containing formamide), then reassemble the **FPU**.
- 7 Add 45  $\mu\text{l}$  of Universal Wash Buffer to each sample well.
- 8 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.

## Extension-Ligation of Bound Oligonucleotides

### Procedure

- 1 Add 45  $\mu\text{l}$  of Extension-Ligation Mix to each sample well of the filter plate.
- 2 Seal the filter plate with adhesive aluminum foil, and then cover with the lid.
- 3 Incubate the **FPU** in the pre-heated  $37^\circ\text{C}$  incubator for 45 minutes.
- 4 While the **FPU** plate is incubating, prepare the AMP (Amplification Plate) as described in the following section.

## PCR Amplification

### Preparation

- 1 Prepare fresh 0.05 N NaOH by adding 25  $\mu\text{l}$  of 10 N NaOH to 4975  $\mu\text{l}$  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 Bring PCR Master Mix and the appropriate index primers to room temperature. Vortex each thawed tube to mix, and then briefly centrifuge the tubes.
- 4 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.
- 5 Set out a new 96-well PCR plate (hereafter referred to as the **AMP** plate).
- 6 Add index primers to the AMP plate as follows:
  - a Add 9  $\mu\text{l}$  of the selected index primers [A–H (A501–A508)] to the appropriate well in a column of the **AMP** plate.
  - b Add 9  $\mu\text{l}$  of the selected index primers [1–12 (A701–A712)] to the appropriate row of the **AMP** plate.

*Tips must be changed after each row to avoid index cross-contamination.*

**NOTE**


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

- 7 Prepare the PCR Master Mix/PCR Polymerase PCR working solution as follows:
  - a For 12 samples/24 libraries, add 21  $\mu\text{l}$  of PCR Polymerase to 1050  $\mu\text{l}$  of PCR Master Mix.
  - b Invert the prepared PCR working solution 20 times to mix.

The PCR working solution is stable at room temperature for 10 minutes and can also be stored on ice if needed during preparation.

### Procedure

- 1 Remove the **FPU** from the incubator, and then remove the aluminum foil seal.
- 2 Cover the filter plate with the lid and centrifuge at  $2400 \times g$  at room temperature for 2 minutes.
- 3 Add 25  $\mu\text{l}$  of 0.05 N NaOH to each sample well on the filter plate. Pipette the NaOH up and down 5–6 times to make sure the NaOH contacts the filter.
- 4 Cover and incubate the filter plate at room temperature for 5 minutes.

- 5 While the filter plate is incubating, transfer 22 µl of the PCR working solution to each well of the AMP plate containing index primers.
  - 6 Transfer samples eluted from the filter to the AMP plate as follows:
    - a 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.
- b Transfer 20 µl from the filter plate to the corresponding column of the **AMP** plate.
  - c Gently pipette up and down 5–6 times to thoroughly combine the DNA with the PCR working solution.
  - d 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.*
- 7 Seal the **AMP** plate and secure with a rubber roller.
  - 8 Centrifuge at 1000 × 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

#### 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

- 1 Combine 5 µl of amplified product with 15 µl 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.
  - } Samples showing little to no amplification in one Oligo Pool or both Oligo Pools 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.



#### NOTE

For more information and examples of valid and invalid results, see the *Praxis Extended RAS Panel Reference Guide (document # 15054440)*.

## PCR Clean-Up

### Preparation

- 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.

## Procedure

- 1 Centrifuge the **AMP** plate at  $1000 \times g$  at room temperature for 1 minute.
- 2 Set out a new MIDI plate (hereafter referred to as the **CLP** plate).
- 3 Invert PCR Clean-Up Beads 10 times. Vortex vigorously and then invert 10 more times. Visually inspect solution to ensure that beads are resuspended.
- 4 Add 55  $\mu\text{L}$  of PCR Clean-Up Beads to each well of the **CLP** plate.
- 5 Transfer the entire PCR product from the **AMP** plate to the **CLP** plate.
- 6 Seal the **CLP** plate and shake on a microplate shaker at 1800 rpm for 2 minutes.
- 7 Incubate at room temperature without shaking for 10 minutes.
- 8 Place the plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 9 With the **CLP** plate on the magnetic stand, carefully remove and discard the supernatant.
- 10 With the **CLP** plate on the magnetic stand, wash the beads as follows:
  - a Add 200  $\mu\text{L}$  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 is clear.
  - c Carefully remove and discard the supernatant.
- 11 Repeat the wash as described in the previous step.
- 12 Use a P20 multi-channel pipette set to 20  $\mu\text{L}$  to remove excess ethanol. Do not disturb or touch the beads.
- 13 Remove the **CLP** plate from the magnetic stand and air-dry the beads for 5 minutes.
- 14 Add 25  $\mu\text{L}$  of Elution Buffer to each sample.
- 15 Seal the **CLP** plate and shake on a microplate shaker at 1800 rpm for 5 minutes. After shaking, verify if samples were resuspended. If not, repeat this step.
- 16 Place the **CLP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
- 17 Set out a new MIDI plate (hereafter referred to as the **LNP** plate).
- 18 Transfer 20  $\mu\text{L}$  of the supernatant from the **CLP** plate to the **LNP** plate.
- 19 [Optional] Transfer the remaining 5  $\mu\text{L}$  of supernatant from the **CLP** plate to a new plate and label the plate with a run name and date. Store this plate at  $-25^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$  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.
- 20 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  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$ .

## Library Normalization

### Preparation

- 1 Prepare fresh 0.1 N NaOH by adding 30  $\mu\text{L}$  of 10 N NaOH to 2970  $\mu\text{L}$  of RNase/DNase-free water.
- 2 Bring Library Normalization Diluent, Library Beads, and Library Normalization Wash to room temperature.
- 3 Vortex Library Normalization Diluent vigorously and ensure that all precipitates have dissolved.
- 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 Mix Library Normalization Diluent and Library Beads in a fresh 15 mL conical tube as follows:
  - a For 12 samples/24 libraries, add 1575  $\mu\text{L}$  of Library Normalization Diluent to a 15 mL conical tube.
  - b To homogenize beads, use a P1000 pipette set to 1000  $\mu\text{L}$  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.

- c For 12 samples/24 libraries, pipette 300 µl of Library Beads to the tube containing Library Normalization Diluent.
  - d Mix by inverting the tube 15–20 times.
- 2 Add 45 µl of the combined Library Normalization Diluent/Library Beads working solution to each well of the **LNP** plate containing libraries.
  - 3 Seal the **LNP** plate and shake on a microplate shaker at 1,800 rpm for 30 minutes.
  - 4 Place the **LNP** plate on a magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
  - 5 With the **LNP** plate on the magnetic stand, carefully remove and discard the supernatant.
  - 6 Remove the **LNP** plate from the magnetic stand and wash the beads with Library Normalization Wash as follows:
    - a Add 45 µl of Library Normalization Wash to each sample well.
    - b Seal the **LNP** plate and shake on a microplate shaker at 1,800 rpm for 5 minutes.
    - c Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes or until the supernatant is clear.
    - d Carefully remove and discard the supernatant.
  - 7 Repeat the Library Normalization Wash procedure as described in the previous step.
  - 8 Seal the **LNP** plate with an adhesive plate seal.
  - 9 Centrifuge the **LNP** plate at 1000 × g at room temperature for 30 seconds to collect residual wash buffer.
  - 10 Place the **LNP** plate on the magnetic stand.
  - 11 Use a P20 multi-channel pipette set to 20 µl to remove excess Library Normalization Wash.
  - 12 Remove the **LNP** plate from the magnetic stand and add 30 µl of 0.1 N NaOH to each well.
  - 13 Seal the **LNP** plate and shake on a microplate shaker at 1,800 rpm for 5 minutes.
  - 14 During the 5-minute shake, set out a new 96-well PCR plate (hereafter referred to as the **SGP** plate.)
  - 15 Add 30 µl Library Storage Buffer to each well to be used in the **SGP** plate.
  - 16 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.
  - 17 [Optional] Centrifuge the **LNP** plate at 1000 × g at room temperature for 30 seconds to collect contents to the bottom of the wells.
  - 18 Place the **LNP** plate on the magnetic stand for a minimum of 2 minutes.
  - 19 Transfer the supernatant from the **LNP** plate to the **SGP** plate. Gently pipette up and down 5 times to mix.
  - 20 Seal the **SGP** plate and then centrifuge at 1,000 × g at room temperature for 1 minute. Proceed immediately to Library Pooling.

## Library Pooling

### 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

- 1 Thaw the MiSeqDx 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.
- 2 Allow the reagent cartridge to thaw in the room temperature water bath for approximately 1 hour or until thawed.



- 3 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 µl)
  - } Stock 10 N NaOH (25 µl)
- 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 µl)
  - } 1X TE Buffer (8 µl)
- 4 Combine the following volumes to make Denatured Diluted PhiX Control:
  - } Diluted PhiX Control (10 µl)
  - } 0.1 N NaOH (10 µl)
- 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 µl)
  - } Pre-chilled Library Dilution Buffer (980 µl)

### 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.
- 3 Set out a fresh microcentrifuge tube (hereafter referred to as the **PAL** tube.)
- 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 Set out 3–6 fresh microcentrifuge tubes (hereafter referred to as the **DAL** tube.)
- 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. 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. 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 Incubate the **DAL** tube on a heat block 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

### 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.
- 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.

## View Analysis Results

- 1 From the Local Run Manager dashboard, click the run name.
- 2 Click the **Samples** and **Results** tab to view the analysis report.  
 } If analysis was repeated, expand the Select Analysis drop-down list and select the appropriate analysis.
- 3 [Optional] Click the **Copy to**  icon to copy the Analysis folder path.

## Interpretation of Results

- 1 The Illumina Praxis Extended RAS Panel is designed to detect the *KRAS* and *NRAS* mutations listed in [Table 1](#).
- 2 Analysis results are summarized on the Samples and Results tab. The report is also available for export.

## Praxis Extended RAS Panel Report

The Praxis Extended RAS Panel Report details if a run passed or failed, and whether each sample contains any of the 56 Extended RAS Panel mutations.

The Run State on the Samples and Results tab shows if the sequencing run passed quality metrics. If the Run State is Fail, all samples are listed as invalid.

## Sample Results

**Table 14** Sample Results Table

| Column Heading | Description   |
|----------------|---|
| Sample Name    | The sample name provided when the run was created.  |
| Sample         | Sample validity results.<br><b>Valid</b> — Presence or absence of panel mutations is reported.<br><b>Invalid</b> — Presence or absence of panel mutations cannot be determined. See <a href="#">Sample Invalid on page 25</a> . |
| Result         | Sample results for panel mutation detection.<br>See <a href="#">Panel Mutation Detected on page 25</a> and <a href="#">Panel Mutation Not Detected on page 25</a> .   |
| Gene           | The gene, <i>KRAS</i> or <i>NRAS</i> , where a mutation is detected, if applicable.   |
| Exon           | The exon number in which the mutation is present, if applicable.  |
| Amino Acid     | Human Genome Variation Society (HGVS) amino acid change notation.   |
| Nucleotide     | HGVS nucleotide change notation.  |

### Sample Invalid

The sample result, Invalid, indicates that the presence or absence of a Praxis Extended RAS Panel mutation cannot be determined. Repeat library preparation, sequencing, and analysis for Invalid samples. If a sample fails 2 times, additional troubleshooting can be necessary. If all samples in a run are Invalid because of a Run State of Fail, contact Illumina Technical Support.

### Panel Mutation Detected

The sample result, Panel Mutation Detected, indicates that a Praxis Extended RAS Panel mutation is detected. The report lists all detected mutations.

### Panel Mutation Not Detected

The sample result, Panel Mutation Not Detected, indicates that no Praxis Extended RAS Panel mutations are detected.

## Quality Control Procedures

Good laboratory practices dictate that control material should be evaluated to detect differences in processing and technical procedures in the user's laboratory that may produce significant variability in results.

- 1 **Positive Controls (Panel Control)**—The Panel Control is required on every run. The Panel Control has 2 mutations, *KRAS* Exon 2 p.Gly12Asp c.35G>A and *NRAS* Exon 3 p.Gln61Lys c.181C>A. The Panel Control must generate the expected genotype to be valid. If the control is invalid, processing errors may have occurred. The software will fail the entire sequencing run and all samples will appear as Invalid.

- 2 **Negative (No Template/No DNA) Control**— The use of a negative (no template/no DNA) control (Blank) is required on every run. For a valid run, the Blank sample should appear as Invalid. If an unexpected outcome occurs in the Blank sample, the software will fail the entire sequencing run and all samples will appear as Invalid.
- 3 Before using this assay in the laboratory, the performance of the assay should be verified by testing a number of positive and negative samples with known performance characteristics.
- 4 All quality control requirements should be performed in accordance with local, state, and/or federal regulations or accreditation requirements.
- 5 If a sequencing run has failed, contact Illumina Technical Support to troubleshoot your run.

## Performance Characteristics

### Definitions Of Calculations Used In Performance Characteristics

**Sample Qualification (Sample QC) Pass Rate:** The Sample QC pass rate is defined as the percent of specimens that meet the sample quality control specification ( $dCq \leq 5$ ).

**Sample First Pass Rate:** Sample first pass rate is defined as the percent of specimens that give a valid result the first time they are processed.

**Agreement Calculations:** The Praxis Extended RAS Panel is designed to specifically detect 56 mutations in the *KRAS* and *NRAS* genes. The assay simultaneously determines the presence or absence of each of the 56 mutations in every sequenced sample. The reference for agreement calculations is bi-directional Sanger sequencing or a control condition where indicated.

- 1 Overall percent agreement (OPA) is calculated as the number of mutant and non-mutant positions that agree with the reference divided by the total number possible mutant and non-mutant positions for all valid replicates. OPA is calculated on a per mutation basis; there are 56 mutations per sample. Overall percent agreement was calculated as follows:  

$$\left\{ \frac{\# \text{ specimens} \times 56 \text{ mutant and non-mutant positions concordant with reference}}{\# \text{ specimens} \times 56 \text{ mutant and non-mutant positions total}} \right\} = \text{OPA}$$
- 2 Positive Percent Agreement (PPA) is calculated as 100% times the number of true positives (mutant positions concordant with reference) divided by the sum of the number of true positives and the number of false negatives on a per mutation basis. Positive Percent Agreement was calculated as follows:  

$$\left\{ \frac{\# \text{ mutant positions} \times \# \text{ replicates}}{\# \text{ true positives} + \# \text{ false negatives}} \right\} = \text{PPA}$$
- 3 Negative Percent Agreement (NPA) is calculated as 100% times the number of true negatives (non-mutant positions concordant with reference) divided by the sum of the number of true negatives and the number of false positives. NPA is calculated on a per mutation basis; there are 56 mutations per sample. Negative Percent Agreement was calculated as follows:  

$$\left\{ \frac{\# \text{ wild type specimens} \times \# \text{ replicates} \times 56 \text{ non-mutant positions}}{\# \text{ true negatives} + \# \text{ false positives}} \right\} = \text{NPA}$$
- 4 Percent Correct Call (PCC) is calculated as 100% times the number of correct calls divided by the number of calls attempted. This term is used for Reproducibility. The same calculation is described as percent agreement (positive or negative percent agreement) in other analytical studies.

**Confidence Interval Calculations:** One-sided 95% lower confidence bounds and 2-sided 95% confidence intervals were calculated using the Clopper-Pearson (exact binomial) method for analytical or clinical studies, or the Wilson Score method (where indicated) in clinical studies.

### Analytical Accuracy – Comparison to an Orthogonal Method

Banked formalin-fixed, paraffin embedded (FFPE) colorectal cancer tissue samples collected during the Amgen panitumumab study (protocol no. 20050203 ['203 study]) were tested with the Praxis Extended RAS Panel. Samples were also evaluated using the reference method, bidirectional Sanger sequencing (Sanger). The agreements between results from Praxis Extended RAS Panel and Sanger were compared to evaluate the accuracy of the Praxis Extended RAS Panel.

Subject-level RAS status was positive if the subject had at least 1 mutation detected. Subject-level RAS status was negative if no mutation was detected. Positive Percent Agreement (PPA) is calculated as 100% times the

number of true positive subjects divided by the sum of the number of true positive subjects and the number of false negative subjects. Negative Percent Agreement (NPA) is calculated as 100% times the number of true negative subjects divided by the sum of the number of true negative subjects and the number of false positive subjects. Two-sided 95% confidence intervals are calculated using the Clopper-Pearson (Exact binomial) method.

Of the 1183 subjects, the following were excluded from the accuracy analysis (combination of Sanger and Praxis Extended RAS Panel): 358 had no tissue available, 127 had insufficient tissue area, and 13 had insufficient neoplastic cells. Of the remaining 685 subjects, 214 were removed due to dCq > 5.0, 1 due to unestimable dCq, another 28 due to MiSeqDx QC failures, and lastly 1 subject was removed due to sample processing error/aborted run. The remaining 441 subjects had valid Sanger and Praxis Extended RAS Panel results, which were compared.

When evaluated at the subject level ([Table 15](#)), 230 of the 441 subjects were RAS positive by Sanger (mutation detected by Sanger); of these, 227 were RAS positive by the Praxis Extended RAS Panel. The remaining 211 of 441 subjects were RAS negative by Sanger (no mutation detected by Sanger); of these, 206 were RAS negative by the Praxis Extended RAS Panel. This resulted in a PPA of 98.7% and an NPA of 97.6%.

**Table 15** Positive and Negative Percent Agreement of Subject-level RAS Results

| Praxis Extended RAS Panel | Sanger   |          | Total |
|---------------------------|----------|----------|-------|
|                           | Positive | Negative |       |
| Positive                  | 227*     | 5        | 232   |
| Negative                  | 3**      | 206      | 209   |
| Total                     | 230      | 211      | 441   |

| Performance Summary |                 |               |
|---------------------|-----------------|---------------|
| Agreement Statistic | Point Estimate  | Exact 95% CI  |
| PPA                 | 227/230 = 98.7% | [96.2%,99.7%] |
| NPA                 | 206/211 = 97.6% | [94.6%,99.2%] |

\*There were 224 exact matches for within-subject, all mutation-level results; for 2 subjects, MiSeqDx detected the Sanger detected mutation and 1 additional mutation; for 1 subject, MiSeqDx and Sanger detected different mutations.

\*\*1 subject had 2 mutations detected by Sanger; 2 subjects had 1 mutation detected by Sanger.

The data were also evaluated at the mutation level and designated true positive (TP), true negative (TN), false positive (FP), and false negative (FN). The PPA and NPA are presented for each mutation along with a 95% Clopper-Pearson (Exact binomial) confidence interval in [Table 16](#) and [Table 17](#).

**Table 16** Positive and Negative Percent Agreement of Mutation-level Results in the *KRAS* Gene (dCq≤5)

| Location           | Mutation       | TP | FN | FP | TN  | Total | PPA (95% CI)       | NPA (95% CI)       |
|--------------------|----------------|----|----|----|-----|-------|--------------------|--------------------|
| <i>KRAS</i> Exon 2 | c.34G>T        | 14 | 0  | 0  | 426 | 440   | 100.0 (76.8-100.0) | 100.0 (99.1-100.0) |
|                    | c.34G>A        | 9  | 1  | 0  | 430 | 440   | 90.0 (55.5-99.7)   | 100.0 (99.1-100.0) |
|                    | c.34G>C        | 0  | 0  | 0  | 440 | 440   | NE <sup>1</sup>    | 100.0 (99.2-100.0) |
|                    | c.34_35GG>TT   | 1  | 0  | 0  | 439 | 440   | 100.0 (2.5-100.0)  | 100.0 (99.2-100.0) |
|                    | c.34_35GG>AA   | 0  | 0  | 0  | 440 | 440   | NE                 | 100.0 (99.2-100.0) |
|                    | c.34_36GGT>TGG | 0  | 0  | 0  | 440 | 440   | NE                 | 100.0 (99.2-100.0) |
|                    | c.35G>A        | 55 | 0  | 1  | 384 | 440   | 100.0 (93.5-100.0) | 99.7 (98.6-100.0)  |
|                    | c.35G>T        | 40 | 2  | 2  | 396 | 440   | 95.2 (83.8-99.4)   | 99.5 (98.2-99.9)   |
|                    | c.35G>C        | 11 | 0  | 2  | 427 | 440   | 100.0 (71.5-100.0) | 99.5 (98.3-99.9)   |
|                    | c.37G>T        | 2  | 0  | 0  | 438 | 440   | 100.0 (15.8-100.0) | 100.0 (99.2-100.0) |
|                    | c.37G>C        | 0  | 0  | 0  | 440 | 440   | NE                 | 100.0 (99.2-100.0) |
|                    | c.38G>A        | 41 | 0  | 0  | 399 | 440   | 100.0 (91.4-100.0) | 100.0 (99.1-100.0) |
|                    | c.38_39GC>TT   | 0  | 0  | 0  | 440 | 440   | NE                 | 100.0 (99.2-100.0) |
|                    | c.38_39GC>AA   | 0  | 0  | 0  | 440 | 440   | NE                 | 100.0 (99.2-100.0) |
|                    | c.38_39GC>AT   | 0  | 0  | 0  | 440 | 440   | NE                 | 100.0 (99.2-100.0) |
| <i>KRAS</i> Exon 3 | c.175G>A       | 1  | 0  | 0  | 440 | 441   | 100.0 (2.5-100.0)  | 100.0 (99.2-100.0) |
|                    | c.176C>G       | 1  | 0  | 0  | 440 | 441   | 100.0 (2.5-100.0)  | 100.0 (99.2-100.0) |
|                    | c.181C>A       | 0  | 1  | 0  | 440 | 441   | 0.0 (0.0-97.5)     | 100.0 (99.2-100.0) |
|                    | c.181C>G       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|                    | c.182A>T       | 1  | 0  | 0  | 440 | 441   | 100.0 (2.5-100.0)  | 100.0 (99.2-100.0) |
|                    | c.182A>G       | 2  | 0  | 0  | 439 | 441   | 100.0 (15.8-100.0) | 100.0 (99.2-100.0) |
|                    | c.183A>C       | 6  | 0  | 0  | 435 | 441   | 100.0 (54.1-100.0) | 100.0 (99.2-100.0) |
|                    | c.183A>T       | 1  | 0  | 0  | 440 | 441   | 100.0 (2.5-100.0)  | 100.0 (99.2-100.0) |
| <i>KRAS</i> Exon 4 | c.351A>C       | 2  | 1  | 0  | 437 | 440   | 66.7 (9.4-99.2)    | 100.0 (99.2-100.0) |
|                    | c.351A>T       | 2  | 0  | 0  | 438 | 440   | 100.0 (15.8-100.0) | 100.0 (99.2-100.0) |
|                    | c.436G>A       | 11 | 0  | 0  | 429 | 440   | 100.0 (71.5-100.0) | 100.0 (99.1-100.0) |
|                    | c.436G>C       | 1  | 0  | 0  | 439 | 440   | 100.0 (2.5-100.0)  | 100.0 (99.2-100.0) |
|                    | c.437C>T       | 3  | 0  | 0  | 437 | 440   | 100.0 (29.2-100.0) | 100.0 (99.2-100.0) |

\* NE = not estimable

**Table 17** Positive and Negative Percent Agreement of Mutation-level Results in the *NRAS* Gene (dCq≤5)

| Location    | Mutation       | TP | FN | FP | TN  | Total | PPA (95% CI)       | NPA (95% CI)       |
|-------------|----------------|----|----|----|-----|-------|--------------------|--------------------|
| NRAS Exon 2 | c.34G>T        | 1  | 0  | 1  | 439 | 441   | 100.0 (2.5-100.0)  | 99.8 (98.7-100.0)  |
|             | c.34G>A        | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.34G>C        | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.34_35GG>TT   | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.34_35GG>AA   | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.34_36GGT>TGG | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.35G>A        | 4  | 0  | 0  | 437 | 441   | 100.0 (39.8-100.0) | 100.0 (99.2-100.0) |
|             | c.35G>T        | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.35G>C        | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.37G>T        | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.37G>C        | 2  | 0  | 0  | 439 | 441   | 100.0 (15.8-100.0) | 100.0 (99.2-100.0) |
|             | c.38G>A        | 0  | 0  | 1  | 440 | 441   | NE                 | 99.8 (98.7-100.0)  |
|             | c.38G>T        | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.38_39GT>AA   | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.38_39GT>AG   | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
| NRAS Exon 3 | c.175G>A       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.176C>G       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.181C>A       | 7  | 0  | 1  | 433 | 441   | 100.0 (59.0-100.0) | 99.8 (98.7-100.0)  |
|             | c.181C>G       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.182A>T       | 2  | 0  | 0  | 439 | 441   | 100.0 (15.8-100.0) | 100.0 (99.2-100.0) |
|             | c.182A>G       | 5  | 0  | 0  | 436 | 441   | 100.0 (47.8-100.0) | 100.0 (99.2-100.0) |
|             | c.183A>C       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.183A>T       | 2  | 0  | 0  | 439 | 441   | 100.0 (15.8-100.0) | 100.0 (99.2-100.0) |
| NRAS Exon 4 | c.351G>C       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.351G>T       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.436G>A       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.436G>C       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |
|             | c.437C>T       | 0  | 0  | 0  | 441 | 441   | NE                 | 100.0 (99.2-100.0) |

\* NE = not estimable

Overall PPA and NPA calculated at the mutation level for each gene, and across both genes (total), are presented in [Table 18](#). Two-sided 95% confidence intervals for PPA and NPA are calculated using the Clopper-Pearson (Exact binomial) method.

**Table 18** Total Positive and Negative Percent Agreement of Mutation-Level Results

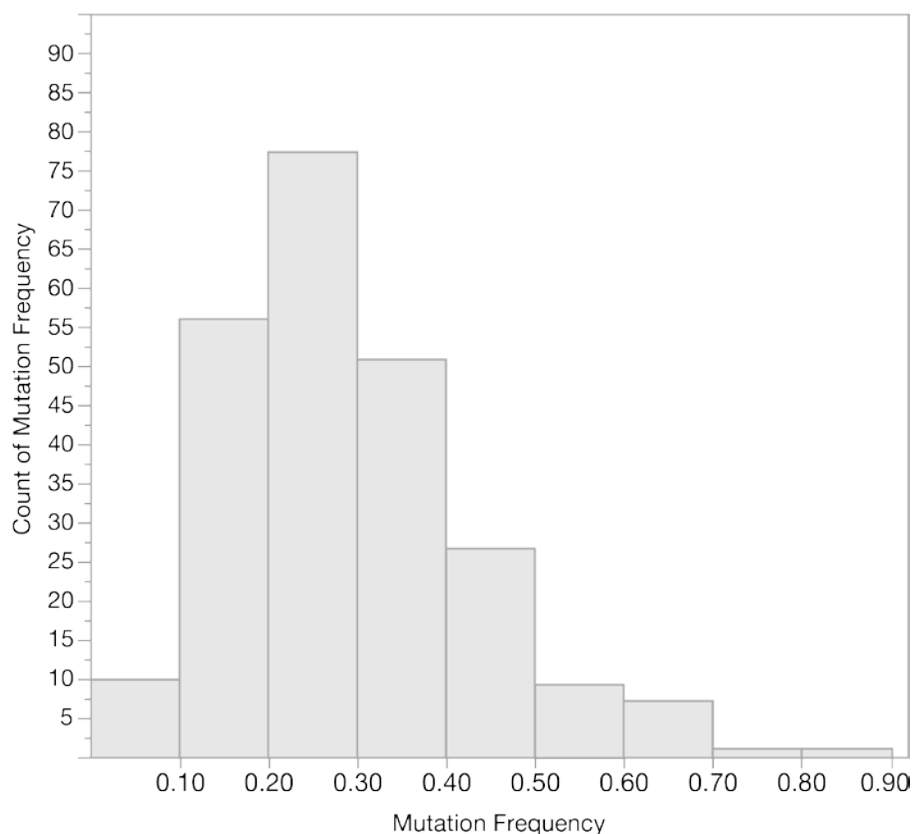
| Location/ Mutation | TP  | FN | FP | TN    | Total | PPA (95% CI)     | NPA (95% CI)       |
|--------------------|-----|----|----|-------|-------|------------------|--------------------|
| KRAS/All           | 204 | 5  | 5  | 12114 | 12328 | 97.6 (94.5-99.2) | 99.96 (99.9-100.0) |
| NRAS/All           | 23  | 0  | 3  | 12322 | 12348 | 100 (85.2-100.0) | 99.98 (99.9-100.0) |
| Total              | 227 | 5  | 8  | 24436 | 24676 | 97.8 (95.0-99.3) | 99.97 (99.9-100.0) |

[Figure 7](#) shows a distribution of the Praxis Extended RAS Panel mutation frequencies. Using ≥ 50% tumor content in the FFPE specimen, the distribution of mutation frequencies observed in the '203 study for samples that tested positive is demonstrated in [Figure 7](#). The majority of specimens showed mutation



frequencies in the 0.15 to 0.35 range. There were 14.9% of subjects with mutation frequency < 0.15, and 55.3% of subjects with mutation frequency  $\geq 0.15$  and  $\leq 0.35$ .

**Figure 7** Praxis Extended RAS Panel Mutation Frequency Distribution for Positive Samples (n=235)



A cross-tabulation of Praxis Extended RAS Panel and Sanger mutation-level results are summarized in the confusion table of Sanger and Praxis Extended RAS Panel Results (see [Table 19](#)). There were 445 possible comparisons between mutations reported by Sanger and/or Praxis Extended RAS Panel for the 441 evaluable subjects in the RAS Evaluable analysis set. Four (4) subjects had two mutations reported by Praxis Extended RAS Panel, by Sanger, or both methods; therefore, the number of mutation-level results (n=445) is greater than the number of subjects (n=441).

Counts along the diagonal in the table represent results that agree at the mutation level (both methods report the same mutation or both methods report no mutation detected). Counts off the diagonal represent results that disagree. Both methods agreed on most results (434/445, 97.5%); 206 of the results that agreed were reported as no mutation detected (negative). In the negative column for Sanger, there were 5 instances (FP) of mutations reported by the Praxis Extended RAS Panel (eg, 1 sample Sanger reported as no mutation, while the Praxis Extended RAS Panel reported *KRAS* Ex2 c35G>A). In the negative row for Praxis Extended RAS Panel, there were 4 instances (FN) of mutations reported for Sanger (eg, 1 sample RAS Panel reported as no mutation, while Sanger reported *KRAS* Ex2 c34G>A).

Results corresponding to the 4 subjects who had 2 mutations detected by Sanger and/or Praxis Extended RAS Panel are noted by footnotes in the confusion table shown in [Table 19](#).

Table 19 Confusion Table of Sanger and Praxis Extended RAS Panel Results

|                            |             |          |               | SANGER          |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         | Negative | Total |          |          |
|----------------------------|-------------|----------|---------------|-----------------|---------|----------------|---------|---------|---------|-------------------|---------|-------------|----------|----------|----------|----------|----------|-------------|----------|----------|----------|-------------|----------------|---------|---------|-------------|---------|----------|-------|----------|----------|
|                            |             |          |               | KRAS Exon 2     |         |                |         |         |         |                   |         | KRAS Exon 3 |          |          |          |          |          | KRAS Exon 4 |          |          |          | NRAS Exon 2 |                |         |         | NRAS Exon 3 |         |          |       |          |          |
|                            |             |          |               | c.34G>T         | c.34C>A | c.34_35GG>TT   | c.35G>A | c.35G>T | c.35G>C | c.37G>T           | c.38G>A | c.175G>A    | c.176C>G | c.181C>A | c.182A>T | c.182A>G | c.183A>C | c.183A>T    | c.351A>C | c.351A>T | c.436G>A | c.436G>C    | c.437C>T       | c.34G>T | c.35G>A | c.37G>C     | c.38G>A |          |       | c.181C>A | c.182A>T |
| MiSeqDx Extended RAS Panel | KRAS Exon 2 |          | c.34G>T       | 14 <sup>8</sup> |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       | 14       |          |
|                            |             |          | c.34G>A       | 9               |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       | 9        |          |
|                            |             |          | c.34_35 GG>TT |                 | 1       |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       | 1        |          |
|                            |             |          | c.35G>A       |                 |         | 55             |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       | 1        |          |
|                            |             |          | c.35G>T       |                 |         |                | 40      |         |         | 1 <sup>C</sup>    |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       | 1        |          |
|                            |             |          | c.35G>C       | 1 <sup>B</sup>  |         |                |         | 11      |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       | 1        |          |
|                            |             |          | c.37G>T       |                 |         |                |         |         | 2       |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.38G>A       |                 |         |                |         |         |         | 41 <sup>C,D</sup> |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            | KRAS Exon 3 |          | c.175G>A      |                 |         |                |         |         |         | 1                 |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.176C>G      |                 |         |                |         |         |         |                   |         | 1           |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.181C>A      |                 |         |                |         |         |         |                   |         |             | 0        |          |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.182A>T      |                 |         |                |         |         |         |                   |         |             |          | 1        |          |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.182A>G      |                 |         |                |         |         |         |                   |         |             |          |          | 2        |          |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            | KRAS Exon 4 |          | c.183A>C      |                 |         |                |         |         |         |                   |         |             |          |          |          | 6        |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.183A>T      |                 |         |                |         |         |         |                   |         |             |          |          |          | 1        |          |             |          |          |          |             |                |         |         |             |         |          |       |          |          |
|                            |             |          | c.351A>C      |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          | 2           |          |          |          |             |                |         |         |             |         |          |       |          |          |
| c.351A>T                   |             |          |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          | 2           |          |          |          |             |                |         |         |             |         |          |       |          |          |
| c.436G>A                   |             |          |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          | 11       |          |             |                |         |         |             |         |          |       |          |          |
| NRAS Exon 2                |             | c.436G>C |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          | 1        |          |             |                |         |         |             |         |          |       |          |          |
|                            |             | c.437C>T |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          | 3        |             |                |         |         |             |         |          |       |          |          |
|                            |             | c.34G>T  |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          | 1           |                |         |         |             |         |          |       |          |          |
|                            |             | c.35G>A  |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             | 4 <sup>D</sup> |         |         |             |         |          |       |          |          |
|                            |             | c.37G>C  |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                | 2       |         |             |         |          |       |          |          |
| NRAS Exon 3                |             | c.38G>A  |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          | 1        |             |          |          |          |             |                |         | 0       |             |         |          |       |          |          |
|                            |             | c.181C>A |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         | 7           |         |          |       |          |          |
|                            |             | c.182A>T |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             | 2       |          |       |          |          |
|                            |             | c.182A>G |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         | 5        |       |          |          |
|                            |             | c.183A>T |               |                 |         |                |         |         |         |                   |         |             |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         | 2        |       |          |          |
|                            |             | Negative |               | 1 <sup>A</sup>  |         | 2 <sup>A</sup> |         |         |         |                   |         | 1           |          |          |          |          |          |             |          |          |          |             |                |         |         |             |         | 206      |       |          |          |
|                            |             | Total    | 15            | 10              | 1       | 55             | 42      | 11      | 2       | 42                | 1       | 1           | 1        | 1        | 2        | 6        | 1        | 3           | 2        | 11       | 1        | 3           | 1              | 4       | 2       | 0           | 7       | 2        | 5     | 2        | 211      |

Footnoted results A, B, C and D indicate the 4 subjects who contributed two results to the cell counts in the table

<sup>A</sup>Subject where Sanger reported 2 mutations (*KRAS* Exon 2 c.34G>A and *KRAS* Exon 2 c.35G>T) and Praxis Extended RAS Panel reported panel mutation not detected.

<sup>B</sup>Subject where Sanger reported 1 mutation (*KRAS* Exon 2 c.34G>T) and Praxis Extended RAS Panel reported the same mutation and an additional mutation (*KRAS* Exon 2 c.34G>T and *KRAS* Exon 2 c.35G>C).

<sup>C</sup>Subject where Sanger reported 1 mutation (*KRAS* Exon 2 c.38G>A) and Praxis Extended RAS Panel reported the same mutation and an additional mutation (*KRAS* Exon 2 c.38G>A and *KRAS* Exon 2 c.35G>T).

<sup>D</sup>Subject where Sanger reported two mutations (*KRAS* Exon 2 c.38G>A and *NRAS* Exon 2 c.35G>A) and Praxis Extended RAS Panel reported the same two mutations (*KRAS* Exon 2 c.38G>A and *NRAS* Exon 2 c.35G>A).

## Analytical Sensitivity (DNA Input Range)

The Praxis Extended RAS Panel workflow involves sample qualification, library preparation and sequencing. Genomic DNA specimens are qualified for use in the assay by measuring amplifiability of the specimen relative to a control DNA template through a real-time quantitative PCR (qPCR) reaction. Measuring amplifiability accounts for both the quantity and quality of the DNA specimen for qualification. A quantification threshold value (Cq) is generated using the Quality Control Template and each genomic DNA specimen. The metric to assess the amplifiability is the change in quantification threshold (Cq) between the specimen and the control template DNA (Delta Cq; dCq). The dCq value is used to determine whether a specimen qualifies for the Praxis Extended RAS Panel assay and is the measure of DNA quality and quantity (DNA Input) for this assay. The acceptable measure for the assay is a Delta Cq  $\leq 5$ .

DNA input was evaluated over the range of delta Cq values from -0.5 to 5 in order to evaluate assay performance. A Delta Cq level of -0.5 to 5 corresponds to approximately 1200 ng to 25 ng, respectively, of intact DNA used in the assay.

A panel of 9 CRC FFPE specimens each containing 1 unique Extended RAS panel mutation, and 1 CRC FFPE specimen wild type for Extended RAS panel mutations was evaluated. The mutations represented were in *KRAS* exons 2, 3, and 4 and *NRAS* exons 2 and 3. The mutation frequency for this panel ranged from approximately 10% to 20%.

The specimen panel was tested in duplicate library preparations at each DNA input level for a total of 20 observations at each DNA input level. Specimens were sequenced at the maximum multiplexing level of 10 samples plus controls per run. Two (2) DNA input levels were tested: 1) dCq -0.5 to 0.5 (nominal high end) 2) dCq 4 to 5 (nominal low end).

All specimens showed a valid result after library preparation and sequencing with a sample first pass rate of 100% (20/20) for each DNA input level. OPA, PPA, and NPA are calculated on a per mutation basis; there are 56 mutations per sample. The overall percent agreement relative to Sanger sequencing was 100% (1120/1120) for each DNA input level. The positive percent agreement was 100% (18/18) and negative percent agreement was 100% (1102/1102) for both DNA input levels relative to Sanger sequencing. Therefore, this study demonstrates the performance of the Praxis Extended RAS Panel assay for the delta Cq (DNA input) range of -0.5 to 5.

To demonstrate the performance with challenging samples that harbor mutations near the limit of detection (LoD) of the mutant allele frequency, an additional study was conducted. Since DNA is qualified based on a dCq cutoff of 5.0, a nominal DNA input concentration range (12.5ng to 50ng) corresponding to approximate values of dCq 6-4 were selected for evaluation. A panel of 5 blended CRC FFPE specimen mixes, each containing 2 unique representative Extended RAS panel mutations, one at a low (approximately 8%) and one at a high (approximately 14%) mutation frequency level, was used. Two (2) of the five (5) sample mixes were spiked with a RAS panel multinucleotide variant (MNV). Two DNA input levels were evaluated for each FFPE blend: a dCq level of 4-5 corresponds to approximately 50-25 ng, respectively, and a dCq level of 5-6 corresponds to approximately 25-12.5 ng, respectively, of intact DNA. Twelve (12) observations of each of the 5 specimen mixes were collected over 3 non-consecutive days per DNA Input/Delta Cq and mutation frequency level. The total number of observations for the study across all specimen mixes was 60 observations; there were 120 observations across all mutation frequency levels per DNA input level

(2 observations  $\times$  2 instruments [sequencing runs]  $\times$  3 non-consecutive days  $\times$  5 mixes). The sample first pass rate was 100% (60/60) for dCq 4 to 5 and 91.7% (55/60) for dCq 5 to 6. For dCq 4 to 5, 100% (120/120) of observations showed the expected Extended RAS Panel mutation result. For dCq 5 to 6, 95% (114/120) of observations showed the expected Extended RAS Panel mutation result. Mean mutation frequencies were

similar between Delta Cq levels, whereas specimens with Delta Cq 5 to 6 had more mutation frequency variability in general than the same specimens with Delta Cq 4 to 5. The study confirmed that the Praxis Extended RAS Panel is able to detect mutants with accuracy at DNA input corresponding to  $dCq \leq 5.0$  and approximately 25 ng of intact DNA for mutations near the LoD.

## Analytical Sensitivity (Limit of Blank [LoB] and Limit of Detection [LoD])

LoD is defined as the minor allele frequency at which 95% of samples are reliably detected. The Limit of Blank (LoB) and the Limit of Detection (LoD) study for the Praxis Extended RAS Panel was conducted using 3 blended CRC FFPE specimen mixes, each containing 2 representative Extended RAS Panel mutations at 5 mutation frequency levels (approx. 10%, 5%, 2.5%, 1.25% and 0%). These mixes were run in duplicate with 2 different reagent lots by 5 operators on 2 nonconsecutive days for a total of 20 observations per specimen mix per lot. The total number of observations for the study across all levels, specimen mixes, and reagent lots is 600 observations (20 observations  $\times$  5 levels  $\times$  3 specimen mixes  $\times$  2 lots = 600 observations). All runs included 10 samples plus controls on a single flow cell to test the LoD at the assay sample throughput limits. The sample mixes were prepared at the low level of DNA input ( $dCq=4-5$ ). The range of results for 95% detection each mutation tested is shown in the table below. Using Probit analysis, the LoD for the 6 representative mutations ranged between 4.2%-5.3% (Table 20). Overall, the data supports the claim of 5%.

**Table 20** Limit of Detection

| Gene | Exon | Mutation      | LoD  |
|------|------|---------------|------|
| KRAS | 2    | c.35 G>A      | 5.0% |
| NRAS | 3    | c.182 A>G     | 4.7% |
| KRAS | 2    | c.38 G>A      | 4.8% |
| KRAS | 4    | c.436 G>A     | 5.3% |
| KRAS | 3    | c.183 A>C     | 4.2% |
| NRAS | 2    | c.38_39 GT>AA | 4.5% |

The limit of blank (LoB) for each of the 56 mutations claimed by this assay was calculated using specimens wild type for RAS panel mutations. Three wild-type (WT) specimens were used in this study. A total of 60 observations were generated per study mutation per lot (20 observations  $\times$  3 WT samples). Two lots were used in the study. No false positive calls were reported. There was one observation with mutation frequency above the threshold, but the mutation was only detected in one oligo pool and not confirmed in the other, and was therefore not reported. The clinical threshold is set at 2.6%.

## Precision

### Lot-to-Lot Precision

A Lot-to-Lot Precision study was conducted to evaluate the performance of the Praxis Extended RAS Panel across manufactured reagent kit lots. Three (3) reagent kit lots were tested using a panel of 5 blended CRC FFPE specimens. Each CRC FFPE specimen contained 2 unique representative Extended RAS panel mutations: one at a low (approximately 8%) mutation frequency level and one at a high (approximately 14%) mutation frequency level. Twelve (12) observations of each of the 5 specimen mixes were collected over 3 nonconsecutive days with 3 reagent kit lots. The total number of observations for the study across all reagent lots was 180 observations across all specimen mixes and 360 observations across all mutation frequency levels. Across all lots and days, 99.7% (359/360) of observations showed the expected Extended RAS Panel mutation result. One low frequency mutation was incorrectly called as wild-type. A variance component analysis was performed for each of the mutations/mutation frequency levels to estimate the variability of the system. The total standard deviations ranged from 0.011 to 0.029. The reagent lot component of the total standard deviation ranged from 0 to 0.015.

## Reproducibility

An external study was performed to assess the reproducibility of the Praxis Extended RAS Panel across 3 external testing sites (2 operators per site), 1 reagent lot, and 3 non-consecutive testing days. Testing was conducted with 6 well-characterized sample panels of genomic DNA samples from FFPE clinical specimens or cell lines. Each panel consisted of 10 members, for a total of 60 members across panels.

The 60 panel members consisted of duplicates of 4 unique wild type (for RAS panel mutations) specimens, 12 unique mutant specimens (with a single mutation) prepared at both high and low mutation frequency levels, and 2 unique mutant specimens (with a single mutation) prepared at a low mutation frequency level only. Each unique specimen/mutation frequency level sample (tested in duplicate in each run) had 36 possible results (2 replicates × 2 operators × 3 days × 3 sites) if all results were valid. The Delta Cq values for each of the reproducibility sample panel members ranged from 3.64-4.08.

Percent Correct Call (PCC) across mutations and wild types was evaluated by comparing the Praxis Extended RAS Panel result to the expected mutation result (expected mutation detected or not detected) in each sample. PCC is calculated as 100% times the number of correct calls divided by the number of calls attempted. Two-sided 95% confidence intervals are calculated using the Wilson score method.

## Qualitative Reproducibility

Combining sites, sample pass rates were ≥ 94.7% for the first run of the sample or in samples tested in runs that were valid on first pass. The mutation-level percent correct call (PCC) across mutations from all mutant samples was 99.6% (905/909) excluding failed sample data. The number of attempted calls across all 56 panel mutations (regardless of whether a detected mutation was expected or not) for all valid samples was 58,856 (56 × 1051). Out of these 58,856 mutation level observations, there were only 6 incidents where the observed and expected results were discordant; the mutation-level PCC across mutations and wild types from all samples combined was 99.99% (58,850/58,856).

Table 21 shows PCCs for each of the 26 unique mutant specimen/mutation frequency level samples (combining replicates across 2 panel members) and corresponding 95% 2-sided score CIs.

**Table 21** Mutation-Level Percent Correct Call for Individual Unique Mutant Specimens/Mutation Frequency Level Samples (Evaluable Analysis Set)

| Mutation Level | Exon                     | Amino Acid Change | Base Change | N   | Percent Correct Call (%) | 95% CI <sup>1</sup> |
|----------------|--------------------------|-------------------|-------------|-----|--------------------------|---------------------|
| Low            | KRAS exon 2              | p.Gly12Cys        | c.34G>T     | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Gly12Arg        | c.34G>C     | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Gly12Ser        | c.34G>A     | 35  | 35/35(100%)              | (90.1, 100)         |
|                |                          | p.Gly12Ala        | c.35G>C     | 35  | 35/35(100%)              | (90.1, 100)         |
|                |                          | p.Gly12Asp        | c.35G>A     | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Gly12Val        | c.35G>T     | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Gly13Asp        | c.38G>A     | 35  | 35/35(100%)              | (90.1, 100)         |
|                | KRAS exon 2(all samples) |                   |             | 249 | 249/249(100%)            | (98.5, 100)         |
|                | KRAS exon 3              | p.Gln61His        | c.183A>C    | 33  | 33/33(100%)              | (89.6, 100)         |
|                | KRAS exon 4              | p.Lys117Asn       | c.351A>T    | 35  | 33/35(94.3%)             | (81.4, 98.4)        |
|                |                          | p.Ala146Thr       | c.436G>A    | 36  | 36/36(100%)              | (90.4, 100)         |
|                | KRAS exon 4(all          |                   |             | 71  | 69/71(97.2%)             | (90.3,              |

| samples)                        |             |          | 99.2) |                |              |
|---------------------------------|-------------|----------|-------|----------------|--------------|
| <i>KRAS</i> (all exons)         |             |          | 353   | 351/353(99.4%) | (98, 99.8)   |
| <i>NRAS</i> exon 2              | p.Gly12Asp  | c.35G>A  | 31    | 31/31(100%)    | (89, 100)    |
| <i>NRAS</i> exon 3              | p.Gln61Arg  | c.182A>G | 33    | 33/33(100%)    | (89.6, 100)  |
| <i>NRAS</i> exon 4              | p.Lys117Asn | c.351G>T | 35    | 35/35(100%)    | (90.1, 100)  |
|                                 | p.Ala146Thr | c.436G>A | 35    | 34/35(97.1%)   | (85.5, 99.5) |
| <i>NRAS</i> exon 4(all samples) |             |          | 70    | 69/70(98.6%)   | (92.3, 99.7) |
| <i>NRAS</i> (all exons)         |             |          | 134   | 133/134(99.3%) | (95.9, 99.9) |
| RAS(all exons)                  |             |          | 487   | 484/487(99.4%) | (98.2, 99.8) |

**Table 21** Mutation-Level Percent Correct Call for Individual Unique Mutant Specimens/Mutation Frequency Level Samples (Evaluable Analysis Set) – Continuation

| Mutation Level | Exon                     | Amino Acid Change | Base Change | N   | Percent Correct Call (%) | 95% CI <sup>1</sup> |
|----------------|--------------------------|-------------------|-------------|-----|--------------------------|---------------------|
| High           | KRAS exon 2              | p.Gly12Arg        | c.34G>C     | 33  | 33/33(100%)              | (89.6, 100)         |
|                |                          | p.Gly12Ala        | c.35G>C     | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Gly12Asp        | c.35G>A     | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Gly12Val        | c.35G>T     | 35  | 35/35(100%)              | (90.1, 100)         |
|                |                          | p.Gly13Asp        | c.38G>A     | 36  | 36/36(100%)              | (90.4, 100)         |
|                | KRAS exon 2(all samples) |                   |             | 176 | 176/176(100%)            | (97.9, 100)         |
|                | KRAS exon 3              | p.Gln61His        | c.183A>C    | 35  | 35/35(100%)              | (90.1, 100)         |
|                | KRAS exon 4              | p.Lys117Asn       | c.351A>T    | 36  | 36/36(100%)              | (90.4, 100)         |
|                |                          | p.Ala146Thr       | c.436G>A    | 35  | 35/35(100%)              | (90.1, 100)         |
|                | KRAS exon 4(all samples) |                   |             | 71  | 71/71(100%)              | (94.9, 100)         |
|                | KRAS(all exons)          |                   |             | 282 | 282/282(100%)            | (98.7, 100)         |
|                | NRAS exon 2              | p.Gly12Asp        | c.35G>A     | 34  | 34/34(100%)              | (89.8, 100)         |
|                | NRAS exon 3              | p.Gln61Arg        | c.182A>G    | 34  | 34/34(100%)              | (89.8, 100)         |
|                | NRAS exon 4              | p.Lys117Asn       | c.351G>T    | 36  | 35/36(97.2%)             | (85.8, 99.5)        |
|                |                          | p.Ala146Thr       | c.436G>A    | 36  | 36/36(100%)              | (90.4, 100)         |
|                | NRAS exon 4(all samples) |                   |             | 72  | 71/72(98.6%)             | (92.5, 99.8)        |
|                | NRAS(all exons)          |                   |             | 140 | 139/140(99.3%)           | (96.1, 99.9)        |
|                | RAS(all exons)           |                   |             | 422 | 421/422(99.8%)           | (98.7, 100)         |
| Overall        | All panel mutations      |                   |             | 909 | 905/909(99.6%)           | (98.9, 99.8)        |

<sup>[1]</sup> 95% two-sided confidence interval calculated using the Wilson score method.

**Table 22** Sample-Level Percent Correct Call Across Wild Type Samples (Evaluable Analysis Set)

| Sample      | N   | Percent Correct Call (%) | 95% CI <sup>1</sup> |
|-------------|-----|--------------------------|---------------------|
| Wild Type 1 | 35  | 35/35(100%)              | (90.1, 100)         |
| Wild Type 2 | 36  | 36/36(100%)              | (90.4, 100)         |
| Wild Type 3 | 36  | 36/36(100%)              | (90.4, 100)         |
| Wild Type 4 | 35  | 35/35(100%)              | (90.1, 100)         |
| Overall     | 142 | 142/142(100%)            | (97.4, 100)         |

<sup>[1]</sup> 95% two-sided confidence interval calculated using the Wilson score method.



Table 23 shows the variance component analysis of mutation frequency results for each source of variation and total variation for the Evaluable analysis set (for mutation frequency). Results are shown for each unique mutant specimen/mutation frequency level sample, combining sites.

Table 23 Variance Component Estimates for Individual Mutant Specimen and Mutation Level (Evaluable Analysis Set for Mutation Frequency)

| Mutation Level | Exon        | Amino Acid Change | Base Change | N  | Mean Mutation Frequency | Site SD (%CV) | Operator (Site) SD (%CV) | Day(Site, Operator) SD (%CV) | Replicate SD (%CV) | Total SD (%CV) |
|----------------|-------------|-------------------|-------------|----|-------------------------|---------------|--------------------------|------------------------------|--------------------|----------------|
| Low            | KRAS exon 2 | p.Gly12Cys        | c.34G>T     | 36 | 0.080                   | 0.003 (4.0)   | 0.003 (4.0)              | 0.000 (0.0)                  | 0.009 (10.8)       | 0.010 (12.2)   |
|                |             | p.Gly12Arg        | c.34G>C     | 36 | 0.078                   | 0.020 (25.2)  | 0.004 (5.3)              | 0.005 (6.6)                  | 0.008 (9.8)        | 0.022 (28.3)   |
|                |             | p.Gly12Ser        | c.34G>A     | 35 | 0.080                   | 0.005 (6.1)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.011 (13.9)       | 0.012 (15.2)   |
|                |             | p.Gly12Ala        | c.35G>C     | 35 | 0.072                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.010 (13.8)                 | 0.013 (17.5)       | 0.016 (22.2)   |
|                |             | p.Gly12Asp        | c.35G>A     | 36 | 0.072                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.002 (3.4)                  | 0.013 (18.7)       | 0.014 (19.0)   |
|                |             | p.Gly12Val        | c.35G>T     | 36 | 0.083                   | 0.002 (3.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.009 (11.1)       | 0.010 (11.5)   |
|                |             | p.Gly13Asp        | c.38G>A     | 35 | 0.092                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.015 (16.7)                 | 0.017 (18.9)       | 0.023 (25.3)   |
|                | KRAS exon 3 | p.Gln61His        | c.183A>C    | 33 | 0.086                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.011 (12.9)       | 0.011 (12.9)   |
|                | KRAS exon 4 | p.Lys117Asn       | c.351A>T    | 33 | 0.090                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.003 (3.1)                  | 0.010 (10.7)       | 0.010 (11.2)   |
|                |             | p.Ala146Thr       | c.436G>A    | 36 | 0.099                   | 0.005 (4.8)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.009 (9.4)        | 0.010 (10.6)   |
|                | NRAS exon 2 | p.Gly12Asp        | c.35G>A     | 31 | 0.078                   | 0.002 (2.9)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.009 (12.1)       | 0.010 (12.4)   |
|                | NRAS exon 3 | p.Gln61Arg        | c.182A>G    | 33 | 0.097                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.002 (1.8)                  | 0.009 (9.5)        | 0.009 (9.6)    |
|                | NRAS exon 4 | p.Lys117Asn       | c.351G>T    | 35 | 0.086                   | 0.001 (1.7)   | 0.002 (2.4)              | 0.003 (3.9)                  | 0.007 (8.4)        | 0.008 (9.7)    |
|                |             | p.Ala146Thr       | c.436G>A    | 35 | 0.080                   | 0.006 (6.9)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.016 (20.5)       | 0.017 (21.7)   |

| Mutation Level | Exon        | Amino Acid Change | Base Change | N  | Mean Mutation Frequency | Site SD (%CV) | Operator (Site) SD (%CV) | Day(Site, Operator) SD (%CV) | Replicate SD (%CV) | Total SD (%CV) |
|----------------|-------------|-------------------|-------------|----|-------------------------|---------------|--------------------------|------------------------------|--------------------|----------------|
| High           | KRAS exon 2 | p.Gly12Arg        | c.34G>C     | 33 | 0.230                   | 0.047 (20.4)  | 0.004 (1.7)              | 0.000 (0.0)                  | 0.019 (8.1)        | 0.051 (22.1)   |
|                |             | p.Gly12Ala        | c.35G>C     | 36 | 0.281                   | 0.009 (3.3)   | 0.000 (0.0)              | 0.008 (2.9)                  | 0.013 (4.7)        | 0.018 (6.5)    |
|                |             | p.Gly12Asp        | c.35G>A     | 36 | 0.259                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.005 (2.0)                  | 0.015 (5.8)        | 0.016 (6.2)    |
|                |             | p.Gly12Val        | c.35G>T     | 35 | 0.206                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.020 (9.9)        | 0.020 (9.9)    |
|                | KRAS exon 2 | p.Gly13Asp        | c.38G>A     | 36 | 0.263                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.024 (9.3)        | 0.024 (9.3)    |
|                | KRAS exon 3 | p.Gln61His        | c.183A>C    | 35 | 0.259                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.013 (5.0)        | 0.013 (5.0)    |
|                | KRAS exon 4 | p.Lys117Asn       | c.351A>T    | 36 | 0.267                   | 0.008 (3.0)   | 0.007 (2.6)              | 0.003 (1.2)                  | 0.014 (5.3)        | 0.018 (6.7)    |
|                |             | p.Ala146Thr       | c.436G>A    | 35 | 0.277                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.015 (5.6)        | 0.015 (5.6)    |
|                | NRAS exon 2 | p.Gly12Asp        | c.35G>A     | 34 | 0.274                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.013 (4.8)        | 0.013 (4.8)    |
|                | NRAS exon 3 | p.Gln61Arg        | c.182A>G    | 34 | 0.281                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.012 (4.3)        | 0.012 (4.3)    |
| High           | NRAS exon 4 | p.Lys117Asn       | c.351G>T    | 36 | 0.258                   | 0.010 (3.9)   | 0.010 (3.7)              | 0.012 (4.5)                  | 0.045 (17.3)       | 0.048 (18.7)   |
|                |             | p.Ala146Thr       | c.436G>A    | 36 | 0.258                   | 0.000 (0.0)   | 0.000 (0.0)              | 0.000 (0.0)                  | 0.014 (5.6)        | 0.014 (5.6)    |

## Analytical Specificity (Interfering Substances)

An Interfering Substances study was conducted to evaluate the impact of potentially interfering substances on the performance of the Praxis Extended RAS Panel Assay. Assay performance was evaluated in the presence of exogenous substances, (paraffin wax, xylene, ethanol and Proteinase K, extraction solutions), as well as endogenous substances (necrotic tissue and hemoglobin).

### Exogenous Substances

The exogenous substances tested are extraction solutions commonly used during the DNA extraction process and are listed with tested quantities in Table 24. Fifteen (15) colorectal FFPE specimens were tested per interfering substance and compared with untreated controls. The specimens represented wild type specimens containing no Extended RAS panel mutations (5/15 specimens), as well as specimens containing prevalent Extended RAS panel mutations (10/15 specimens). Specimens were sequenced at the maximum multiplexing level of 10 samples plus controls per run.

**Table 24** Substances Tested

| Interfering substance      | Actual Amount [ul/25uL eluate] |
|----------------------------|--------------------------------|
| Deparaffinization Solution | $1.69 \times 10^{-04}$         |
| Paraffin Wax (in Xylene)   | $2.50 \times 10^{-05}$         |
| Xylene                     | $2.50 \times 10^{-05}$         |
| Ethanol                    | $1.69 \times 10^{-04}$         |
| Proteinase K               | $3.30 \times 10^{-06}$         |
| Wash Solution, N           | $6.25 \times 10^{-01}$         |
| 1X Wash Solution, P        | $6.25 \times 10^{-01}$         |
| Wash Buffer AW1            | $6.25 \times 10^{-02}$         |
| Wash Buffer AW2            | $6.25 \times 10^{-01}$         |

For all exogenous substances tested, all 15 specimens passed the sample qualification requirement (15/15, 100% Sample QC Pass Rate) and showed a valid result after library preparation and sequencing (15/15, 100% Sample First Pass Rate).

OPA, PPA, and NPA are calculated on a per mutation basis; there are 56 mutations per sample. All 15 specimens for all 9 exogenous substances showed agreement with the untreated control condition at all mutant and non-mutant positions. No false positives and no false negatives were observed. None of the potentially interfering substances evaluated at the maximum concentrations expected to be encountered in the process of genomic DNA (gDNA) extraction from FFPE tissue impacts performance of the Praxis Extended RAS Panel Assay.

### Endogenous substances (Hemoglobin)

Fifteen (15) CRC FFPE specimens were each tested in the presence or absence of 2 mg/mL hemoglobin, a CLSI “high” amount of hemoglobin. The specimens represented wild type specimens containing no Extended RAS panel mutations (5/15 specimens), as well as specimens containing prevalent Extended RAS Panel mutations (10/15 specimens). Specimens were sequenced at the maximum multiplexing level of 10

samples plus controls per run. All 15 specimens passed the sample qualification requirement (15/15, 100% Sample QC Pass Rate) and showed a valid result after library preparation and sequencing (15/15, 100% Sample First Pass Rate). All 15 specimens showed agreement at all mutant and non-mutant positions with the untreated control condition. No false positives or false negatives were observed. The concentration of hemoglobin tested does not affect performance of the Praxis Extended RAS Panel.

### Necrotic tissue

Fifteen (15) CRC FFPE specimens composed of wild type specimens containing no Extended RAS panel mutations (10/15 specimens), as well as specimens containing prevalent Extended RAS Panel mutations (5/15 specimens) and between 10–80% necrotic tissue as determined by pathology review, were utilized for evaluation of endogenous necrotic specimens. Specimens were sequenced at the maximum multiplexing level of 10 samples plus controls per run. 14/15 specimens gave a valid result after library preparation and sequencing (93.3% Sample First Pass Rate). The overall percent agreement was 99.9% (783/784) relative to Sanger sequencing. PPA was 100% (4/4) and NPA was 99.9% (779/780). The 1 false positive detected was likely due to a mutation frequency below the limit of detection of Sanger sequencing. Overall, the Praxis Extended RAS Panel meets performance characteristics with tissue containing 10–80% necrosis.

## Guard banding Studies

The Praxis Extended RAS Panel workflow guard banding studies evaluated selected workflow steps of Sample Qualification, Library Preparation, and Sequencing. Test samples included FFPE genomic DNA and cell line genomic DNA to increase mutation diversity. The Library preparation conditions were tested at the maximum multiplexing level of the assay at 10 specimens per run.

### Sample Qualification (qPCR step)

Five genomic DNA samples were tested at various storage times of qPCR master mix and reaction mix. Since both the qPCR master mix and reaction mix are used right away, the testing conditions included holding the qPCR master mix at room temperature for 1 hour in ambient light, and the reaction mix for 10 minutes on ice. Average dCq differences of less than 0.25 were observed indicating stability of the qPCR master mix and reaction mixes.

### Library Preparation

Hybridization and Extension-Ligation incubation times and temperatures were tested at approximately +/- 5% of the specified value. 100% of specimen tested showed a valid result with the expected mutations.

During PCR Amplification, sodium hydroxide is used to remove the extension-ligation product from the filter plate. The concentration and incubation times were tested at approximately +/- 5% of the specified value. 90% of specimens tested at low sodium hydroxide concentration and 100% of specimen tested at all other conditions showed a valid result with the expected mutations.

Storage times of PCR working solution and hold times of the PCR plate (post-amplification) were tested at approximately +/- 5% of the specified value. All specimens tested at all conditions showed a valid result with the expected mutations.

For the PCR clean-up and Library Normalization steps, the following conditions were tested at approximately +/- 5% of the specified value: a) incubation times, b) shaking times, and concentrations of c) ethanol (PCR clean-up), d) sodium hydroxide solution (Library Normalization). 90% of specimens tested for the longer PCR clean up elution incubation and 100% of specimens tested at all other conditions showed a valid result with the expected mutations.

For the Library Pooling step, times for heat denaturation and cooling were tested at approximately +/- 5% of the specified values. 90% of specimens tested at the longer cooling, and 100% of specimens tested at all other heat denaturation and cooling conditions showed a valid result with the expected mutations.

DAL (Diluted Amplicon Library) storage was tested up to 84 days (stored at -20°C). 100% of specimens tested showed a valid result with the expected mutations.

### Sequencing

Robustness of deep sequencing is achieved with a minimum sequencing depth requirement for the samples to be called "valid". A total of 40 specimens plus controls were tested by 2 operators for meeting the minimum coverage of 1800X across both pools (900x per pool) per sample at the maximum multiplexing

level of the assay at 10 specimens per run. All specimens met the minimum coverage requirement with an average coverage of approximately 31000x across both pools per sample.

## Stability

### Real-Time Stability

The objective of the real-time stability study was to establish the shelf life of the Praxis Extended RAS Panel kit by evaluating the product throughout the proposed shelf life when stored per label conditions. The study design was based on the testing of three Praxis Extended RAS Panel kit lots and utilized the classic stability study design described in CLSI EP25-A. The kits were stored in its final kitted configuration for the duration of the study, at storage conditions defined on the product label. Frozen kit components were stored at -15°C to -25°C. Refrigerated kit components were stored at 2°C to 8°C. Room-temperature components were stored at 15°C to 30°C. Each kit has the appropriate expiry date printed on the box level label.

### Open-Tube Stability

The open-tube, in-use stability of the sample qualification and library preparation reagents was evaluated under standard use conditions. The reagents are expected to be used two (2) times which corresponds to opened tubes for the room temperature and 2°C to 8°C components and two (2) freeze/thaw cycles for the reagents stored between -15°C and -25°C. The frozen reagents were subjected to 5 and 6 freeze/thaw cycles between execution control testing and final testing. Room temperature and 2°C to 8°C components were tested under the standard use conditions per label requirements for two separate library preparations with opened reagents closed and returned to their indicated storage conditions between uses. The study showed that all quality control acceptance criteria were met for all kits tested, both in the execution tests and after the freeze/thaw cycles.

## Summary of Analytical Studies

When evaluated at the specimen level at external sites using study specimens, and after exclusion of Praxis invalids, the Praxis Extended RAS Panel demonstrated a PPA of 98.7% (227/230) and an NPA of 97.6% (206/211), as compared to Sanger.

The Praxis Extended RAS Panel can detect 56 mutations ([Table 1](#)) in the *KRAS* and *NRAS* genes. The performance of the Praxis Extended RAS Panel assay was demonstrated for the delta Cq (DNA input) range of -0.5 to 5. The LoD for each mutation tested ranged from 4.2% to 5.3%. Assay performance was not negatively impacted by interfering substances and necrotic tissue. No false positive results were detected due to well-to-well or run-to-run sample carryover. Precision and reproducibility of assay performance was demonstrated across multiple sources of variation with minimal contribution of reagent lot and other factors to overall variability.

## Clinical Performance

A clinical study was conducted to generate data to support the clinical effectiveness of the Praxis Extended RAS Panel as a companion diagnostic test that enables selection of patients for treatment with panitumumab (Vectibix). Banked formalin-fixed, paraffin embedded (FFPE) colorectal cancer tissue samples collected during the Amgen panitumumab study (protocol no. 20050203 ['203 study]) were tested with the Praxis Extended RAS Panel. The '203 study assessed the effectiveness of panitumumab plus FOLFOX versus FOLFOX alone in a multicenter, prospective, open-label, randomized phase 3 study that enrolled 1183 subjects with previously untreated, metastatic colorectal cancer. Note: Samples evaluated in this Clinical Performance study were from the same sample set evaluated in the Clinical Accuracy study described above. The first subject was enrolled August 2006. The study was closed to randomization September 2008.

Of the 1183 subjects, 200 (17%) had no tissue available and 983 (83%) had tissue potentially eligible for Praxis Extended RAS Panel testing. Of the 983 subjects with tissue available, 159 (16%) had specimens that did not meet tissue requirements. Of these 159 subjects, 13 (8%) had specimens with no colorectal cancer tissue present on the available remaining slides, 100 (63%) had specimens with insufficient tissue area, 4 (3%) had specimens with an insufficient number of neoplastic cells, and 42 (26%) had specimens that were not evaluable by H&E.

There were 824 subjects who had samples that met the assay tissue requirements, had DNA extracted, and were processed and tested with Praxis Extended RAS Panel reagents. Of the 824 subjects, 567 had dCq values  $\leq 5.0$ . Of these, 528 subjects had a valid RAS result by the Praxis Extended RAS Panel and were evaluable for the primary analyses.

Of the 528 subjects with dCq values  $\leq 5.0$  and valid sequencing results, 217 (41%) had wild type RAS status and 311 (59%) had mutant RAS status. Of the 311 mutant RAS subjects, 57 (18%) had wild type *KRAS* Exon 2 mutant RAS status, and 254 (82%) had mutant *KRAS* Exon 2 status.

The primary endpoints for the [Clinical Performance](#) study were progression free survival (PFS) and overall survival (OS). Once the RAS mutation status was determined, the clinical outcome and safety data for the trial cohorts was added, allowing assessment of therapeutic efficacy:

### PFS (cutoff date 02 August 2010)

- } *Wild Type RAS*: For subjects with wild type RAS, the median PFS time was 10.0 months for the panitumumab plus FOLFOX group, and 9.2 months for the FOLFOX alone group. The hazard ratio (HR) for PFS from a stratified Cox proportional hazards model, as measured by panitumumab plus FOLFOX:FOLFOX alone, was 0.700 (95% CI: 0.516 to 0.948), indicating longer PFS for the panitumumab plus FOLFOX group than for the FOLFOX alone group. The stratified log-rank test revealed a statistically significant difference in PFS between the panitumumab plus FOLFOX group versus the FOLFOX alone group ( $P = .0206$ ). (See [Table 25](#).)
- } *Mutant RAS*: For subjects with a RAS mutation detected, the HR for PFS was 1.242 (95% CI: 0.976 to 1.582), indicating a longer PFS for the FOLFOX alone group than for the panitumumab plus FOLFOX group ( $P = .0757$ ). (See [Table 25](#).)
- } *Interaction*: Quantitative interaction testing indicated the treatment effect, as measured by panitumumab plus FOLFOX:FOLFOX alone, differed when comparing subjects with wild type RAS to subjects with mutant RAS ( $P = .0038$ ).

**Table 25** Primary Efficacy Parameters – PFS for Wild Type RAS and Mutant RAS

| Endpoint/ Data Cutoff date | Parameter            | Wild Type RAS         |                      | Mutant RAS            |                      |
|----------------------------|----------------------|-----------------------|----------------------|-----------------------|----------------------|
|                            |                      | Pmab + FOLFOX (n=106) | FOLFOX Alone (n=111) | Pmab + FOLFOX (n=163) | FOLFOX Alone (n=148) |
| PFS 2010                   | Events n (%)         | 86 (81)               | 93 (84)              | 147 (90)              | 133 (90)             |
|                            | Median <sup>a</sup>  | 10.0                  | 9.2                  | 7.4                   | 9.2                  |
|                            | (95% CI)             | (9.2, 12.8)           | (7.7, 10.3)          | (6.3, 9.0)            | (7.7, 10.1)          |
|                            | HR <sup>b</sup>      | 0.700                 |                      | 1.242                 |                      |
|                            | (95% CI)             | (0.516, 0.948)        |                      | (0.976, 1.582)        |                      |
|                            | P-value <sup>c</sup> | 0.0206                |                      | 0.0757                |                      |

Pmab = panitumumab

<sup>a</sup> Kaplan-Meier estimate of median time (months) to event; 95% CI is based on a sign test (Brookmeyer and Crowley, 1982).

<sup>b</sup> HR presented as panitumumab plus FOLFOX:FOLFOX alone is estimated from Cox proportional hazard model stratified by region (Western Europe, Canada and Australia vs. Rest of World) and ECOG score (0 or 1 vs. 2).

<sup>c</sup> P-value is based on a 2-sided log-rank test stratified by region and ECOG score.

OS (cutoff date 24 January 2013):

- } *Wild Type RAS*: For subjects with wild type RAS, the median OS time was 26.0 months for the panitumumab plus FOLFOX group and 23.1 months for the FOLFOX alone group. The HR (panitumumab plus FOLFOX:FOLFOX alone) for OS from a stratified Cox proportional hazards model was 0.754 (95% CI: 0.555 to 1.024), indicating longer survival for the panitumumab plus FOLFOX group than for the FOLFOX alone group. The stratified log-rank test did not reveal a statistically significant difference in OS between the panitumumab plus FOLFOX group versus the FOLFOX alone group (P = 0.0694). (See [Table 26](#).)
- } *Mutant RAS*: For subjects with mutant RAS, the HR was 1.156 (95% CI: 0.906, 1.476), indicating a longer OS for the FOLFOX alone group than for the panitumumab plus FOLFOX group (P = 0.2441). (See [Table 26](#).)
- } *Interaction*: Quantitative interaction testing indicated that the treatment effect differed, as measured by panitumumab plus FOLFOX:FOLFOX alone, when comparing subjects with wild type RAS to subjects with mutant RAS (P = 0.0323).

**Table 26** Primary Efficacy Parameters – OS for Wild Type RAS and Mutant RAS

| Endpoint/ Data Cutoff date | Parameter            | Wild Type RAS         |                      | Mutant RAS            |                      |
|----------------------------|----------------------|-----------------------|----------------------|-----------------------|----------------------|
|                            |                      | Pmab + FOLFOX (n=106) | FOLFOX Alone (n=111) | Pmab + FOLFOX (n=163) | FOLFOX Alone (n=148) |
| OS 2013                    | Events n (%)         | 81 (76)               | 91 (82)              | 140 (86)              | 132 (89)             |
|                            | Median <sup>a</sup>  | 26.0                  | 23.1                 | 17.4                  | 19.6                 |
|                            | (95% CI)             | (19.9, 32.5)          | (17.2, 26.9)         | (13.8, 20.2)          | (15.9, 22.6)         |
|                            | HR <sup>b</sup>      | 0.754 (0.555, 1.024)  |                      | 1.156 (0.906, 1.476)  |                      |
|                            | (95% CI)             |                       |                      |                       |                      |
|                            | P-value <sup>c</sup> | 0.0694                |                      | 0.2441                |                      |

Pmab = panitumumab

<sup>a</sup> Kaplan-Meier estimate of median time (months) to event; 95% CI is based on a sign test (Brookmeyer and Crowley, 1982).

<sup>b</sup> HR presented as panitumumab plus FOLFOX:FOLFOX alone is estimated from Cox proportional hazard model stratified by region (Western Europe, Canada and Australia vs. Rest of World) and ECOG score (0 or 1 vs. 2).

<sup>c</sup> P-value is based on a 2-sided log-rank test stratified by region and ECOG score.



In the Mutant RAS group and the wild type *KRAS* Exon 2/Mutant RAS subset (subjects who did not have a mutation in *KRAS* Exon 2 but did have a mutation in *KRAS* exons 3, 4; or *NRAS* exons 2, 3, 4; or nucleotide positions in Table 1, respectively), the trends were in the opposite (unfavorable) direction: PFS and OS are longer in the FOLFOX alone group compared to the panitumumab plus FOLFOX group (see Table 27).

**Table 27** Primary Efficacy Parameters – PFS and OS for Wild Type *KRAS* Exon 2/Mutant RAS

| Endpoint/ Data Cutoff date | Parameter                    | Wild Type <i>KRAS</i> Exon 2 / Mutant RAS <sup>c</sup> |                     |
|----------------------------|------------------------------|--|---------------------|
|                            |                              | Pmab + FOLFOX (n=30)                                   | FOLFOX Alone (n=27) |
| PFS 2010                   | Events n (%)                 | 26 (87)  | 20 (74)             |
|                            | Median <sup>a</sup> (95% CI) | 9.1 (6.2, 10.8)  | 11.1 (6.4, 13.8)    |
|                            | HR <sup>b</sup> (95% CI)     | 1.477 (0.796, 2.741)                                   |                     |
| OS 2013                    | Events n (%)                 | 25 (83)  | 21 (78)             |
|                            | Median <sup>a</sup> (95% CI) | 20.9 (14.8, 30.5)                                      | 22.4 (13.0, 39.4)   |
|                            | HR <sup>b</sup> (95% CI)     | 1.346 (0.728, 2.488)                                   |                     |

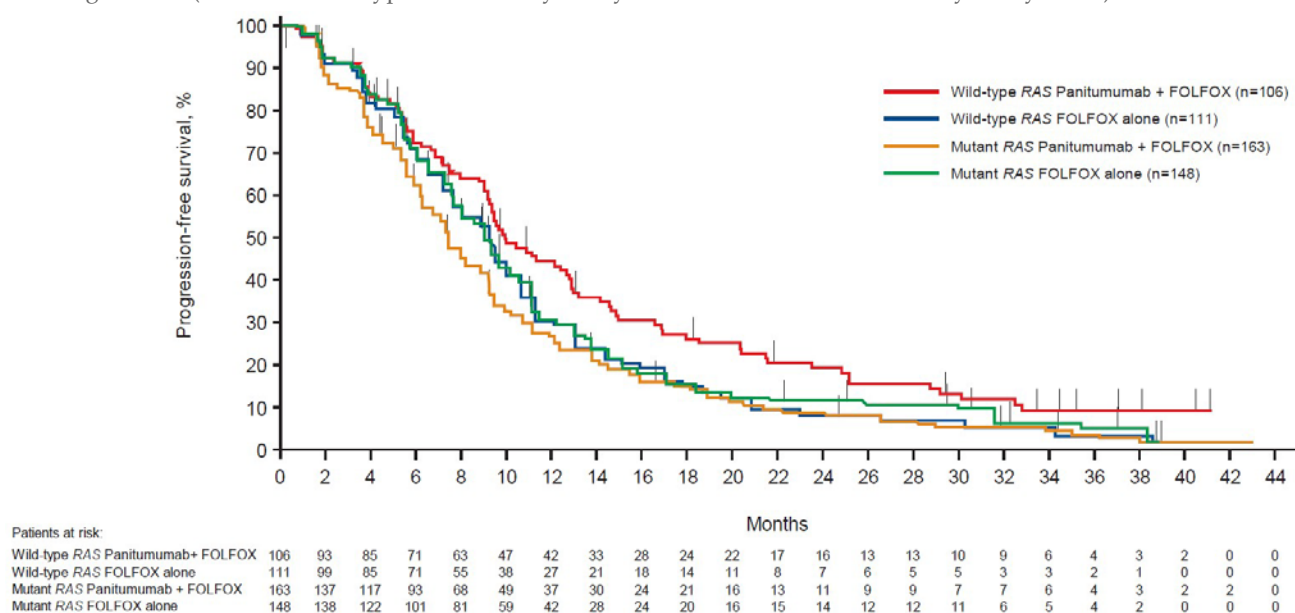
Pmab = panitumumab

<sup>a</sup>Kaplan-Meier estimate of median time (months) to event; 95% CI is based on a sign test (Brookmeyer and Crowley, 1982).

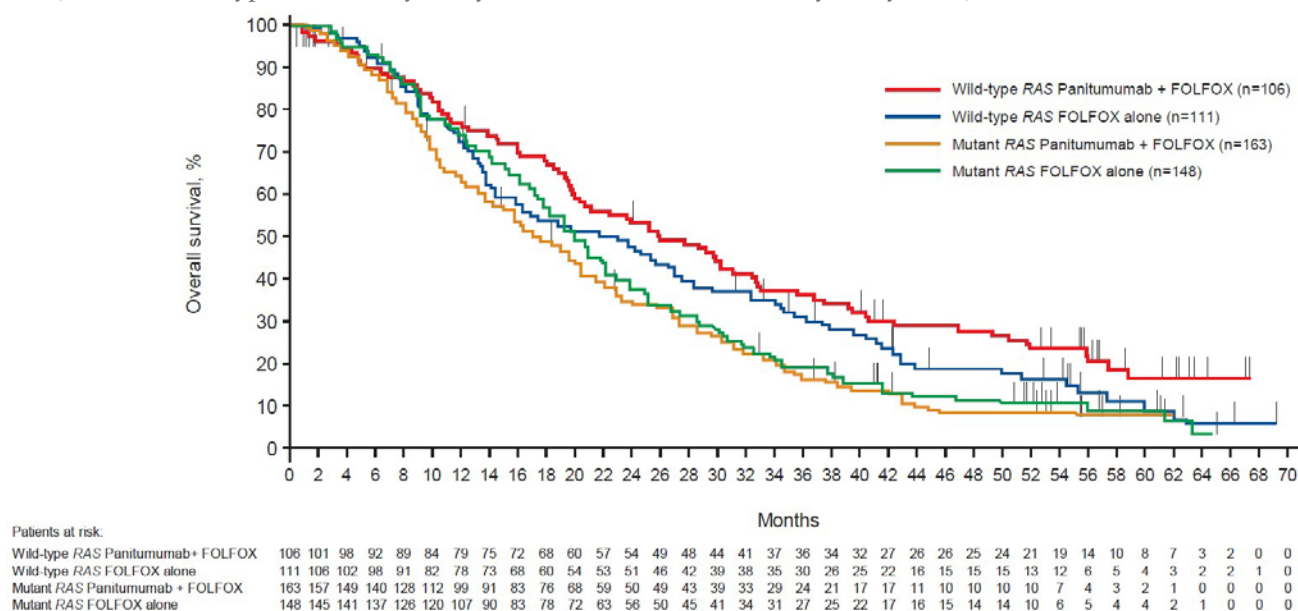
<sup>b</sup>HR presented as panitumumab plus FOLFOX:FOLFOX alone is estimated from Cox proportional hazard model stratified by Region (Western Europe, Canada and Australia vs. Rest of World) and ECOG score (0 or 1 vs. 2).

<sup>c</sup>Subset of Mutant RAS; subjects who did not have a mutation in *KRAS* Exon 2 but did have an extended RAS panel mutation.

**Figure 8** Kaplan-Meier Plot of Progression-free Survival Time by RAS Wild Type vs Mutant and by Treatment, 02 August 2010 (Union of Wild Type RAS Efficacy Analysis Set and Mutant RAS Efficacy Analysis Set)



**Figure 9** Kaplan-Meier Plot of Survival Time by RAS Wild Type vs. Mutant and by Treatment, 24 January 2013  
(Union of Wild Type RAS Efficacy Analysis Set and Mutant RAS Efficacy Analysis Set)



## Summary of Clinical Performance

### Clinical Performance

The latest cutoff date PFS results (02 August 2010) demonstrated that, among subjects with wild type RAS mCRC, panitumumab plus FOLFOX improved PFS, as compared to FOLFOX alone. In addition, the treatment effect is more favorable for wild type RAS subjects in the panitumumab plus FOLFOX group over the FOLFOX alone group when compared to the treatment effect for mutant RAS subjects.

The latest cutoff date OS results (24 January 2013) showed a favorable trend among subjects with wild type RAS mCRC: panitumumab plus FOLFOX improved OS compared to FOLFOX alone. In addition, the treatment effect is more favorable for wild type RAS subjects in the panitumumab plus FOLFOX group over the FOLFOX alone group when compared to the treatment effect for mutant RAS subjects.

In the Mutant RAS group and the wild type *KRAS* Exon 2/Mutant RAS subset (subjects who did not have a mutation in *KRAS* Exon 2 but did have a mutation in *KRAS* exons 3, 4 or *NRAS* exons 2, 3, 4), the trends were in the opposite (unfavorable) direction: PFS and OS are longer in the FOLFOX alone group compared to the panitumumab plus FOLFOX group.

The safety profile for panitumumab in subjects with wild type RAS mCRC was similar to that in subjects with wild type *KRAS* exon 2 mCRC.

The data validated use of the companion diagnostic, Praxis Extended RAS Panel, as an aid in the identification of patients eligible for treatment with panitumumab.

Refer to the drug label at [Drugs@FDA](http://Drugs@FDA) for the most current information about Vectibix (panitumumab).

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