

Praxis[®] Extended RAS Panel

Lab Tracking Form

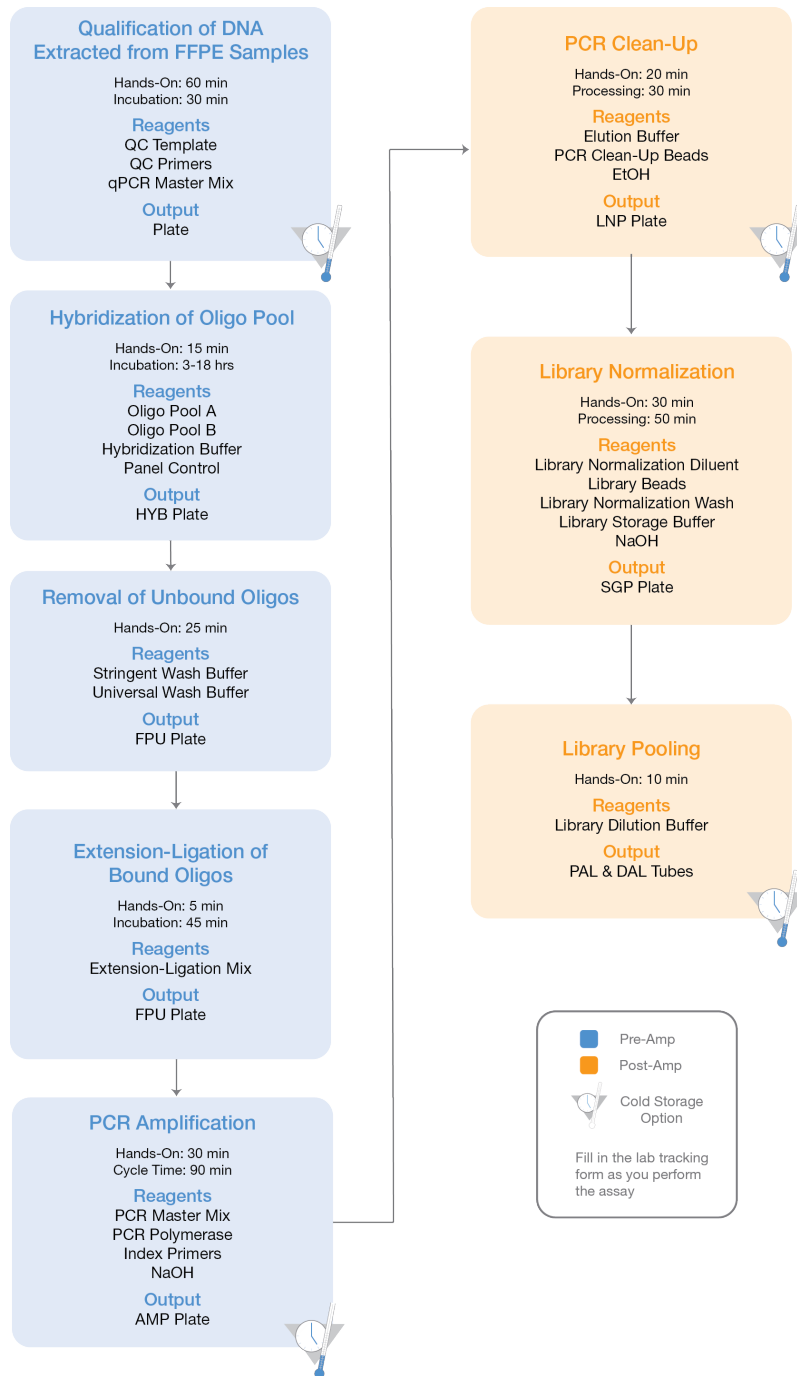
FOR *IN VITRO* DIAGNOSTIC USE

Date: _____

Description: _____

Illumina Kit Lot #: _____

Operator: _____



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Qualification of FFPE DNA Samples

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

Estimated Time

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

Consumables

Illumina Kit Lot #: _____

Item	Lot Number
QC Primers	Lot #: _____
QC Template	Lot #: _____
qPCR Master Mix	Lot #: _____
RNase/DNase-free water	Lot #: _____

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 μ l of QC Template into different PCR strip tubes for long-term storage to avoid freeze-thawing.

Procedure

- 1 Determine the plate layout of the qPCR reaction.



NOTE

Samples, QC Template, and No Template Control (NTC) all must be tested in triplicate. See the *Praxis Extended RAS Panel Reference Guide (document # 1000000037191)* for more information on plate layouts.

- 2 Add 5 μ l of QC Template to 495 μ l of RNase/DNase-free water in a microcentrifuge tube.
- 3 Vortex the dilution to mix the sample.
- 4 Add 1.5 μ l of extracted genomic DNA to 148.5 μ l 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 μ l 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 μ l 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.

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- ▶ 384-Well Format
 - ▶ qPCR Master Mix: 5.0 μ l/well (205 μ l for 36 reactions)
 - ▶ QC Primers: 0.8 μ l/well (33 μ l for 36 reactions)
 - ▶ RNase/DNase-free Water: 2.2 μ l/well (90 μ l for 36 reactions)
- ▶ 96-Well Format
 - ▶ qPCR Master Mix: 10.0 μ l/well (410 μ l for 36 reactions)
 - ▶ QC Primers: 1.6 μ l/well (66 μ l for 36 reactions)
 - ▶ RNase/DNase-free Water: 4.4 μ l/well (180 μ l for 36 reactions)



NOTE

See the *Praxis Extended RAS Panel Reference Guide* (document # 1000000037191) 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 μ l (384-well format) or 16 μ l (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 μ l (384-well format) or 4 μ l (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 \times 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.

qPCR Machine ID: _____

Start time: _____

Stop time: _____



NOTE

The Cq 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 Cq of approximately 15–22 cycles. Exclude replicates from a triplicate group that are > 0.5 Cq different from the rest of the group. At least two of the three replicates less than or equal to 0.5 Cq apart must be

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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 # 1000000037191)* 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 # 1000000037191)*.

- 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.

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Sample ID	Delta Cq	Dilution Factor (i.e. 0, 2x, 4x)	Result (circle one)
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail

Sample ID	Delta Cq	Dilution Factor (i.e. 0, 2x, 4x)	Result (circle one)
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail

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Date/Time: _____

Operator: _____

Sample ID	Delta Cq	Dilution Factor (i.e. 0, 2x, 4x)	Result (circle one)
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail
Sample ID: _____	Delta Cq: _____	Dilution Factor: _____	Pass Fail

Pipette IDs: _____

Comments

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Post-Qualification Consumables

Illumina Kit Lot #: _____

Item	Lot Number
Panel Control	Lot #: _____
TE Buffer	Lot #: _____
Oligo Pool A	Lot #: _____
Oligo Pool B	Lot #: _____
Hybridization Buffer	Lot #: _____
Stringent Wash Buffer	Lot #: _____
Universal Wash Buffer	Lot #: _____
Filter Plate	Lot #: _____
Extension-Ligation Mix	Lot #: _____
PCR Master Mix	Lot #: _____
PCR Polymerase	Lot #: _____
0.05 N NaOH	10 N NaOH
Date Prepared: _____	Lot #: _____
	Template-free Water
	Lot #: _____
Elution Buffer	Lot #: _____
PCR Clean-Up Beads	Lot #: _____
80% Ethanol	100% Ethanol
Date Prepared: _____	Lot #: _____
	Template-free Water
	Lot #: _____
Library Normalization Diluent	Lot #: _____
Library Beads	Lot #: _____
Library Normalization Wash	Lot #: _____
Library Storage Buffer	Lot #: _____
0.1 N NaOH	10 N NaOH
Date Prepared: _____	Lot #: _____
	Template-free Water
	Lot #: _____
Library Dilution Buffer	Lot #: _____
PhiX Control	Lot #: _____
TE Buffer	Lot #: _____
MiSeqDx Cartridge	Lot #: _____
	Lot #: _____
MiSeqDx Flow Cell	Serial #: _____
	Serial #: _____
MiSeqDx SBS Solution (PR2)	Lot #: _____
	Lot #: _____

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Index Primers	
Index Primer A (A501) Lot #: _____	Index Primer 1 (A701) Lot #: _____
Index Primer B (A502) Lot #: _____	Index Primer 2 (A702) Lot #: _____
Index Primer C (A503) Lot #: _____	Index Primer 3 (A703) Lot #: _____
Index Primer D (A504) Lot #: _____	Index Primer 4 (A704) Lot #: _____
Index Primer E (A505) Lot #: _____	Index Primer 5 (A705) Lot #: _____
Index Primer F (A506) Lot #: _____	Index Primer 6 (A706) Lot #: _____
Index Primer G (A507) Lot #: _____	Index Primer 7 (A707) Lot #: _____
Index Primer H (A508) Lot #: _____	Index Primer 8 (A708) Lot #: _____
	Index Primer 9 (A709) Lot #: _____
	Index Primer 10 (A710) Lot #: _____
	Index Primer 11 (A711) Lot #: _____
	Index Primer 12 (A712) Lot #: _____

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Operator: _____

Hybridization of Oligonucleotide Pool

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


Estimated Time

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

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.
Heat Block ID: _____
- 6 Pre-heat an incubator to 37°C.
Incubator ID: _____
- 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.
Run Name: _____
Run Name: _____

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 # 100000037191) for more information on suggested **HYB** plate layout.
Plate ID: _____
- 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 1.

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Table 1 Delta Cq Dilution Table

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

- 6 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 # 1000000037191).

- 7 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.
- 8 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.

- 9 Seal the **HYB** plate and centrifuge 1000 × g at room temperature for 1 minute.

Centrifuge ID: _____

- 10 Place the **HYB** plate in the pre-heated block at 95°C and incubate for 1 minute.

Start time: _____

Stop time: _____

- 11 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.

Start time: _____

Stop time: _____

Pipette IDs:

Comments

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Removal of Unbound Oligonucleotides

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

Estimated Time

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

Preparation

- 1 Bring Extension-Ligation Mix, Stringent Wash Buffer, and Universal Wash Buffer to room temperature, and then vortex briefly.
- 2 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.
Filter Plate ID: _____
- 3 Label the filter plate **FPU_Plate_ID**.
- 4 Pre-wash the filter plate membrane as follows:
 - a Add 50 μ l of Stringent Wash Buffer to each well.
 - b Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.
 - c If the wash buffer does not drain completely, centrifuge the filter plate again at $2400 \times g$ at room temperature for 5 minutes.

Centrifuge ID: _____

Procedure

- 1 Remove the **HYB** plate from the heat block and centrifuge at $1000 \times g$ at room temperature for 1 minute.
- 2 Transfer the entire volume (approximately 65 μ l) of each sample to the corresponding wells of the filter plate.
- 3 Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.
- 4 Wash the filter plate as follows:
 - a Add 50 μ l of Stringent Wash Buffer to each sample well.
 - b Cover the filter plate with the lid and centrifuge at $2400 \times g$ at room temperature for 5 minutes.
 - c If the wash buffer does not drain completely, centrifuge the filter plate again at $2400 \times g$ at room temperature for 5 minutes.



NOTE

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

- 5 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).

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- 6 Discard all the flow-through (containing formamide), then reassemble the **FPU**.
- 7 Add 45 μ 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.

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Operator: _____

Extension-Ligation of Bound Oligonucleotides

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

Estimated Time

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

Procedure

- 1 Add 45 µl 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°C incubator for 45 minutes.
Start time: _____ Stop time: _____
- 4 While the **FPU** plate is incubating, prepare the AMP (Amplification Plate) as described in the following section.

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Operator: _____


PCR Amplification

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

Estimated Time

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

Preparation

- 1 Prepare fresh 0.05 N NaOH by adding 25 μ l of 10 N NaOH to 4975 μ 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 μ l of the selected index primers [A-H (A501-A508)] to the appropriate well in a column of the **AMP** plate.
 - b Add 9 μ 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 μ l of PCR Polymerase to 1050 μ 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 μ 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.

Start time: _____


Stop time: _____

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- 5 While the filter plate is incubating, transfer 22 μ l 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 μ l 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 \times g at room temperature for 1 minute.
- 9 Transfer the AMP plate to the post-amplification area.
- 10 Perform PCR using the following program on a thermal cycler:
 - ▶ 95°C for 3 minutes
 - ▶ 32 cycles of:
 - ▶ 95°C for 30 seconds
 - ▶ 66°C for 30 seconds
 - ▶ 72°C for 60 seconds
 - ▶ 72°C for 5 minutes
 - ▶ Hold at 10°C

PCR Machine ID: _____

Start time: _____

Stop time: _____

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.

Comments

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Operator: _____

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 # 1000000037191).

Lane	Sample ID	Oligo Pool (A or B)	Valid/Invalid

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Date/Time: _____

Operator: _____

Lane	Sample ID	Oligo Pool (A or B)	Valid/Invalid

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Date/Time: _____

Operator: _____

PCR Clean-Up

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

Estimated Time

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

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 × g at room temperature for 1 minute.
Centrifuge ID: _____
- 2 Set out a new MIDI plate (hereafter referred to as the **CLP** plate).
Plate ID: _____
- 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 µl 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.
Start time: _____ Stop time: _____
Microplate Shaker ID: _____
- 7 Incubate at room temperature without shaking for 10 minutes.
Start time: _____ Stop time: _____
- 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 µl 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 µl 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.
Start time: _____ Stop time: _____
- 14 Add 25 µl of Elution Buffer to each sample.

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- 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.

Start time: _____

Stop time: _____

- 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).

Plate ID: _____

- 18 Transfer 20 μ l of the supernatant from the **CLP** plate to the **LNP** plate.

- 19 [Optional] Transfer the remaining 5 μ l of supernatant from the **CLP** plate to a new plate and label the plate with a run name and date. Store this plate at -25°C to -15°C until completion of the sequencing run and data analysis. The cleaned up PCR products can be used for troubleshooting efforts in the event of sample failures.

- 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°C to 8°C.

Pipette IDs:

Comments

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Library Normalization

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


Estimated Time

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

Preparation

- 1 Prepare fresh 0.1 N NaOH by adding 30 μ l of 10 N NaOH to 2970 μ 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 μ l of Library Normalization Diluent to a 15 ml conical tube.
 - b To homogenize beads, use a P1000 pipette set to 1000 μ 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 μ l of Library Beads to the tube containing Library Normalization Diluent.
 - d Mix by inverting the tube 15–20 times.
- 2 Add 45 μ l 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.
Start time: _____ Stop time: _____
- 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 μ l 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.
Start time: _____ Stop time: _____

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- 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 $1000 \times g$ 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.
Start time: _____ Stop time: _____
- 14 During the 5-minute shake, set out a new 96-well PCR plate (hereafter referred to as the **SGP** plate.)
Plate ID: _____
- 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 $1000 \times g$ 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 $1,000 \times g$ at room temperature for 1 minute. Proceed immediately to Library Pooling.

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Library Pooling

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

Estimated Time

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

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.

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- 8 Combine the following volume of pre-chilled Library Dilution Buffer and Denatured Diluted PhiX Control to make PhiX/Library Dilution Buffer solution.
 - ▶ Denatured Diluted PhiX Control (20 μ l)
 - ▶ Pre-chilled Library Dilution Buffer (980 μ l)

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 \times g at room temperature for 1 minute.
- 3 Set out a fresh microcentrifuge tube (hereafter referred to as the **PAL** tube.)
Tube ID: _____
Tube ID: _____
- 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 μ l 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.)
Tube ID: _____ Tube ID: _____
Tube ID: _____ Tube ID: _____
Tube ID: _____ Tube ID: _____
- 8 Add 784 μ l of Library Dilution Buffer to each **DAL** tube.
- 9 Add 8 μ l 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 μ l 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.

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- 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.

Start time: _____

Stop time: _____

- 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.

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Library Sequencing

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

Estimated Time

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

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.

MiSeqDx Serial Number: _____

Date of Last Preventive Maintenance: _____

MiSeqDx Serial Number: _____

Date of Last Preventive Maintenance: _____

- 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.

Run ID: _____

DAL Tube ID: _____

Run ID: _____

DAL Tube ID: _____

- 13 Perform a post-run wash.

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Operator: _____

Comments



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