**Overview of full protocol**

This protocol describes the method for performing dual-barcoding of a tageted amplicon via a two-step PCR process. The first PCR step isolates the target-specific region whereas the second PCR step adds on Illumina sequencing adapters and indexing barcodes. Both PCR primers of the first PCR include universal complementary sequence tags (CS1 and CS2) that amplify the product in the second PCR step. These allow for maximum flexibility in target specific primer usage and the ability to separately barcode or include multiple targets in the same sequencing reaction without needing to purchase a large number of barcoded primers. Barcodes are included in both adapters, giving 24x24 adapter combinations to uniquely identify 576 samples.

The protocol assumes DNA samples are clean and of relatively high quality, with no additional contaminants or salts.

**Ordering FL1 and FL2 sequencing primers for Illumina MiSeq**

The protocol for sequencing dual-barcoded amplicons is dependent upon the FL1 and FL2 primers as designed and explained in the Fluidigm Access ArrayTM System for Illumina Sequencing Systems User Guide (pp.126-136). The following details the steps for ordering and preparing the primers up to the sequencing step.

|  |  |  |
| --- | --- | --- |
| **Name** | **Oligo Name** | **Sequence (5'-3')** |
| FL1 | CS1 | A+CA+CTG+ACGACATGGTTCTACA |
| CS2 | T+AC+GGT+AGCAGAGACTTGGTCT |
| FL2 | CS1rc | T+GT+AG+AACCATGTCGTCAGTGT |
| CS2rc | A+GAC+CA+AGTCTCTGCTACCGTA |

The “+” indicates the following nuleotide is LNA. Go to Exiqon’s website order page for Custom LNATM Oligos (<http://www.exiqon.com/order-lna-oligos>).

Under **Enter an oligonucleotide sequence below**, copy Oligo Name “CS1” from Table 1 for Oligo name.

* Select **DNA oligo** from Oligo type drop-down menu.
* Select **100 nmole** from Synthesis scale drop-down menu.
* Select **HPLC** **Purification** from Purification drop-down menu.
* Copy the sequence for the oligo CS1 from the above table for Sequence (5'-3').
* Repeat steps as above to order CS2, CS1rc, and CS2rc oligos.
* Complete checkout process.

**Preparation of FL1 and FL2 primer mixes for sequencing**

1. Spin tubes down before opening.

2. Calculate the resuspension volume required for each oligo using the following formula:

(X nmol oligo)\* 10 = μL volume needed to resuspend oligo.

3. Add correct volume of low EDTA TE (10 mM Tris pH 8. 0.1mM EDTA) buffer calculated from step 2 to each primer tube such that the final concentration of each primer is 100 μM.

4. Vortex and spin down briefly all components after resuspension in low EDTA TE buffer.

5. Prepare FL1 primer mix. Mix CS1 oligo and CS2 oligo as described below to a final concentration of 50 μM for each oligo. Vortex after mixing to ensure complete mixing. Spin down.

|  |  |  |
| --- | --- | --- |
| **Oligo Name** | **Volume** | **Final Concentration** |
| CS1 (100 μM) | 30 μL | 50 μM |
| CS2 (100 μM) | 30 μL | 50 μM |
| Total | 60 μL |  |

6. Prepare FL2 primer mix. Mix CS1rc oligo and CS2rc oligo as described below to a final concentration of 50 μM for each oligo. Vortex after mixing to ensure complete mixing. Spin down.

|  |  |  |
| --- | --- | --- |
| **Oligo Name** | **Volume** | **Final Concentration** |
| CS1rc (100 μM) | 30 μL | 50 μM |
| CS2rc (100 μM) | 30 μL | 50 μM |
| Total | 60 μL |  |

Store FL1 and FL2 stocks at -20C.

**Protocol**

Materials required:

- Fragment Analyzer 12- capillary array (Advanced Analytical Technologies)

- High sensitivity next-generation DNA Kit (Advanced Analytical Technologies DNF-474)

- PCR grade water, nuclease-free water

- Qubit 2.0 dsDNA Brad Range Assay kit (Life Technologies Q32850)

- Qubit 2.0 Fluorometer (Life Technologies)

- 96-well plate reader (Molecular Devices SPECTRA max GEMINI XPS)

- 96-well plates and adhesive sealing for 96-well plates

- Thermocycler (e.g. Applied Biosystems 4314445 and N8050200)

- PCR reagent kit (New England Biolabs E5000S)

- Magnetic beads (Ampure XP, MagBio)

- Magnetic stand or plate (Life Technologies 12027)

**PCR 1**

1. Quantify template DNA, preferably using fluorometry.

2. Use 100ng of DNA for the PCR 1 reaction, adding more template when necessary. Avoid excessive cycling, which may bias the downstream products with a false signal from PCR duplicates.

3. Prepare a reaction mix for PCR 1 using the following components:

|  |  |
| --- | --- |
| **Reagent** | **Volume** |
| 10X PCR Buffer | 5.0 |
| 25mM MgCl2 | 6.0 |
| 10mM dNTP mix | 1.0 |
| 10uM TS-CS1 forward primer | 0.25 |
| 10uM TS-CS2 forward primer | 0.25 |
| *Taq* DNA polymerase 5000U/mL | 0.25 |
| PCR-grade water | 36.25 |
| Template DNA | 1.0 |

Total reaction volume is 50 uL. Cycle the reaction with the following parameters:

Denaturation: 95C - 2 minutes

Cycle 20 times: 95C – 1 minute

51C – 1 minute

68C – 1 minute

Extention: 68C – 10 minutes

Hold at 4C until ready to proceed.

4. Run 2uL of the product through the Fragment Analyzer to check amplification of the target region. It is not necessary to check each product if the number of samples is too large, but a representative sample should be taken to ensure the amplicon amplified properly without excessive primer-dimers or non-specific amplification. Band sizes should incorporate the length of the CS-tags.

– Stopping point: PCR 1 products may be stored for up to 3 days at 4C, or at -20C for longer-term storage.

**PCR 2**

5. Prepare a 15-fold dilution of the PCR product from PCR 1 as follows: add 2µl of product from first PCR to 28µl of PCR grade water. Vortex well to mix, then centrifuge briefly for 30 seconds to spin down all components. The 1:15 dilute PCR 1 product is now ready to be used as template for PCR 2 below.

6. Prepare the reaction mix for PCR 2 using the following components:

|  |  |
| --- | --- |
| **Reagent** | **Volume** |
| 10X PCR Buffer | 2.0 |
| 25mM MgCl2 | 3.6 |
| 10mM dNTP mix | 0.40 |
| 2uM Barcoded Illumina primer | 0.75 |
| *Taq* DNA polymerase 5000U/mL | 0.20 |
| PCR-grade water | 36.25 |
| Template DNA (PCR 1 product) | 1.0 |

Total reaction volume is 20uL. Cycle the reaction with the following parameters:

Denaturation: 95C – 1 minute

Cycle 10 times: 95C – 30 seconds

60C – 30 seconds

68C – 1 minute

Extention: 68C – 5 minutes

Hold at 4C until ready to proceed.

– Stopping point: PCR 2 products may be stored for up to 3 days at 4C, or at -20C for longer.

**QC, pooling, and cleaning**

1. Quantify all PCR 2 products using fluorometry. Check the size and amplification of a representative sample of products by running on the Fragment Analyzer. If the amplicon is of the expected size (~69bp increased from PCR 1 products if barcoded adapters were successfully added) the sample is ready to be cleaned, pooled, and prepared for sequencing. If necessary, adjust DNA amounts in the previous PCR to optimize DNA yield.

2. Pool amplicon samples together based on DNA mass from fluorometry. Accurate quantification is crucial in this step to correctly measure the amount of double-stranded DNA in each sample without bias from unincorporated adapters. If the samples span a large range of mass, a qPCR quantification step of similarly massed pools may be necessary to determine absolute library masses and to normalize before all samples can be pooled.

3. Clean the amplicon pool(s) of small fragments with magnetic bead-based chemistry using a 0.8X volume of beads to the total volume of the pool (e.g. a 100uL pool will require 80uL of beads for cleaning). Confirm cleaning by running samples on the Fragment Analyzer and determine the average fragment size within the targeted peak.

4. Quantify DNA pool(s) with the KAPA Library Quantification DNA Kit for Illumina (KAPA Biosystems KK4824) according to instructions from the KAPA Library Quantification Technical Guide (v1.14). Serial dilutions are recommended as per protocol specifications.

5. Calculate library concentrations of each pool and normalize all libraries to 10nM. Pooling will be dependent on the concentration and volume of the smallest sample, so this protocol recommends normalizing using the following procedure:

- Transfer 10uL from each pool that was quantified using qPCR into a clean 1.5mL microcentrifuge tube. Calculate the nanomolar concentration of each pool using the masses quantified from the qPCR and the average fragment size from the Fragment Analyzer, and adjust volumes to obtain 10nM.

- If **dilution** is required:

* Determine the appropriate amount of buffer EBT to add for 10nM.
* Vortex 2X and centrifuge tube. Keep on ice

- If **concentration** is required:

* Determine the total volume of sample that is required for 10nM.
* Prepare a vacuum centrifuge to dry samples at 30C.
* Pipette measure sample frequently to check proper volume. Add additional EBT if necessary

6. Pool all normalized libraries together to make a working stock of 10nM. Pool according to the lowest sample volume and desired proportion of reads to come from each sample (e.g. if the smallest sample volume is 5uL and equimolar pooling is desired, pool 5uL from each sample to make the working stock).

Repeat KAPA qPCR quantification as above on the pooled samples.

**Preparing for MiSeq sequencing**

Please refer to the Illumina MiSeqTM User Guide for proper maintenance and operation of the MiSeq.

**\*Note: This protocol recommends at least 15% of the sequencing run to consist of diverse library. Amplicon sequences are not as diverse as general shotgun libraries, and too many clusters with the same sequence may cause the machine to have increased error rates and may even cause a run failure.**

Before beginning:

* Complete all necessary wash steps for the Miseq
* Prepare a sample sheet for the run
* Set a heat block at 96C for the last step heat shock
* Remove the frozen tray component of the 600 cycle MiSeq kit from -20C and allow to thaw at room temperature. Protect from light until ready to sequence.
* Thaw HT1 on ice and keep chilled for the duration of the setup.
* Prepare a fresh dilution of 0.1N NaOH
* Thaw FL1 and FL2 sequencing primers on ice.

1. Combine the following volumes of DNA and freshly diluted 0.1N NaOH in a 1.5μL tube:

* 5 μL 10 nM pooled sample DNA
* 5 μL 0.1N NaOH

Vortex briefly to mix and spin down.

2. Incubate library for 5 minutes at room temperature to denature DNA.

3. Add 990 μL of pre-chilled HT1 to the tube. This results in a 50pM denatured library in 1 mM NaOH. Vortex to mix and keep tube on ice.

4. Determine appropriate library concentration for optimal cluster generation using the below table as a guide. Low to mid-range clustering within the abilities of the MiSeq kit is recommended for low diversity dual-barcoded amplicons so that the sequencer can differentiate individual clusters. 18-20pM concentration for V3 chemistry is the most commonly used library concentration with the technique outlined in this protocol. Add appropriate amounts of denatured library and pre-chilled HT1 in a separate 1.5mL tube.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Final library concentration (pM) | 15pM | 18pM | 20pM | 25pM |
| Volume of 50 pM denatured DNA | 180 | 216 | 240 | 300 |
| **Volume of pre-chilled HT1** | 420 | 384 | 360 | 300 |

5. Vortex the mixture and spin down. Place on ice until ready for the final heat shock and loading. The final input volume is 600μL. If a PhiX control is added it must be in the same final concentration as the DNA and must be added within the final volume of 600μL for loading.

6. Prepare the Read 1 sequencing primer FL1:

* Ensure the reagent tray has fully thawed.
* Dilute 7 uL of sequencing reagent FL1 to a final concentration of 500 nM with 693 uL HT1 buffer. Vortex to mix and spin down.
* Pierce the foil seal covering reservoir 18 with a long pipette tip and load 680uL of diluted FL1 for Read 1.

7. Prepare the Read 2 sequencing primer FL2:

* Dilute 7 uL of sequencing reagent FL2 to a final concentration of 500 nM with 693 uL HT1 buffer. Vortex to mix and spin down.
* Pierce the foil seal covering reservoir 19 with a long pipette tip and load 680uL of diluted FL2 for Read 2.

8. Prepare the Read 3 sequencing primer FL1:

* Dilute 7 uL of sequencing reagent FL1 to a final concentration of 500 nM with 693 uL HT1 buffer. Vortex to mix and spin down.
* Pierce the foil seal covering reservoir 20 with a long pipette tip and load 680uL of diluted FL1 for Read 3.

9. Place the tube containing 600uL of denatured DNA in a 96C heat block for 2 minutes. Remove immediately and chill on ice until ready to load. Pierce foil covering sample reservoir with a long pipette tip and load 600uL into the well. Load reagent tray into MiSeq.

10. Load all necessary reagents into the MiSeq, including flow cell and P1 buffer. Start the run and monitor on BaseSpace.

**Preparing a custom sample sheet for the run**

Please refer to the Illumina MiSeqSample Sheet Quick Reference Guide for information on setting up the sample sheet for a double-barcoded sequencing run with custom sequencing primers. The simplest way to set up a sheet uses the Illumina Experiment Manager available on the Illumina site: (<http://support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.html>)

1. Open the Illumina Experiment Manager

\*Add assay to IEM? Via appendix C of the Schloss protocol