

Access Array™ System for Illumina Sequencing Systems



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Contents

Preface	About this User Guide	
	How to Use This Guide	11
	Document Conventions	11
	Instrument Warnings	12
	Related Documents	12
Chapter 1	Overview	
	Access Array System Overview	14
	4-Primer Amplicon Tagging Overview	16
	Access Array System Components	19
	48.48 Access Array IFC	20
	Access Array System Workflow	21
	Before Getting Started	22
	Required Reagents	22
	Required Consumables	22
	Required Lab Equipment	22
	Example sample and primer layout in 96-well plates	23
Chapter 2	Designing Targeted Sequencing Primers	
	Designing Targeted Sequencing Primers	26
	Reference Documents	26
	Primer Design Procedure	26
Chapter 3	Targeted Sequencing Primer Validation	
	Target-Specific Primer Validation for 4-Primer Amplicon Tagging on the 48.48 Access Array IFC	36
	Reference Documents	36
	Required Reagents	36
	Stored at -20°C	36
	Stored at 4°C	36
	Stored at Room Temperature	36
	Preparing Primer Validation Reactions	37
	Setting up the PCR Reactions in a 384-well PCR Plate	39

Running the PCR Reactions	40
Checking PCR Products on the Agilent 2100 BioAnalyzer	40
Chapter 4 Sample Qualification and Quantification	
Sample Quantitation and Normalization	44
Reference Documents	44
Required Reagents	44
Stored at 4°C	44
Required Equipment	44
Sample Quantitation Procedure	44
Sample Normalization Procedure	44
Chapter 5 4-Primer Amplicon Tagging on the 48.48 Access Array IFC	
4-Primer Amplicon Tagging 48.48 Access Array IFC Introduction	48
Running the 48.48 Access Array IFC	50
Reference Documents	50
Required Supplies	50
Stored at -20°C	50
Store at 4°C	50
Stored at Room Temperature	50
48.48 Access Array IFC Workflow	51
Priming the 48.48 Access Array IFC	51
Preparing the 20X Primer Solutions	52
Preparing Sample Master Mix Solutions	53
Prepare the Sample Pre-Mix Solution	53
Prepare the Sample Mix Solutions	54
Loading the 48.48 Access Array IFC	54
Thermal Cycling the 48.48 Access Array IFC	56
Harvesting the 48.48 Access Array IFC	56
Chapter 6 Multiplex Amplicon Tagging on the 48.48 Access Array IFC	
Multiplex Amplicon Tagging for Illumina on the 48.48 Access Array IFC Introduction	60
Multiplex PCR on the 48.48 Access Array IFC	61
Target-Specific Primer Pooling	61
Reference Documents	62
Required Reagents	62
48.48 Access Array IFC Workflow	63
Priming the 48.48 Access Array IFC	63
Preparing the 20X Primer Solutions	64
Preparing Sample Pre-Mix and Samples	65

Prepare the Sample Pre-Mix Solution	65
Prepare the Sample Mix Solutions	66
Loading the 48.48 Access Array IFC	66
Thermal Cycling the 48.48 Access Array IFC	68
Harvesting the 48.48 Access Array IFC	68
Attaching Sequence Tags and Sample Barcodes	70
Preparing the Sample Pre-Mix Solution	70
Preparing a 100-Fold Dilution of the Harvested PCR Products	70
Preparing the Sample Mix Solutions	71
Thermal Cycling the 96-Well PCR Plate	72
Checking PCR Products on the Agilent 2100 BioAnalyzer	72
Chapter 7 Bidirectional Amplicon Tagging on the 48.48 Access Array IFC	
Bidirectional Amplicon Tagging using the 48.48 Access Array IFC Introduction	74
Reference Documents	78
Required Reagents	78
Multiplex PCR on the 48.48 Access Array IFC	79
2-Primer Target-Specific PCR on the 48.48 Access Array IFC	79
Barcode PCR Products in Two 96-Well Plates for Bidirectional Amplicon Tagging	79
.	79
Chapter 8 Post-PCR Amplicon Purification and Quantitation	
PCR Product Purification and Quantification	82
Reference Documents	82
Required Equipment	82
Procedure	82
Required Reagents	82
Stored at 4°C	82
Stored at Room Temperature	82
Qualification of PCR Products	83
Pooling Products from Multiple Access Array IFCs	83
Purification of Harvested PCR Products	83
Agilent 2100 Bioanalyzer Qualification:	84
PCR Product Library Quantification Procedure	85
Picogreen Fluorimetry Quantification:	85
PCR Product Library Concentration	86
Appendix A Using the Fluidigm FC1 Cycler	
Using the Fluidigm FC1 Cycler	88
Powering On the FC1 Cycler	89
Login	89

Prepare Chip for Thermal Cycling	90
Running a Protocol	90
Cleaning Protocol	91
Troubleshooting	92

Appendix B 4-Primer Amplicon Tagging on the BioMark System

Running the 48.48 Access Array IFC on the BioMark System	94
Required Reagents	94
Stored at -20°C	94
Stored at 4°C	94
Stored at Room Temperature	94
Required Equipment	94
Priming the 48.48 Access Array IFC	95
Preparing the 20X Primer Solutions	96
Preparing Sample Master Mix Solutions	96
Prepare the Sample Pre-Mix Solutions	96
Preparing the Sample Mix Solutions	98
Loading the 48.48 Access Array IFC	98
Data Acquisition	99
Running the Access Array IFC with Detection Chemistry	99
Running the Access Array IFC without Detection Chemistry	100
Harvesting the 48.48 Access Array IFC	100
PCR Data Analysis	101

Appendix C 2-Primer Target-Specific PCR Amplification

2-Primer Target-Specific PCR Amplification Overview	104
Target-Specific Primer Validation for	
2-Primer Reactions on the 48.48 Access Array IFC	107
Reference Documents	107
Required Reagents	107
Stored at -20°C	107
Stored at 4°C	107
Stored at Room Temperature	107
Preparing the Primer Validation Reaction	107
Setting up the PCR Reactions in a 384-well PCR Plate	110
Running the PCR Reactions	111
Checking PCR Products on the Agilent 2100 BioAnalyzer	111
2-Primer Target-Specific PCR Amplification on the 48.48 Access Array IFC ..	114
Reference Documents	114
Required Supplies	114
48.48 Access Array IFC Workflow	115
Priming the 48.48 Access Array IFC	115

Preparing the 20X Primer Solutions	116
Preparing Sample Mix Solutions	116
Loading the 48.48 Access Array IFC	118
Thermal Cycling the 48.48 Access Array IFC	119
Harvesting the 48.48 Access Array IFC	119
Appendix D Electropherogram Examples	
Examples of Electropherograms	122
Examples of an Agilent 2100 BioAnalyzer Electropherogram of a harvested PCR product pool	122
Appendix E Ordering Instructions for FL1 and FL2 Sequencing Primers	
Ordering and Preparing FL1 and FL2 Sequencing Primers for the Illumina Sequencing Systems	126
Intended Use	126
Ordering Oligos for FL1 and FL2 Primers	126
Preparation of FL1 and FL2 Primer Mixes	128
Appendix F Sequencing Workflow Using Fluidigm FL1 and FL2 Sequencing Primers	
Sequencing Workflow Using Fluidigm FL1 and FL2 Sequencing Primers	132
Reference Documents	132
Preparing Reagents for Sequencing on the Illumina GAIi and HiSeq Sequencing Systems	133
Preparing Read 1 Sequencing Primer HT1/FL1 for the cBot	133
Adding Read 2 Indexing Primer FL2 to HP8	133
Adding Read 3 Sequencing Primer FL1 to HP7 (for Paired-End Sequencing)	133
Preparing Reagents for Sequencing on the Illumina MiSeq Sequencer	134
Preparing Read 1 Sequencing Primer FL1	134
Preparing Read 2 Indexing Primer FL2	134
Preparing Read 3 Sequencing Primer FL1 (for Paired-End Sequencing only)	135
Performing a Sequencing Run	136
Index Read	136
Appendix G Guidelines for Using FFPE Genomic DNA on the 48.48 IFC	
Section 1: Introduction	140
Section 2: Extraction of Genomic DNA from FFPE tissue Samples	141
Reference Documents	141

Required Reagents	141
Required Equipment	141
Guidelines	141
Section 3: Sample Quality Assessment	143
Reference Documents	143
Required Reagents	143
Required Equipment	143
Absolute copy number determination with a 180 bp TaqMan assay	145
Prepare Standard Curve DNA	146
Prepare the PCR Master Mix	146
Prepare the Sample Mix Solutions	146
Thermal Cycling of the 384-Well Plate	147
Section 4: Amplicon Tagging on the Access Array IFC	148
Reference Documents	148
Required Reagents	148
Required Equipment and Consumables	149
General Considerations	149
Specific Target Amplification (Preamplification)	152
Prepare the Preamplification Primer Pool	152
ExoSAP-IT Treatment of the Preamplification Products	154
Prepare a 5-fold Dilution of the Preamplification Products	154
Amplicon Tagging on the 48.48 Access Array IFC	155
Priming the 48.48 Access Array IFC	155
Enrichment PCR with Target-Specific Primers on the 48.48 Access Array IFC	156
Prepare the 20X Primer Solutions	156
Prepare the Sample Mix Solutions	157
Prepare the Sample Pre-Mix Solution	158
Prepare the Sample Mix Solutions	158
Load the Sample Inlets of the 48.48 Access Array IFC	159
Thermal Cycling the 48.48 Access Array IFC	159
Harvest PCR Products from the 48.48 Access Array IFC	159
Attaching Sequence Tags and Sample Barcodes	161
Prepare the Sample Pre-Mix Solution	161
Prepare a 100-Fold Dilution of the Harvested PCR Products	161
Prepare the Sample Mix Solutions	162
Thermal Cycling of the 96-Well PCR Plate	162
PCR Product Quantitation and Qualification	163
PCR Product Pooling and Purification	164
Section 5: BioAnalyzer Gel Image Example	165

Appendix H Access Array Barcodes for the Illumina Sequencing Systems

Access Array Barcode Library for Illumina Sequencers	168
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About this User Guide

How to Use This Guide

The following chapters provide information about protocols of the Access Array for Illumina Sequencing Systems.

Document Conventions

This guide uses specific conventions for presenting information that may require your attention. Please refer to the conventions below.



CAUTION! This convention highlights potential bodily injury or potential equipment damage upon mishandling of the Access Array System. Read and follow instructions and/or information in a caution note very carefully to avoid any potential hazards.



WARNING! This convention highlights situations that may require your attention. May also indicate correct usage of instrument or software.



IMPORTANT: This convention highlights situations or procedures that are important to the successful outcome of your experiments.



NOTE: This convention highlights useful information.

Instrument Warnings



HOT SURFACE! The FC1 Cycler chuck gets hot and can burn your skin.
Please use caution when working near the chuck.

Related Documents

This document is intended to be used in conjunction with these related documents:

- *Fluidigm® Access Array™ - Generate Tagged Primers Workbook* (PN 100-3873)
- *Fluidigm® IFC Controller for Access Array™ System User Guide* (PN 68000157)
- *Fluidigm® FC1™ Cycler User Guide* (PN 100-1279)
- *Fluidigm Control Line Fluid Loading Procedure Quick Reference* (PN 68000132)
- *Fluidigm Access Array IFC 2-Primer Workflow Quick Reference* (PN 68000148)
- *Fluidigm Access Array IFC 4-Primer Amplicon Tagging Workflow Quick Reference* (PN 68000161)

Overview

1

Access Array System Overview.	14
4-Primer Amplicon Tagging Overview	16
Access Array System Components.	19
Access Array System Workflow	21
Before Getting Started.	22

Access Array System Overview

Amplicon tagging on the Access Array™ System significantly reduces the time required for enrichment of targeted sequences by combining amplicon generation with library preparation. The Access Array System workflow consists of four major phases: 1) designing and validating target-specific primers for targeted resequencing, 2) qualifying and quantifying samples, 3) running an Access Array Integrated Fluidic Circuit (IFC), and 4) qualifying and quantifying harvested PCR products for sequencing. The chapters in this user guide detail this workflow chronologically.

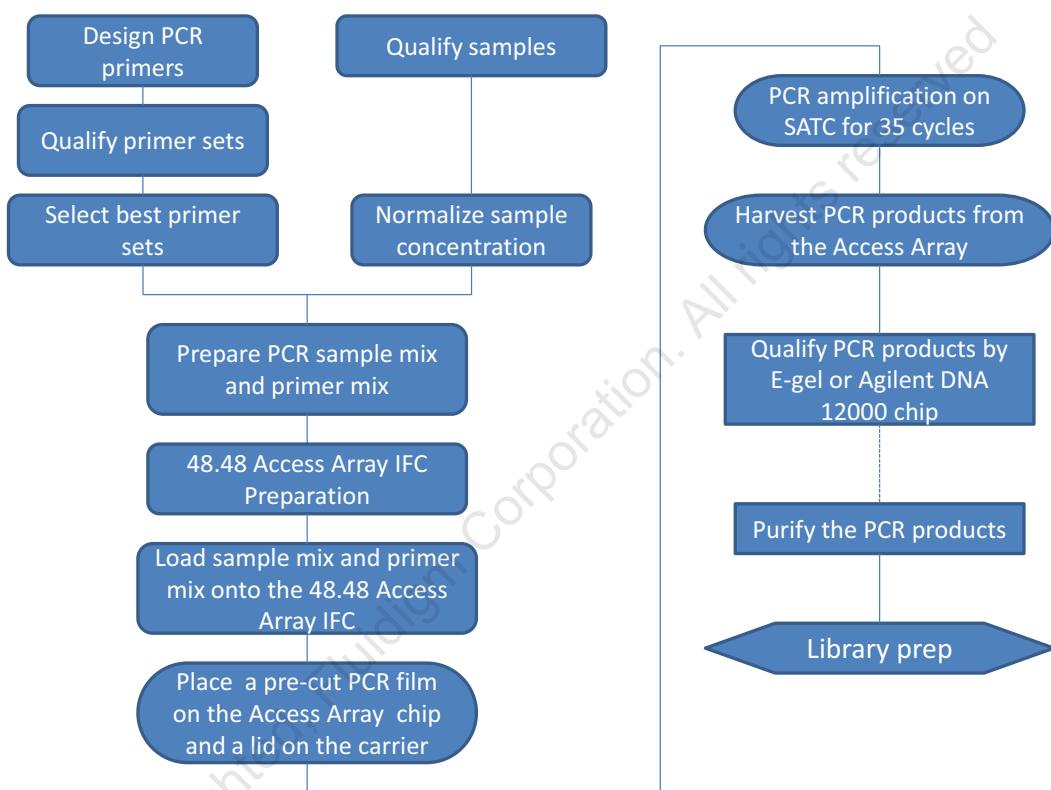


Figure 1 Overview

Depending on the type of experiment, there are several Access Array amplicon tagging approaches for generating single-direction or bidirectional amplicon libraries to sequence on next generation sequencers such as the Illumina MiSeq™, Genome Analyzer II™ (GAII) or HiSeq™. The figure below illustrates some of the various amplicon tagging strategies and sequencing options.

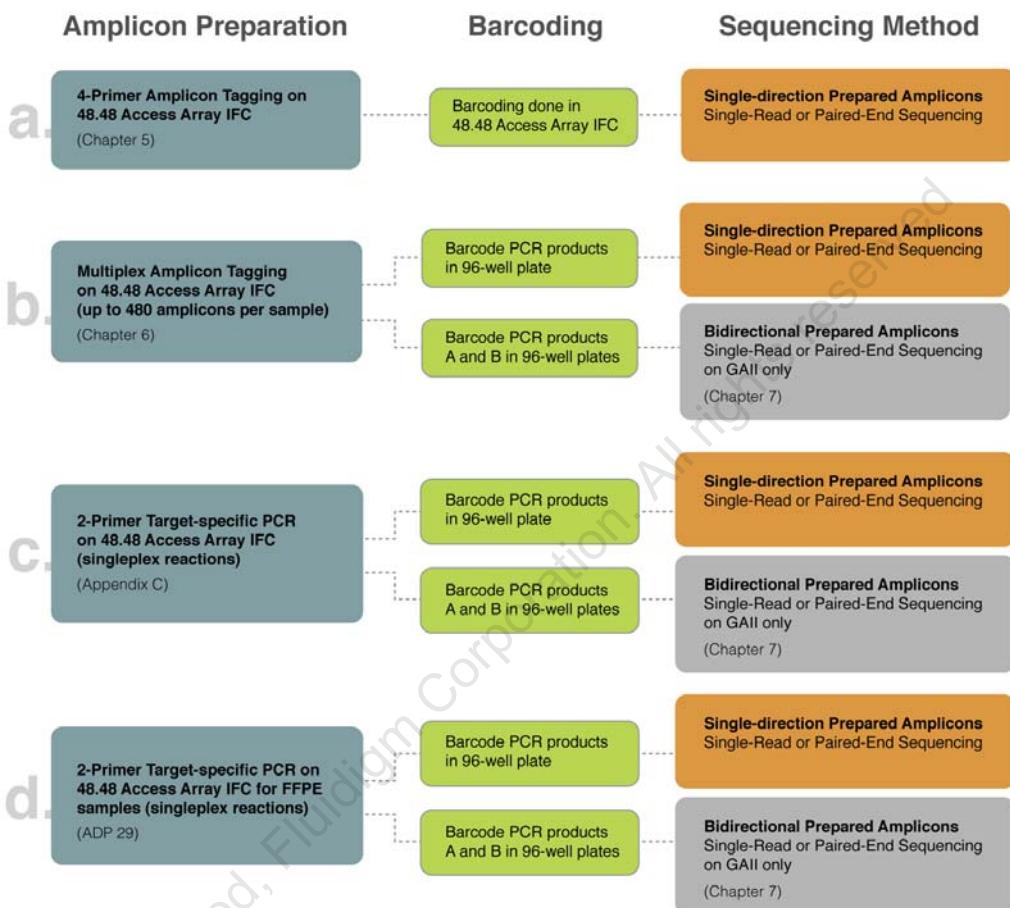


Figure 2 Access Array amplicon tagging approaches



WARNING! Bidirectional prepared amplicons should only be sequenced on a GAII at this time.



NOTE: For FFPE samples, refer to *Advanced Development Protocol 29 (ADP 29): Guidelines for Using FFPE Genomic DNA on the 48.48 Access Array IFC*. ADP 29 is not included in this user guide. Please contact Technical Support for more information.

4-Primer Amplicon Tagging Overview

The Access Array 4-primer amplicon tagging scheme is based on a step-out four primer PCR strategy where tagged target-specific (TS) primer pairs are combined with sample-specific primer pairs that contain a barcoding sequence and the adaptor sequences used by Illumina sequencing systems. By incorporating sample-specific barcodes, all 2,304 PCR products generated from a 48.48 Access Array Integrated Fluidic Circuit (IFC) are unique and can be pooled together to run in a single sequencing experiment.

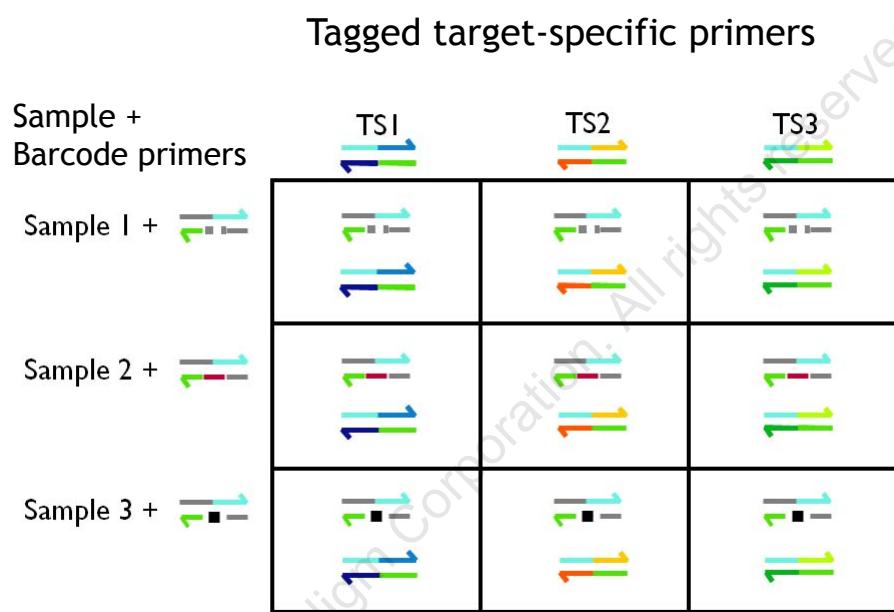


Figure 3 Combination of target-specific primers and sample-specific barcodes using the Access Array IFC. In this example, adaptor sequences and barcodes appropriate for use on the Illumina are shown. A 3 x 3 grid of reaction wells is shown before any reagents are loaded into the 48.48 Access Array IFC.

Tagged target-specific primers

Sample +
Barcode primers

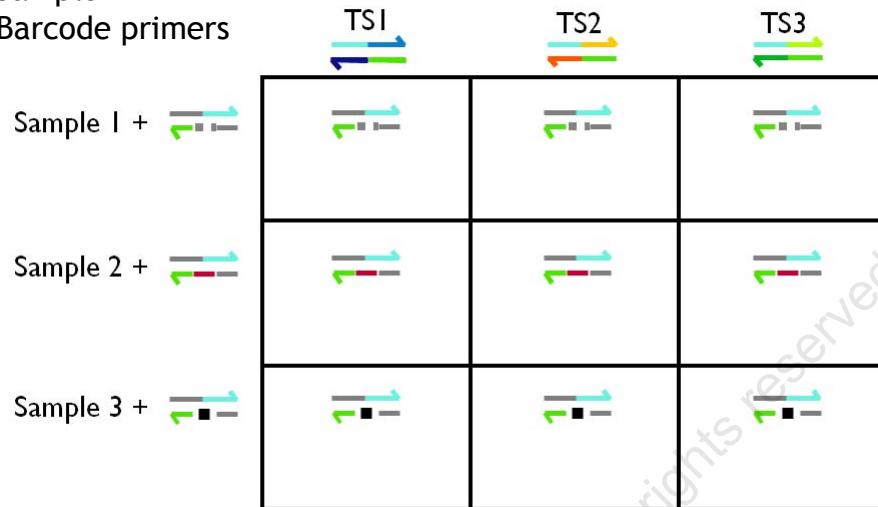


Figure 4 Load the samples and the Illumina sample-specific primers into each row of reaction wells on the 48.48 Access Array IFC.

Tagged target-specific primers

Sample +
Barcode primers

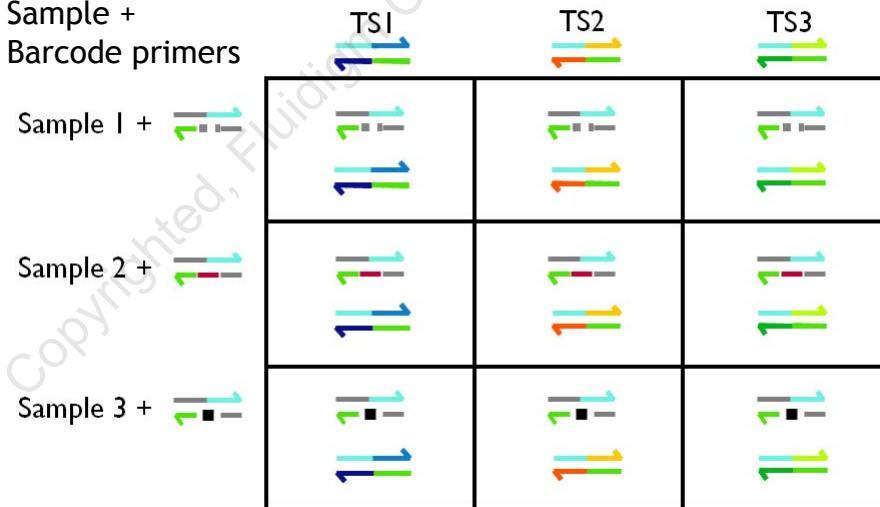


Figure 5 Load the tagged TS forward and reverse primers into each column of reaction wells on the 48.48 Access Array IFC.

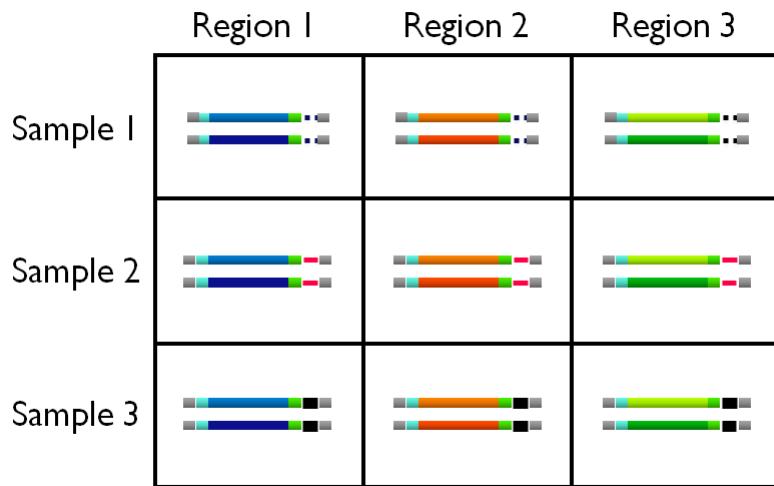


Figure 6 After loading the 48.48 Access Array IFC, each reaction well contains a unique combination of TS Primer pairs and Samples. Each row of reaction wells also contains an Illumina barcode primer pair.

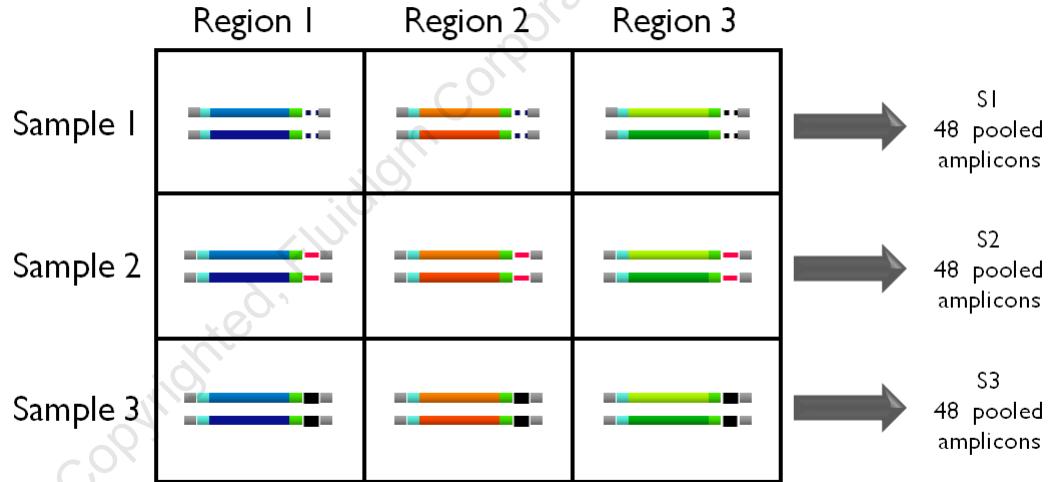


Figure 7 Resultant PCR Products

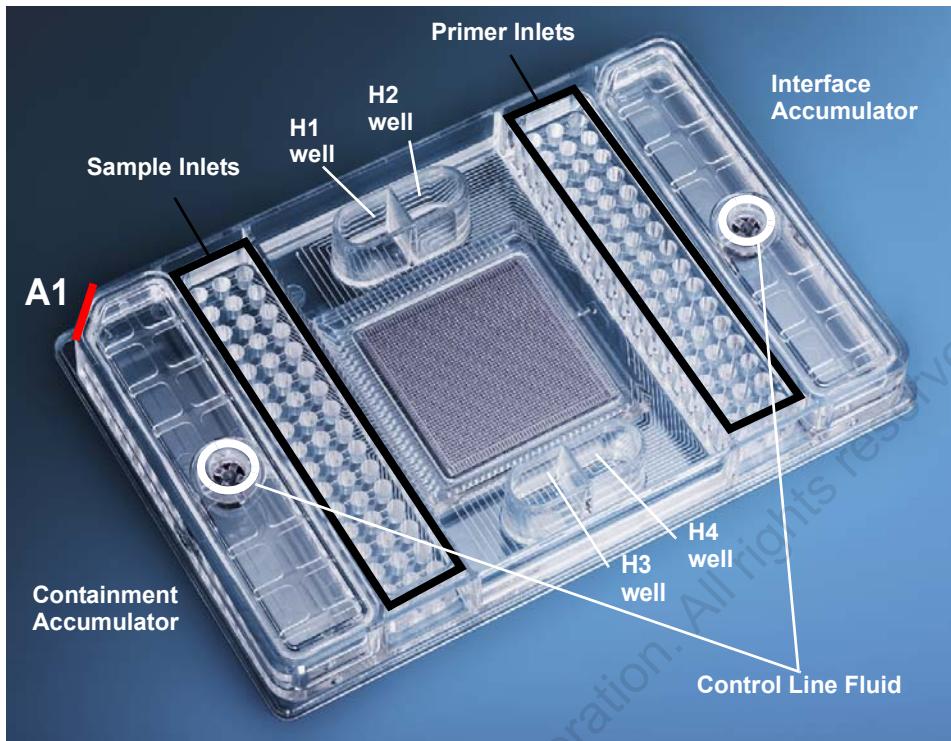
Access Array System Components

The Access Array System consists of the following components:

- 48.48 Access Array Integrated Fluidic Circuits (IFC)
- IFC Controller AX (2 quantity, for **Pre-PCR** and **Post-PCR**)
- FC1™ Cycler or Stand-Alone Thermal Cycler



48.48 Access Array IFC



The high-throughput 48.48 Access Array IFC enables target enrichment of 48 unique samples at the same time. See the table below for specifications.

Specifications

Description and Parameter(s)	
Footprint dimensions	128 x 85 x 14 mm
Inlet spacing on input frame	4.5 mm pitch
Primer inlets	48
Sample inlets	48
Reaction chambers	2,304
Reaction volume	35 nL
Instrument compatibility	IFC Controller AX, FC1 Cycler, Stand-Alone Thermal Cycler, BioMark, BioMark HD system

Access Array System Workflow

The simplicity of running experiments is illustrated in the process below. For more details, see the *Access Array IFC 2-Primer Workflow Quick Reference Card*, (Fluidigm, PN 68000148).

The Access Array System workflow consists of five major steps:

1 Dispense

Transfer samples and primers to the inlets on the 48.48 Access Array IFC from a standard 96-well plate, using an 8-channel pipette.

2 Load

Reaction mixtures are automatically loaded and assembled on the IFC using the **Pre-PCR** IFC Controller AX.

3 Thermal Cycle

PCR amplification is performed using the FC1 Cycler or Stand-alone Thermal Cycler.

4 Harvest

PCR products from each sample are automatically pooled and harvested using the **Post-PCR** IFC Controller AX.

5 Recover

Recover the PCR products from each of the 48 sample inlets, using an 8-channel pipette.

Before Getting Started

This section describes the materials required to perform an Access Array experiment including the supported reagents and consumables.

Required Reagents

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)
- Access Array Barcode Library for Illumina Sequencers-384 (Single Direction) (Fluidigm, PN 100-4876)

OR

- Access Array Barcode Library for Illumina Sequencers-384 (Bidirectional) (Fluidigm, PN 100-3771)
- Agilent DNA 1000 Kit (Agilent, PN 5067-1504)
- PCR Certified Water (TEKnova, PN W330)
- DNA Suspension Buffer (10mM TRIS, pH 8.0, 0.1mM EDTA) (TEKnova, PN T0221)
- Agencourt® AMPure® XP Reagent Beads (Beckman Coulter Genomics, PN A63880)
- 100% Ethanol

Required Consumables

- 48.48 Access Array IFC (Fluidigm)
- Control line fluid syringes (Fluidigm, PN 890000020)
- Microcentrifuge tubes, 1.5 mL
- 96- and 384-well PCR plates
- Adhesive seals for PCR plates
- P2-P1000 pipet tips (Rainin recommended)
- Agilent DNA 1000 Kit (Agilent, PN 5067-1504)

Required Lab Equipment

- IFC Controller AX (2 quantity, **Pre-PCR** and **Post-PCR**) (Fluidigm)
- FC1™ Cycler or Stand-Alone Thermal Cycler (Fluidigm)
- Microcentrifuge
- Vortex mixer
- Plate centrifuge
- 96- and 384-well PCR thermal cycler
- Single-channel P2-P1000 pipettes (Rainin recommended)
- 8-channel P20 pipette (Rainin recommended)

- Agilent 2100 Bioanalyzer
- DynaMag™-2 Magnet (Invitrogen, PN 123-210)

Example sample and primer layout in 96-well plates

For ease of pipetting, fill the 96-well plates by columns, not rows.

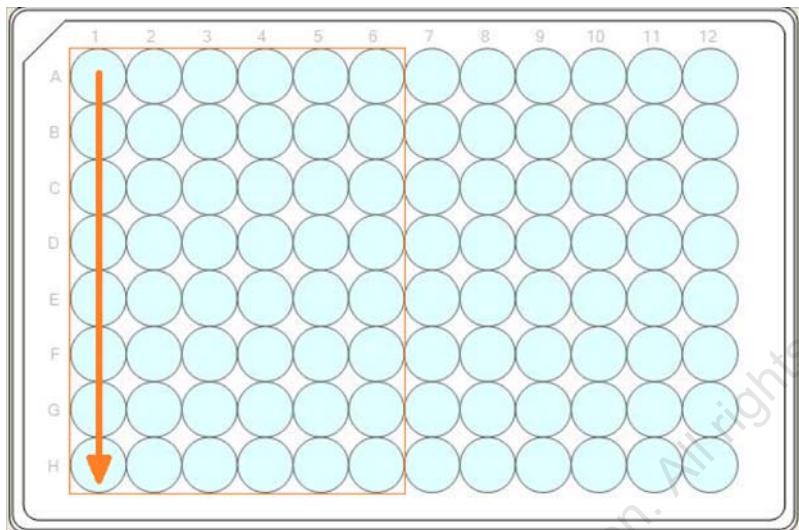


Figure 8

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Designing Targeted Sequencing Primers

2

Designing Targeted Sequencing Primers	26
Reference Documents	26
Primer Design Procedure	26

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Designing Targeted Sequencing Primers

This chapter describes how to design primers for targeted sequencing on the Illumina platforms.



NOTE: If you are using primers provided by the Fluidigm Assay Design Group, please refer to the order informatics packet for primer sequences.

This document uses the EGFR exon 11 sequence as an example for primer design.

EGFR Exon	Chromosome	Chromosome Start Position	Chromosome Stop Position	Size	Fragment Position
11	7	55225356	55225446	91	chr7:55225356-55225446



NOTE: In order to find the fragment position of the targeted gene, go to the UCSC website: <http://genome.ucsc.edu/cgi-bin/hgGateway>, for example, and search for the target gene name to find the target region for primer design.

Reference Documents

- Access Array-Generate Tagged Primers.xls Workbook (Fluidigm, PN 100-3873)

Primer Design Procedure



NOTE: The optimal size range to obtain uniform coverage across amplicons is to design assays targeting >150 bp regions. However, amplicon lengths should be within 20% of the average in a given library.

- 1 Find the targeted DNA sequence.
 - a Go to the UCSC website to submit a query of the **fragment position** of the targeted gene:
<http://genome.ucsc.edu/cgi-bin/hgGateway>

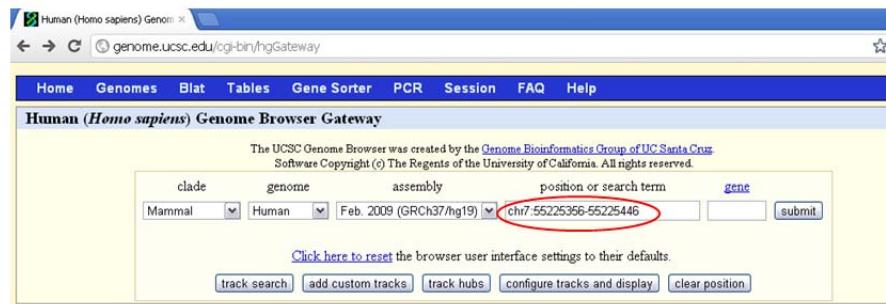


Figure 1

- b** Submit the query and find the targeted gene fragment in the UCSC database. Click the DNA link circled below to get the sequence of the targeted DNA fragment.

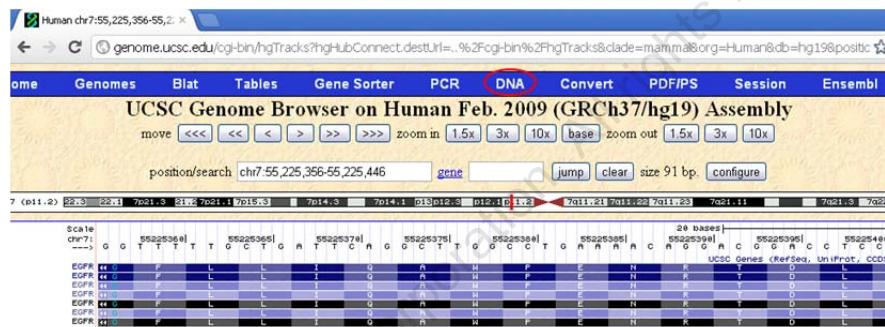


Figure 2

- c In the new window, find the **Sequence Retrieval Region Options** section and enter **50** for both upstream and downstream bases. Next, click the **get DNA** button circled below to get the DNA sequence with 50 base pairs (bp) of upstream and downstream flanking sequence.

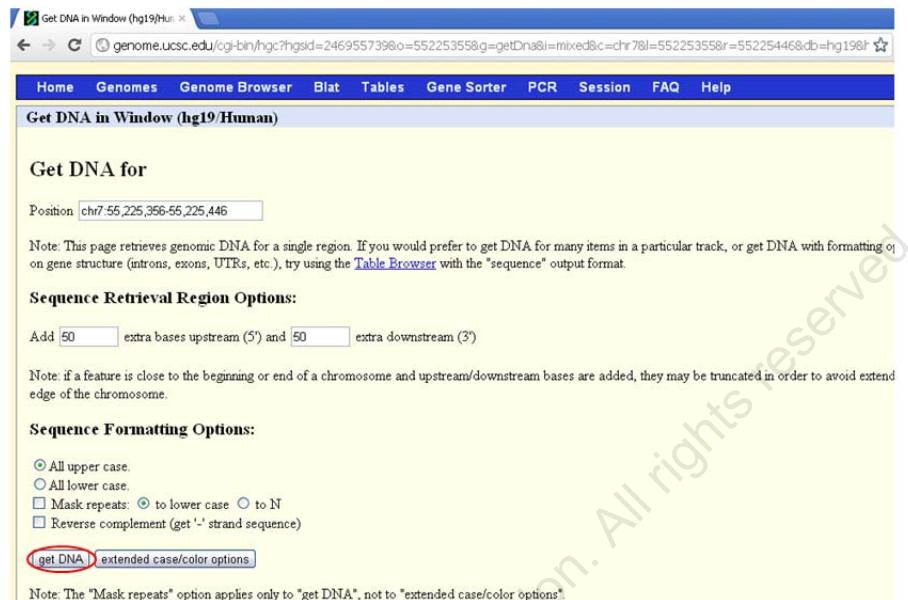


Figure 3

- d The extra flanking sequence will ensure the PCR product covers the entire targeted DNA fragment. The primers should be designed within the flanking regions of the target fragment (see Figure 4 below). A shorter flanking sequence can be selected, such as 25 bp, if a primer can be designed within that flanking sequence.

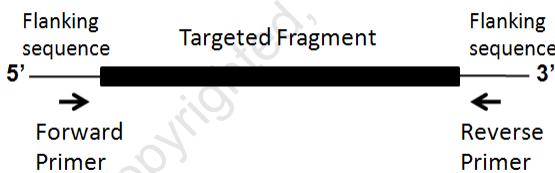


Figure 4

- e If the targeted gene fragment of interest is longer than 180-200 bp, the fragment should be split into multiple segments with 70 bp of overlapping sequence included in each segment. The 70 bp of overlapping sequence in each segment will ensure the primers are designed to produce PCR products that cover the entire targeted gene sequence.

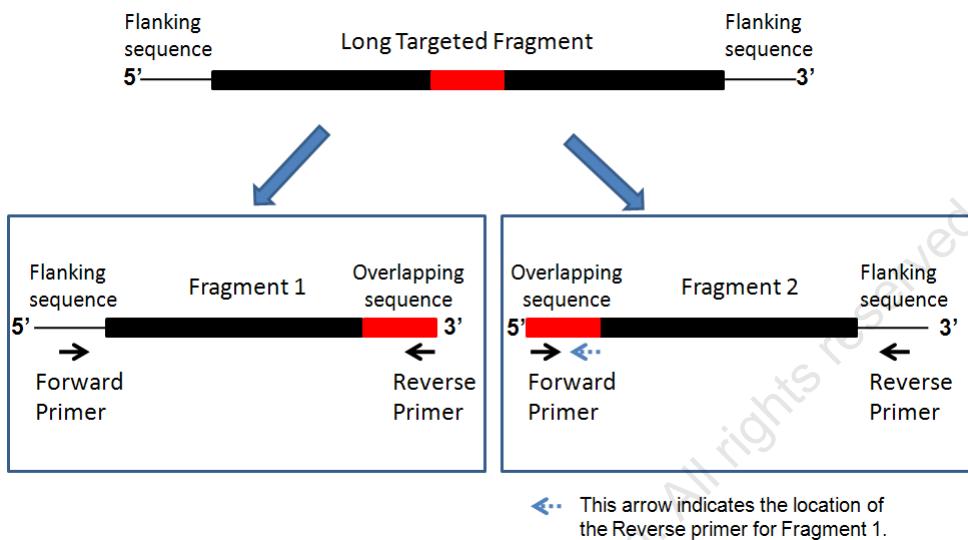


Figure 5



NOTE: The Reverse Primer for Fragment 1 should be located downstream of the Forward Primer for Fragment 2 without overlapping. This will ensure complete coverage of the entire overlapping sequence region.

- f Copy the fragment sequence from the new window below and save it in a text file for the primer design.

A screenshot of a web browser window showing a DNA sequence from the UCSC Genome Browser. The URL in the address bar is genome.ucsc.edu/cgi-bin/hgc?hg19_dna range=chr7:55225306-55225496 5'pad=50 3'pad=50 strand=+ repeatMasking=none. The sequence itself is as follows:

```
>hg19_dna range=chr7:55225306-55225496 5'pad=50 3'pad=50 strand=+ repeatMasking=none
AAAGA&ACTCTACGTGGTGTGCTGAAGCTTTATCTGCCCTACAG
GGTTTTGCTGATTCAAGCTTGGCCTGAAAACAGGACGGACCTCCATGCC
TTTGAGAACCTAGAAATCATACGCGGGAGGACCAACAGTAAGTTGA
CCACAGCCTAAAGCCTGGTAGATTACATTGCCCTTTAGTT
```

Figure 6

2 Design the TS Primers.

- a Go to the Primer3 website to design the TS Forward and Reverse Primers:
<http://frodo.wi.mit.edu>

- b** Paste the fragment sequence from Step 1f into the Primer3 window.

Primer3 (v. 0.4.0) Pick primers from a DNA sequence.

Paste source sequence below (5'->3', string of ACGTNaCgn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUS, LINEs, etc.) or use a [Mismapping library/repeat library](#): **NONE**

```
!NG19_dna range=chr7:55225306-55225496 S'pad=50 3'pad=50 strand=+ repeatMasking=none
AAAGAAACTCCATGGCTGTTGTCATGCTTGAAAGTCCTTCATCTGCCCTACAG
GGTTTTGCTGATTCAAGCTTGGCCCTGAAAAACAGGACGGACCTCATGCC
TTTGAGACCTAGAAAATCATACGGCCAGGACCAAACACAGTAAGTTGA
CCACAGCCAAGCCTGGTAGATTACATTGGCTTTTAGTT
```

Pick left primer, or use left primer below Pick hybridization probe (internal oligo), or use oligo below Pick right primer, or use right primer below (5' to 3' on opposite strand)

Pick Primers **Reset Form**

Figure 7

- c** Change the following parameters to get more stringent primer conditions as shown in Figure 8 below:

- 1 In the **Targets** parameter, put in the *start, length* where *start* is the index of the first base of a Target, and *length* is its length. For this EGFR example, put 51,91, since the gene target region started at position 51 after the 50 bases 5' flanking sequence and the fragment length is 91.
- 2 Change **Product Size Ranges** to cover the target region (target length + 60 bp) up to 180 bp. For this EGFR fragment (91bp), choose 120-200.
- 3 Change **Primer T_m**, Min: **59.0**, Opt: **60.0** and Max: **61.0**.
- 4 Change **Max Poly-X** to 3.



NOTE: These changes will optimize the primers for PCR. If the options are too stringent to design primers for the target sequence, change **Primer T_m** and **Max Poly-X** back to the default settings.

Sequence A string to identify your output.
 Id
Targets: E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the [source sequence](#) with [and]. e.g. ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.
Excluded Regions: E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the [source sequence](#) with < and >. e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.
Product Size Range:
 Number To Return Max 3' Stability
 Max Repeat Mispriming Pair Max Repeat Mispriming
 Max Template Mispriming Pair Max Template Mispriming

General Primer Picking Conditions

Primer Size Min: Opt: Max:
Primer Tm Min: Opt: Max: Max Tm Difference: [Table of thermodynamic parameters](#): Breslauer et al. 1986
 Product Tm Min: Opt: Max:
 Primer GC% Min: Opt: Max:
 Max Self Complementarity Max 3' Self Complementarity
 Max #N's: Max Poly-X:
 Inside Target Penalty: Outside Target Penalty: Note: you can set Inside Target Penalty to allow primers inside a target sequence.
 First Base Index: CG Clamp:
 Concentration of monovalent cations: Salt correction formula: [Schildkraut and Lifson 1965](#)
 Concentration of divalent cations: Concentration of dNTPs:
 Annealing Oligo Concentration: (Not the concentration of oligos in the reaction mix but of those annealing to template.)
 Liberal Base Show Debugging Info Do not treat ambiguity codes in libraries as consensus Lowercase masking

Figure 8

- d Click **Pick Primers** to design the primers.
- e Select the primer pairs circled in Figure 9 below. The >>> arrows indicate the location of the primers. The **** asterisks indicate the location of the targeted gene fragment. Use the first default set of primers. More choices are available from the same web page if needed.

PRIMER PICKING RESULTS FOR hg19_dna range=chr7:55225306-55225496 5' pad=50 3' pad=50 strand=+ repeatMasking=none

No mispriming library specified
Using 1-based sequence positions

OLIGO	start	len	tm	gc%	any	3' seq
LEFT PRIMER	9	21	60.21	52.38	4.00	3.00 TCCTACGTGGTGTGTC
RIGHT PRIMER	163	20	60.30	50.00	3.00	3.00 GCTTTGGCTGTGGTCAACTT

SEQUENCE SIZE: 191
INCLUDED REGION SIZE: 191

PRODUCT SIZE: 155, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00
TARGETS (start, len)*: 51,91

```

1 AAAGAAACTCCTACGTGGTGTGTCAGAGTCCTTCATCTGCCTTACAGGGTTTTGCT
>>>>>>>>>>>>>
61 GATTCAAGGCTTGGCCTGAAAAACAGGACGGACCTCCATGCCTTGAGAACCTAGAAATCAT
*****
121 ACGGGGAGGACCAAGCACAGTAAGTTGACCACAGCCAAGGCTGGTAGATTACATTG
***** <<<<<<<<<<<<<<
181 CCTTTTAGTT

```

Figure 9

- f If Primer 3 fails to design primers with the current settings, additional flanking sequences should be added upstream or downstream of the fragment used for primer design. Add the additional flanking sequences and repeat Steps 2-3e.
- 3 Predict the PCR product using an In-Silico PCR Tool.**
- a Go to the UCSC website:
<http://genome.ucsc.edu/cgi-bin/hgPcr?hgsid=136961442>
 - b Copy the Forward and Reverse Primers from the Primer3 window and paste the primers into the In-Silico PCR page. Then click Submit.

UCSC In-Silico PCR

Genome: Human Assembly: Feb. 2009 (GRCh37/hg19) Target: genome assembly

Forward Primer: TCCTACGTGGTGTGTC (circled)
Reverse Primer: GCTTTGGCTGTGGTCAACTT (circled)

Max Product Size: 4000 Min Perfect Match: 15 Min Good Match: 15 Flip Reverse Primer:

Figure 10

- c Click the circled link as shown in Figure 11 below. Make sure the PCR product matches the targeted gene and is located within the targeted sequence fragment region.

The screenshot shows a web browser window with the URL http://genome.ucsc.edu/cgi-bin/hgPcr?hgSID=246962433&org=Human&db=hg19&wp_target=genome&wp_f=TCCTACGTGGTGTGTC&wp_r=AAAGC. The page title is "UCSC In-Silico PCR". The main content area displays a sequence of DNA with a red box highlighting a specific region: "chr7:55225314+55225468 155bp TCCTACGTGGTGTGTCAGA GCTTTGGCTGTGGTCAACTT". Below this, several lines of sequence are shown: "TCCTACGTGGTGTGTCAGA GCTTTGGCTGTGGTCAACTT", "ctgattcaggcttgccgtgaaacacggacggacccatgcctttagaa", "cctagaaatcatacgcggcaggaccacaaacagtAAAGTGACCAAGCC", and "AAAGC".

Figure 11

NOTE: If the in-silico PCR search predicted multiple products and not a unique one that matches the target gene region, a new set of primers will need to be designed repeating the steps described above.

- d Record the good primer pairs in a spreadsheet with the target name and the forward/Reverse Primer sequences. These TS Forward and Reverse Primer pairs can be used directly for standard PCR amplification experiments.
- 4 Add universal sequence tags to the TS Forward and Reverse Primers for Access Array amplicon tagging experiments.**
- a Use the **Access Array-Generate Tagged Primers.xls Workbook** (Fluidigm, PN 100-3873) to add universal sequencing tags to the Forward and Reverse TS primers (see Figure 12). Refer to the “Instructions” worksheet in the Access Array-Generate Tagged Primers Workbook for details.



IMPORTANT: Add universal sequence tags to the TS Forward and Reverse Primers before moving forward.

Contents of this workbook

This workbook has been designed to assemble primer sequences containing the appropriate tag sequences for use with the Fluidigm 4-primer tagging protocol for Access Array.

The workbook contains 5 worksheets:

- 1) Example Barcode Primers
- 2) Example Target Specific Primers
- 3) Forward Primer Worksheet
- 4) Reverse Primer Worksheet

This sheet contains examples of the barcode primers designed for use in the Fluidigm 4-Primer tagging protocol for Access Array.

This sheet contains examples of forward and reverse target specific primers containing the appropriate tag sequence with the Fluidigm 4-primer tagging protocol for Access Array.

This worksheet will generate tagged forward primers.

This worksheet will generate tagged reverse primers.

How to use this workbook

Design forward and reverse primers for your target regions according to the guidelines provided in the "Primer Design for Access Array" protocol.

Generating Forward Primers (Forward Primer Worksheet)

Copy the forward, target-specific sequences into the "Target-Specific Sequence (Forward)" column.

Enter the name of the target gene in the "Target Name" column.

Up to 96 target primer sequences can be entered into the worksheet.

The tagged sequences are generated in the "Oligonucleotide Sequence" column.

Names for the tagged constructs are generated in the "Oligo Name" column.

Instructions Example Target Specific Primers Forward Primer Worksheet Reverse Primer Worksheet

Figure 12

The universal Forward tag is called common sequence 1 (CS1), and the universal Reverse tag is called common sequence 2 (CS2). The CS1 and CS2 universal tags are designed to be used with the Access Array Barcode Library for Illumina Sequencers - 384. The sequences of these barcodes are provided in [Appendix H](#).

Targeted Sequencing Primer Validation

3

Target-Specific Primer Validation for 4-Primer Amplicon Tagging on the 48.48 Access Array IFC	36
Preparing Primer Validation Reactions	37
Setting up the PCR Reactions in a 384-well PCR Plate	39
Running the PCR Reactions.	40
Checking PCR Products on the Agilent 2100 BioAnalyzer	40

Target-Specific Primer Validation for 4-Primer Amplicon Tagging on the 48.48 Access Array IFC

This chapter describes the validation procedure for the Tagged TS primers designed in Chapter 2.



IMPORTANT: Access Array Barcode 1 Primers for Illumina must be ordered prior to primer validation. Please see Table 1 for oligo sequences and order through an oligonucleotide vendor.

Reference Documents

- *Agilent DNA 1000 Kit Guide*

Required Reagents

Stored at -20 °C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- Access Array Barcode 1 Primers for Illumina with CS1/CS2 tags
- Target-specific primer pairs tagged with universal tags (CS1 Forward tag, CS2 Reverse tag)
 - 50 µM CS1-Tagged TS Forward Primer
 - 50 µM CS2-Tagged TS Reverse Primer
- 60 ng/µL Genomic DNA (Coriell, PN NA17317, optional)

Stored at 4 °C

- Agilent DNA 1000 Kit Reagents (PN 5067-1504)

Stored at Room Temperature

- PCR certified water (TEKnova, PN W3330)

Preparing Primer Validation Reactions

The Primer Validation protocol prepares enough reagents to perform 48 primer validation reactions.



IMPORTANT: It is essential to have Access Array Barcode 1 Primers before proceeding with the primer validation. Please see Table 1 below for the oligo sequences.

Name	Sequence (5'-3')	Total Length
PE1_CS1 Forward Primer	AATGATAACGGCGACCACCGAGATCTACACTGACGACATG GTTCTACA	47bp
PE2_BC_CS2 Reverse primer	CAAGCAGAAGACGGCATACGAGATGTATCGTCGTTACGG TAGCAGAGACTTGGTCT	56bp

Table 1 Access Array Barcode 1 Primers for Illumina

- 1 Prepare the 5X Target-Specific Primer Solutions for 48 individual primer pairs as shown in the table below.

Component	Volume (μ L)	Final Concentration
50 μ M CS1-Tagged TS Forward Primer	1	250 nM
50 μ M CS2-Tagged TS Reverse Primer	1	250 nM
PCR Certified Water (TEKnova)	198	
Total	200	

Table 2 5X Target-Specific Primer Solution Preparation

- 2 Vortex the 5X Primer Solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final Tagged TS Forward and Reverse Primer concentrations are 250 nM in the 5X Primer Solution. The final TS Forward and Reverse Primer concentrations in the PCR reaction are 50 nM.

3 Prepare 2 µM Access Array Barcode 1 Primer Solution.

Component	Volume (µL)	Final Concentration
100 µM PE1_CS1 Forward Primer	2.0	2 µM
100 µM PE2_BC_CS2 Reverse Primer	2.0	2 µM
PCR Certified Water (TEKnova)	96.0	
Total	100	

Table 3 2 µM Access Array Barcode 1 Primers for Illumina

4 Prepare the primer validation reaction components.



IMPORTANT: Warm up the 20X Access Array Loading Reagent to room temperature before use.

Component	Volume per reaction (µL)	Volume for 60 reactions (µL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl ₂ (Roche)	0.5	30.0	1X
25 mM MgCl ₂ (Roche)	0.9	54.0	4.5 mM
DMSO (Roche)	0.25	15.0	5%
10mM PCR Grade Nucleotide Mix (Roche)	0.10	6.0	200 µM ea
5 U/µL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/µL
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
2 µM Access Array Barcode 1 Primers for Illumina (from step 3)	1.0	60.0	400 nM
60 ng/µL Genomic DNA (Coriell)	0.83	49.8	10 ng/µL
PCR Certified Water (TEKnova)	0.12	7.2	
Total	4.0	240.0	

Table 4 4-Primer Amplicon Tagging Primer Validation Reaction Preparation

5 Vortex the Primer Validation Reaction Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The volumes provided in the table above are sufficient for 60 5 µL reactions on a 384-well plate. We recommend preparing this amount to provide sufficient reagent to minimize errors due to pipetting.

Setting up the PCR Reactions in a 384-well PCR Plate

- 1 Prepare the Primer Validation PCR reactions:
 - a Add 4 µL of Primer Validation Reaction Mix to 48 wells.
 - b Add 1 µL of the 5X Target-Specific Primer Solution.
The total PCR reaction volume is 5 µL.
- 2 Vortex the 384-well PCR reaction plate for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Running the PCR Reactions

- 1 Load the 384-well plate onto the PCR thermal cycler.
- 2 Run 35 cycles of PCR using the protocol described below:

PCR Stages	Number of Cycles
50°C 2 minutes	1
70°C 20 minutes	1
95°C 10 minutes	1
95°C 15 seconds 60°C 30 seconds 72°C 1 minute	10
95°C 15 seconds 80°C 30 seconds 60°C 30 seconds 72°C 1 minute	2
95°C 15 seconds 60°C 30 seconds 72°C 1 minute	8
95°C 15 seconds 80°C 30 seconds 60°C 30 seconds 72°C 1 minute	2
95°C 15 seconds 60°C 30 seconds 72°C 1 minute	8
95°C 15 seconds 80°C 30 seconds 60°C 30 seconds 72°C 1 minute	5

Table 5 PCR Protocol

Checking PCR Products on the Agilent 2100 BioAnalyzer

- 1 Use the Agilent DNA 1000 chips from the Agilent DNA 1000 Kit to check 1 μ L of PCR product from each of the PCR reactions described above. Follow the *Agilent DNA 1000 Kit Guide* for details.
- 2 Check the results of the chip to determine if the PCR product in the DNA reaction has the expected size.

-
- 3 On-target products should account for a minimum of 50% of the total yield (by mass) produced for a particular primer pair. If on-target products comprise <50% of total yield, primers may have to be redesigned.



NOTE: The product size should be the sum of the target region and the length of the Access Array Barcode 1 Primers for Illumina (103 bp).

- 4 Figure 1 shows an example of 48 primer pairs checked on an Agilent chip. The red labeled reactions did not generate products with the correct size or generated multiple products in the PCR reactions. Therefore these primers will need to be redesigned following the instructions in Chapter 2 and validated before continuing.

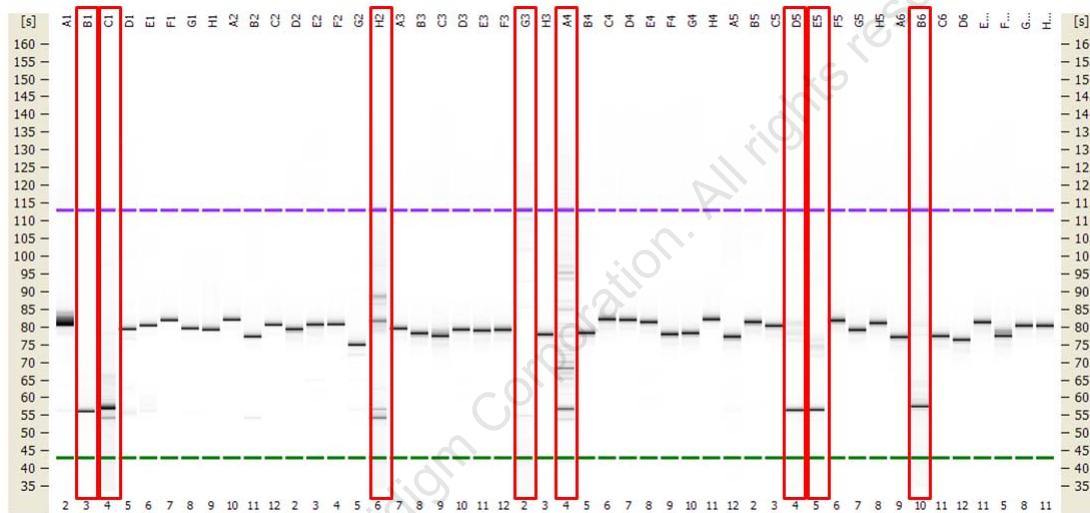


Figure 1 48 primer pairs checked on an Agilent chip

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Sample Qualification and Quantification

4

Sample Quantitation and Normalization	44
Reference Documents	44
Required Reagents	44
Required Equipment	44
Sample Normalization Procedure	44

Sample Quantitation and Normalization

This chapter provides a standard procedure to determine sample concentration using fluorimetry. Sample concentrations need to be normalized before proceeding with the 48.48 Access Array IFC protocol.

The Quant-iT™ PicoGreen® fluorescent assay requires 1 μ L of sample DNA to determine sample concentration.

Reference Documents

Quant-iT™ PicoGreen® User Guide

Required Reagents

Stored at 4°C

- Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, P11496)

Required Equipment

- Fluorimeter-compatible 96- or 384-well microtiter plates

Sample Quantitation Procedure

Quantitate the Samples by fluorimetry, using the Quant-iT PicoGreen dsDNA Assay Kit, following the manufacturer's instructions.

Sample Normalization Procedure

The following recommendations apply to Human genomic DNA samples. For other samples, the recommendations on sample concentrations may be different. Please contact Fluidigm Technical Support for assistance in this case.

- 1 If the sample concentration is between 25-50 ng/ μ L, the sample is ready for amplification on the 48.48 Access Array IFC.
- 2 If the sample concentration is below 25 ng/ μ L, we recommend concentrating the sample before amplification takes place on the Access Array.
- 3 If the sample concentration is above 50 ng/ μ L, we recommend that the sample is diluted to 50 ng/ μ L using DNA Suspension Buffer before proceeding.

-
- a Use the following formula to determine the correct volume of DNA Suspension Buffer required to dilute each sample to 50 ng/ μ L:

$$Y = X (B/50 - 1)$$

Y is the DNA Suspension Buffer volume (μ L) needed to dilute X μ L of the original sample to 50 ng/ μ L

Where X is the volume of the original sample (μ L) to be used in the dilution

B is the sample concentration (ng/ μ L) measured by fluorimetry
50 is the desired sample concentration (ng/ μ L)

For example: If a 10 μ L sample (X = 10 μ L) has a concentration of 200 ng/ μ L (B = 200 ng/ μ L):

$$Y = 10 \mu L * ((200 \text{ ng}/\mu L)/(50 \text{ ng}/\mu L) - 1)$$

$$Y = 30 \mu L$$

Therefore: Dilute 10 μ L of the 200 ng/ μ L sample in 30 μ L of DNA Suspension Buffer to obtain a 50 ng/ μ L sample concentration.

- 4 Normalize all sample concentrations to 50 ng/ μ L using step 3a.
Samples are now ready for amplification using the 48.48 Access Array IFC.

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4-Primer Amplicon Tagging on the 48.48 Access Array IFC

5

4-Primer Amplicon Tagging 48.48 Access Array IFC Introduction	48
4-Primer Amplicon Tagging 48.48 Access Array IFC Introduction	49
48.48 Access Array IFC Workflow	51

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4-Primer Amplicon Tagging 48.48 Access Array IFC Introduction

Amplicon tagging on the Access Array™ System provides a simple path to generating sequencer-ready PCR products. During PCR, sequencer-specific tags and sample-specific barcodes are added to each of the PCR products, reducing the time required for enrichment of target sequences ahead of sequencing. This protocol describes the generation of Illumina sequencer compatible libraries. The 4-primer amplicon tagging scheme is based on a 4-primer PCR strategy where tagged target-specific (TS) primer pairs are combined with sample-specific primer pairs that contain a barcoding sequence and the adaptor sequences used by the Illumina sequencing systems. By incorporating sample-specific barcodes, all 2,304 PCR products generated from a 48.48 Access Array IFC are unique and can be pooled together and run in a single sequencing experiment. The location of the barcode is such that standard single-read or paired-end sequencing can be used following Illumina guidelines.

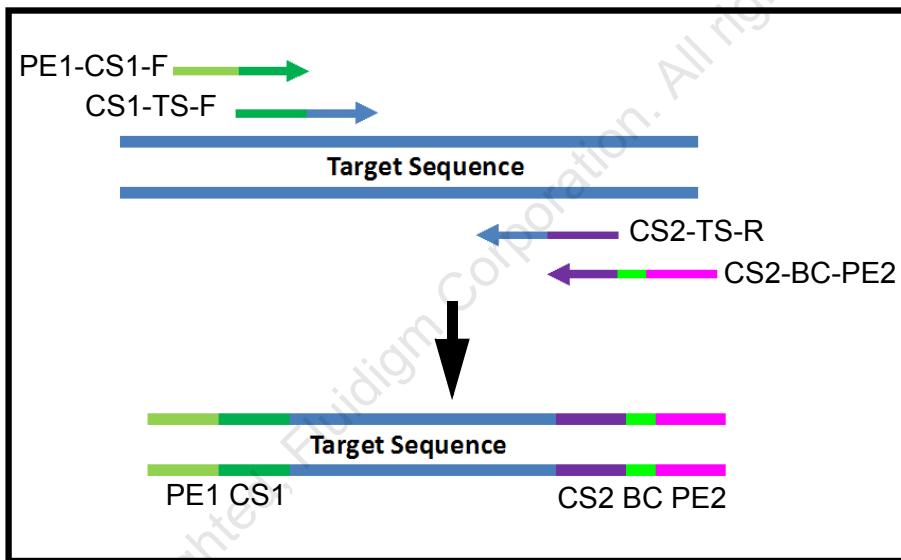


Figure 1 Overview of the 4-primer PCR strategy
CS1 = tag 1; CS2 = tag 2; TS = Target-specific primer sequence; PE1 = Paired end sequence 1; PE2 = Paired end sequence 2; BC = Barcode sequence

# of Samples	Access Array #	Barcode Plate Number	Barcode Plate Part Number	Reagent Description	Portion of Plate
1-48	1	A1	100-4897	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 1-96 at 2 µM per oligo	Left Half
49-96	2	A1	100-4897	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 1-96 at 2 µM per oligo	Right Half
97-143	3	A2	100-4898	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 97-192 at 2 µM per oligo	Left Half
144-192	4	A2	100-4898	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 97-192 at 2 µM per oligo	Right Half
193-240	5	A3	100-4899	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 193-288 at 2 µM per oligo	Left Half
241-288	6	A3	100-4899	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 193-288 at 2 µM per oligo	Right Half
289-336	7	A4	100-4900	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 289-384 at 2 µM per oligo	Left Half
337-384	8	A4	100-4900	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2 289-384 at 2 µM per oligo	Right Half

Table 2 Suggested barcode configuration for up to 384 samples using the Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, PN 100-4876) for single direction amplicon preparation.

Running the 48.48 Access Array IFC

Reference Documents

- *Fluidigm® IFC Controller for Access Array™ System User Guide* (PN 68000157)
- *Fluidigm® Control Line Fluid Loading Procedure Quick Reference* (PN 68000132)
- *Agilent DNA 1000 Kit Guide*

Required Supplies

Stored at -20°C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)
- Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, PN 100-4876)
- Target-specific primer pairs tagged with universal tags (CS1 forward tag, CS2 reverse tag)
 - 50 µM CS1-Tagged TS Forward Primer
 - 50 µM CS2-Tagged TS Reverse Primer
- Template DNA at 50ng/µL



NOTE: 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) is not packaged for individual sale. It can be purchased in units of 10, under the name Access Array Harvest Pack, PN 100-3155, or as a component in the 48.48 Access Array Loading Reagent Kit, PN 100-1032.

Store at 4°C

- Agilent DNA 1000 Kit Reagents (Agilent, PN 5067-1504)

Stored at Room Temperature

- PCR Certified Water (TEKnova, PN W330)

48.48 Access Array IFC Workflow

Priming the 48.48 Access Array IFC



CAUTION! Use the 48.48 Access Array IFC within 24 hours of opening the package.

CAUTION! Control Line Fluid on the IFC or in the inlets makes the IFC unusable. Use only 48.48 syringes with 300 µL of Control Line Fluid (Fluidigm, PN 89000020).

CAUTION! Load the IFC into the **Pre-PCR** IFC Controller AX in the Pre-PCR lab within 60 minutes of priming.

- 1 Inject Control Line Fluid into each accumulator on the IFC.
- 2 Add 500 µL of 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) into the H1-H4 wells on the IFC.
- 3 Remove and discard the blue protective film from the bottom of the AA IFC.
- 4 Load the IFC into the **Pre-PCR** IFC Controller AX located in the Pre-PCR lab.
- 5 Press **Eject** to move the tray out of the IFC Controller AX.
- 6 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 7 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 8 Select **Prime (151x)** and **Run Script** to prime the IFC.
- 9 Once the script is complete, press **Eject** to remove the IFC.

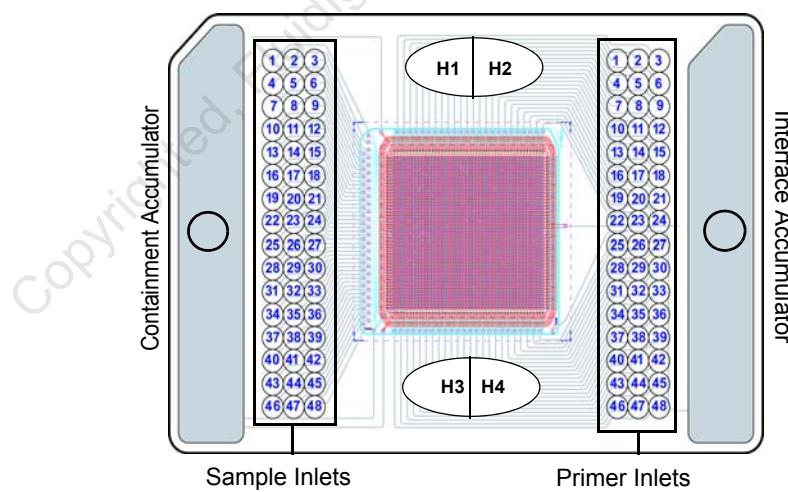


Figure 2 48.48 Access Array IFC overview

Preparing the 20X Primer Solutions



NOTE: If you are using primers provided by the Fluidigm Assay Design Group, please refer to the order informatics packet for correct primer concentrations.

- 1 Prepare the 20X Primer Solutions for 48 individual primer pairs as shown in the table below. These will be loaded into the Primer Inlets of an 48.48 Access Array IFC.



IMPORTANT: Warm up the 20X Access Array Loading Reagent to room temperature before use.

Component	Volume (μL)	Final Concentration
50 μM CS1-Tagged TS Forward Primer	2.0	1 μM
50 μM CS2-Tagged TS Reverse Primer	2.0	1 μM
20X Access Array Loading Reagent (Fluidigm)	5.0	1X
PCR Certified Water (TEKnova)	91.0	
Total Volume	100.0	

Table 3 20X Target-Specific Primer Solution

- 2 Vortex the 20X Primer Solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final Tagged TS forward and reverse primer concentrations are 1 μM in the 20X Primer Solution. The final TS forward and reverse primer concentrations in the Access Array reaction chamber are 50 nM.

Preparing Sample Master Mix Solutions

All DNA samples and the 384 barcodes need to be added into the Sample Pre-Mix individually, prior to loading the Sample Mix solutions into the Sample Inlets of an 48.48 Access Array IFC.

- 1 Working in a DNA-free hood, combine the components listed in the table below.

Prepare the Sample Pre-Mix Solution

This protocol prepares enough Sample Pre-Mix for 60 reactions. This is enough reagent to load one 48.48 Access Array IFC with 16 additional reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 60 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl ₂ (Roche)	0.50	30.0	1X
25 mM MgCl ₂ (Roche)	0.90	54.0	4.5 mM
DMSO (Roche)	0.25	15.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.10	6.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/ μL
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
PCR-Certified Water (TEKnova)	0.95	57.0	
Total Volume	3.0	180.0	

Table 4 Sample Pre-Mix Solution

- 1 Vortex the Sample Pre-Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare the Sample Mix Solutions

- 1 Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume per Reaction (μ L)	Final Concentration
Sample Pre-Mix	3.0	
50 ng/ μ L Genomic DNA	1.0	10 ng/ μ L
Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction), Plate A1	1.0	400 nM
Total	5.0	

Table 5 Sample Mix Solutions

- 2 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



IMPORTANT: It is essential to vortex all components to ensure complete mixing.



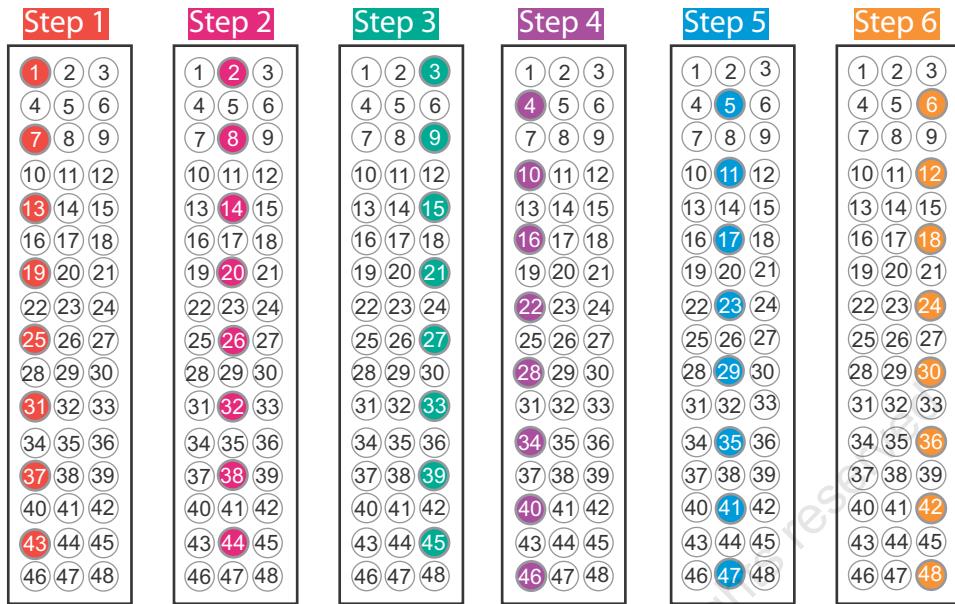
NOTE: Each well should receive a uniquely barcoded primer pair. The final Access Array Barcode Library forward and reverse primer concentrations are 400 nM in the Sample Mix solutions.

Loading the 48.48 Access Array IFC

- 1 Pipette 4 μ L of 20X Primer Solution into each of the Primer Inlets.
- 2 Pipette 4 μ L of Sample Mix solution into each of the Sample Inlets.



NOTE: An 8-channel pipette is recommended to load the Sample Mix and 20X Primer Solutions. The recommended pipetting order is shown below.

**Figure 3** Pipetting guide

- 3 Load the 48.48 Access Array IFC into the Pre-PCR IFC Controller AX in the Pre-PCR lab.
- 4 Press **Eject** to move the tray out of the IFC Controller AX.
- 5 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 6 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 7 Select **Load Mix (151x)** and **Run Script**.
- 8 Once the script is complete, press **Eject** to remove the IFC.

Thermal Cycling the 48.48 Access Array IFC

- 1 Place the 48.48 Access Array IFC onto the FC1 Cycler and start PCR by selecting the protocol **AA 48x48 Standard v1**.

See the FC1 Cycler appendix at the end of this manual for more detailed instructions about using the cycler.

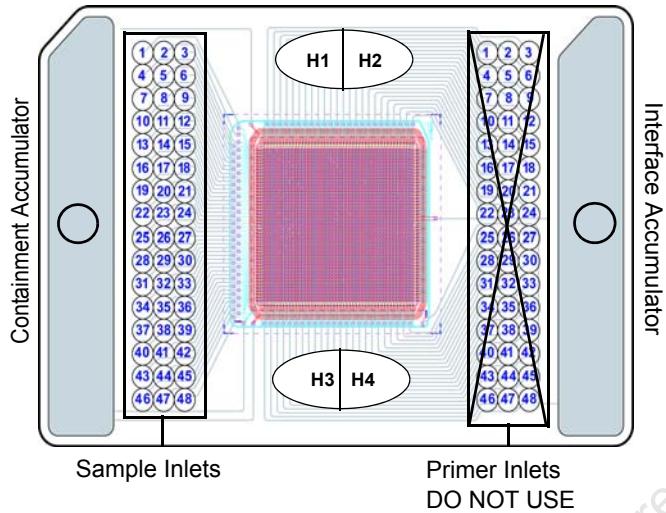
For Stand-Alone Thermal Cycler, select protocol **AA48v1**.



NOTE: The protocol as programmed into the Fluidigm Stand-Alone Thermal Cycler takes undershooting and overshooting of target temperatures into consideration and is therefore not identical to the PCR protocol as written out in tables 3 and 6. Contact Fluidigm Technical Support (1-866-358-4353 or techsupport@fluidigm.com) if you need assistance in programming your Fluidigm FC1 Cycler or Stand-Alone Thermal Cycler.

Harvesting the 48.48 Access Array IFC

- 1 After the PCR has finished, move the 48.48 Access Array IFC into the Post-PCR lab for harvesting.
- 2 Remove the remaining 1X Access Array Harvest Reagent from the H1-H4 wells.
- 3 Pipette **600 µL** of fresh 1X Access Array Harvest Reagent into the H1-H4 wells.
- 4 Pipette **2 µL** of 1X Access Array Harvest Reagent into each of the Sample Inlets on the IFC.
- 5 Load the IFC into the **Post-PCR** IFC Controller AX located in the Post-PCR lab.
- 6 Press **Eject** to move the tray out of the IFC Controller AX.
- 7 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 8 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 9 Select **Harvest (151x)** and **Run Script**.
- 10 Once the script is complete, press **Eject** to remove the IFC.
- 11 Label a 96-well plate with the 48.48 Access Array IFC barcode. Carefully transfer **10 µL** of harvested PCR products from each of the Sample Inlets into columns 1-6 of a 96-well PCR plate, using an 8-channel pipette.



NOTE: Remove PCR products from the IFC in the same order as the IFC was loaded, using an 8-channel pipette.

Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
4 5 6	4 5 6	4 5 6	4 5 6	4 5 6	4 5 6
7 8 9	7 8 9	7 8 9	7 8 9	7 8 9	7 8 9
10 11 12	10 11 12	10 11 12	10 11 12	10 11 12	10 11 12
13 14 15	13 14 15	13 14 15	13 14 15	13 14 15	13 14 15
16 17 18	16 17 18	16 17 18	16 17 18	16 17 18	16 17 18
19 20 21	19 20 21	19 20 21	19 20 21	19 20 21	19 20 21
22 23 24	22 23 24	22 23 24	22 23 24	22 23 24	22 23 24
25 26 27	25 26 27	25 26 27	25 26 27	25 26 27	25 26 27
28 29 30	28 29 30	28 29 30	28 29 30	28 29 30	28 29 30
31 32 33	31 32 33	31 32 33	31 32 33	31 32 33	31 32 33
34 35 36	34 35 36	34 35 36	34 35 36	34 35 36	34 35 36
37 38 39	37 38 39	37 38 39	37 38 39	37 38 39	37 38 39
40 41 42	40 41 42	40 41 42	40 41 42	40 41 42	40 41 42
43 44 45	43 44 45	43 44 45	43 44 45	43 44 45	43 44 45
46 47 48	46 47 48	46 47 48	46 47 48	46 47 48	46 47 48

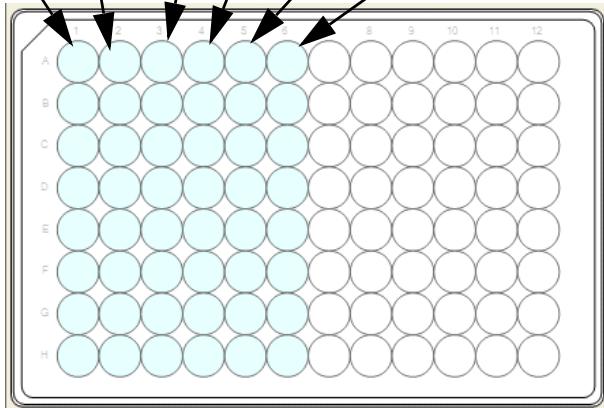


Figure 4 PCR product transfer map from the 48.48 Access Array IFC to a 96-well PCR plate.

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Multiplex Amplicon Tagging on the 48.48 Access Array IFC

6

Multiplex Amplicon Tagging for Illumina on the 48.48 Access Array IFC Introduction	60
48.48 Access Array IFC Workflow	63
Preparing the 20X Primer Solutions	64
Preparing Sample Pre-Mix and Samples.	65
Loading the 48.48 Access Array IFC	66
Attaching Sequence Tags and Sample Barcodes	70
Thermal Cycling the 96-Well PCR Plate.	72

Multiplex Amplicon Tagging for Illumina on the 48.48 Access Array IFC Introduction

Amplicon tagging on the Access Array™ System provides a simple path to generating sequencer-ready PCR products. During PCR, sequencer-specific tags and sample-specific barcodes are added to each of the PCR products, reducing the time required for enrichment of target sequences ahead of sequencing. The number of PCR products generated per sample on an Access Array IFC can be increased by an order of magnitude by multiplexing primer pairs in each PCR reaction on the chip. This protocol describes the generation of Illumina compatible libraries, containing up to 480 amplicons per sample. The procedure follows a two-step approach: In the first step (Figure 1), carried out on the 48.48 Access Array IFC, target regions are amplified with target-specific (TS) primer pairs that have been tagged with common sequence tags (Table 1). Each PCR reaction contains up to 10 primer pairs. The amplification is carried out on a FC1 Cycler. After harvesting the PCR products from the 48.48 Access Array IFC, the second PCR step is carried out in a 96-well PCR plate which introduces a sample-specific barcode and the Illumina adaptor sequences. By incorporating sample-specific barcodes, all 23,040 PCR products from a 48.48 Access Array IFC are unique and thus can be pooled.

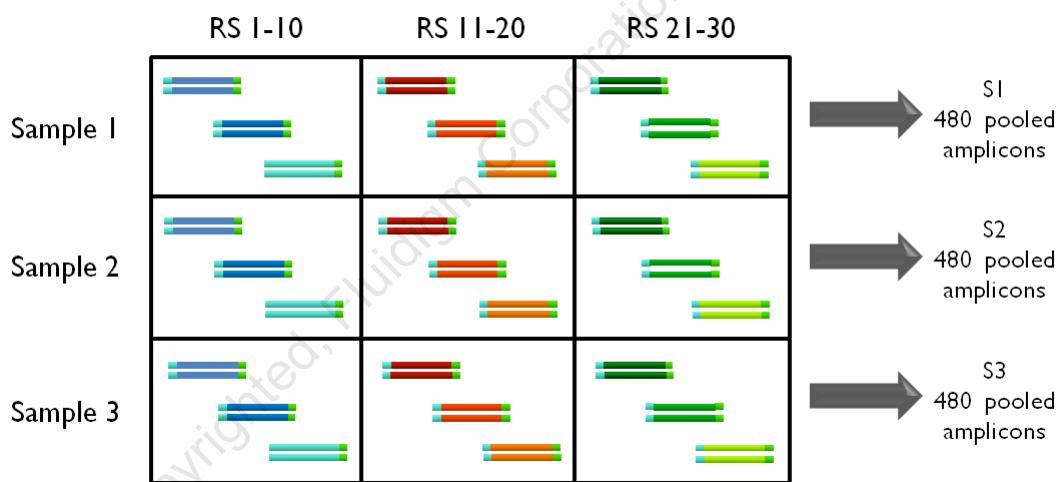


Figure 1 Multiplex Amplicon Tagging

Primer	Sequence
CS1-TS-F	5'-ACACTGACGACATGGTTCTACA - [TS-For] - 3'
CS2-TS-R	5'-TACGGTAGCAGAGACTTGGTCT - [TS-Rev] - 3'
PE1-CS1	5'-AATGATAACGGCGACCACCGAGATCTACACTGACGACATGGTTCTACA-3'
PE2-BC-CS2	5'-CAAGCAGAACGGCATACGAGAT-[BC]-TACGGTAGCAGAGACTTGGTCT-3'

Table 1 Target-specific primer pairs with CS1/CS2 tags and complement CS1/CS2 tags with sample-specific barcodes and Illumina adapters

Multiplex PCR on the 48.48 Access Array IFC

Target-Specific Primer Pooling

In order to minimize generating undesirable PCR products, please keep the following considerations in mind during assignment of primer pairs to primer pools:

- 1 Avoid combining primer pairs that produce overlapping PCR products. For example, where two sets of primers are required to cover a single exon, these primer pairs should be located in separate multiplex pools.
- 2 Combine primer pairs that anneal to target regions separated by at least 5 kb.
- 3 Use primer pairs that produce PCR products within 20% of the average size. For example, for a pool with an average size of 180 bp, amplicon sizes should fall within 140 and 210 bp.
- 4 Avoid combining primer pairs that produce PCR products with high GC content with those that produce PCR products with low GC content.
- 5 Do not include primers that have multiple annealing sites within the template DNA.
- 6 It is recommended to screen primer pools for primer dimer formation and priming within PCR products.



NOTE: Due to the nature of multiplex PCR, it is possible that up to 20% of all PCR products generated can be hybrid products produced by inter-primer pair pairing and mispriming elsewhere in the template DNA. This fraction may vary depending on the number of primer pairs combined in a pool and the complexity of the template DNA.
In silico screening of combined primer pairs against the entire sequence of the template DNA may reduce this population.

Reference Documents

- *Fluidigm® IFC Controller for Access Array™ System User Guide* (PN 68000157)
- *Fluidigm® Control Line Fluid Loading Procedure Quick Reference* (PN 68000132)
- *Agilent DNA 1000 Kit Guide*

Required Reagents

Stored at -20°C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)
- Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, PN 100-4876)
- Target-specific primer pairs tagged with universal tags (CS1 forward tag, CS2 reverse tag)
 - 50 µM CS1-Tagged TS Forward Primer
 - 50 µM CS2-Tagged TS Reverse Primer
- Template DNA at 50ng/µL



NOTE: 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) is not packaged for individual sale. It can be purchased in units of 10, under the name Access Array Harvest Pack, PN 100-3155, or as a component in the 48.48 Access Array Loading Reagent Kit, PN 100-1032.

Stored at 4°C

- Agilent DNA 1000 Kit Reagents (Agilent, PN 5067-1504)

Stored at Room Temperature

- PCR Certified Water (TEKnova, PN W330)

48.48 Access Array IFC Workflow

Priming the 48.48 Access Array IFC



CAUTION! Use the 48.48 Access Array IFC within 24 hours of opening the package.

CAUTION! Control Line Fluid on the IFC or in the inlets makes the IFC unusable. Use only 48.48 syringes with 300 µL of Control Line Fluid (Fluidigm, PN 89000020).

CAUTION! Load the IFC into the **Pre-PCR** IFC Controller AX in the Pre-PCR lab within 60 minutes of priming.

- 1 Inject Control Line Fluid into each accumulator on the IFC.
- 2 Add 500 µL of 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) into the H1-H4 wells on the IFC.
- 3 Remove and discard the blue protective film from the bottom of the AA IFC.
- 4 Load the IFC into the **Pre-PCR** IFC Controller AX located in the Pre-PCR lab.
- 5 Press **Eject** to move the tray out of the IFC Controller AX.
- 6 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 7 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 8 Select **Prime (151x)** and **Run Script** to prime the IFC.
- 9 Once the script is complete, press **Eject** to remove the IFC.

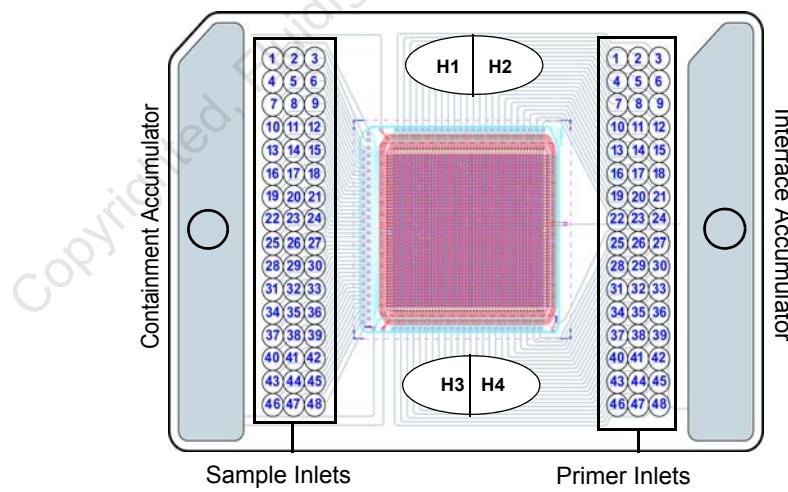


Figure 2 48.48 Access Array IFC overview

Preparing the 20X Primer Solutions

- 1 In a DNA-free hood, prepare the 20X Primer solutions for up to 480 individual primer pairs as shown in the table below. The table shows the primer dilution for one well with 10 primer pairs and needs to be repeated for all 48 wells. These will be loaded into the primer inlets of a 48.48 Access Array IFC.



NOTE: Each well can contain up to 10 forward primers and 10 reverse primers.

Component	Volume (μL)	Final Concentration
CS1-TS-F (50 μM)	2.0 per primer	1 μM per primer
CS2-TS-R (50 μM)	2.0 per primer	1 μM per primer
20X Access Array Loading Reagent	5.0	1X
DNA Suspension Buffer	To a final volume of 100.0	

Table 2 Dilution of Target-specific primers

- 2 Vortex the 20X Primer solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final Tagged TS forward and reverse primer concentrations are 1 μM in the 20X Primer solutions. The final TS forward and reverse primer concentrations in the Access Array reaction chamber are 50 nM per primer.

Preparing Sample Pre-Mix and Samples

All DNA samples need to be added into the Sample Pre-Mix individually, prior to loading the Sample Mix solutions into the sample inlets of an 48.48 Access Array IFC.

Prepare the Sample Pre-Mix Solution

- 1 Working in a DNA-free hood, combine the components listed in the table below. This protocol prepares enough Sample Pre-Mix for 60 reactions. This is enough reagent to load one 48.48 Access Array IFC with 16 additional reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 60 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl ₂ (Roche)	0.5	30.0	1X
25 mM MgCl ₂ (Roche)	0.9	54.0	4.5 mM
DMSO (Roche)	0.25	15.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.1	6.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/ μL
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
PCR Certified Water (TEKnova)	1.95	117.0	
Total	4.0	240.0	

Table 3 Sample Pre-Mix solution

- 2 Vortex the Sample Pre-Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare the Sample Mix Solutions

- 1 Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume (μ L)
Sample Pre-Mix	4.0
Genomic DNA (50 ng/ μ L)	1.0
Total	5.0

Table 4 Sample Mix solutions

- 2 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



IMPORTANT: It is essential to vortex all components to ensure complete mixing.

Loading the 48.48 Access Array IFC

- 1 Pipette 4 μ L of 20X Primer solution into each of the primer inlets.
- 2 Pipette 4 μ L of Sample Mix solution into each of the sample inlets.



NOTE: An 8-channel pipette is recommended to load the Sample Mix and 20X Primer solutions. The recommended pipetting order is shown below.

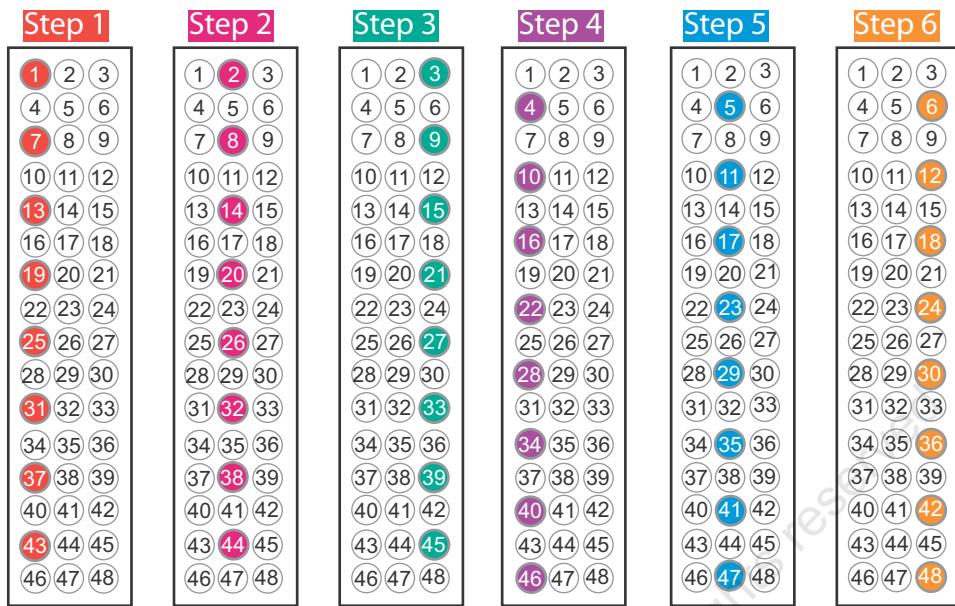


Figure 3 48.48 Access Array IFC pipetting scheme

- 3 Load the 48.48 Access Array IFC into the **Pre-PCR IFC Controller AX** in the Pre-PCR lab.
- 4 Press **Eject** to move the tray out of the IFC Controller AX.
- 5 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 6 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 7 Select **Load Mix (151x)** and **Run Script**.
- 8 Once the script is complete, press **Eject** to remove the IFC.

Thermal Cycling the 48.48 Access Array IFC

- 1 Place the 48.48 Access Array IFC onto the FC1 Cycler and start PCR by selecting the protocol **AA 48x48 Standard v1**.

See the FC1 Cycler appendix at the end of this manual for more detailed instructions about using the cycler.

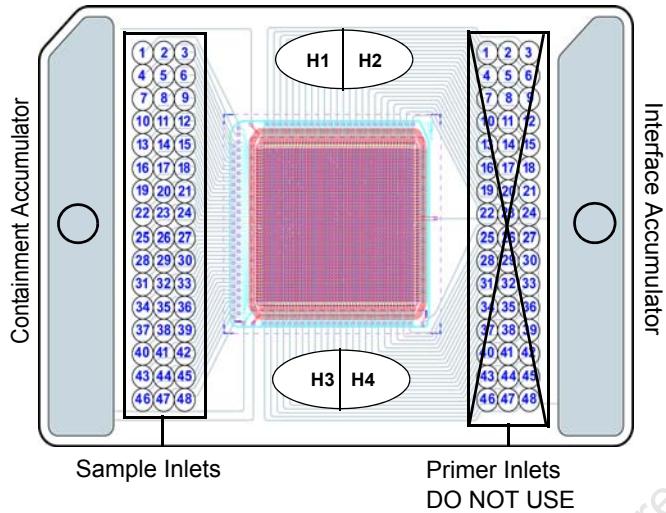
For Stand-Alone Thermal Cycler, select protocol **AA48v1**.



NOTE: The protocol as programmed into the Fluidigm Stand-Alone Thermal Cycler takes undershooting and overshooting of target temperatures into consideration and is therefore not identical to the PCR protocol as written out in tables 3 and 6. Contact Fluidigm Technical Support (1-866-358-4353 or techsupport@fluidigm.com) if you need assistance in programming your Fluidigm FC1 Cycler or Stand-Alone Thermal Cycler.

Harvesting the 48.48 Access Array IFC

- 1 After the PCR has finished, move the 48.48 Access Array IFC into the Post-PCR lab for harvesting.
- 2 Remove the remaining 1X Access Array Harvest Reagent from the H1-H4 wells.
- 3 Pipette **600 µL** of fresh 1X Access Array Harvest Reagent into the H1-H4 wells.
- 4 Pipette **2 µL** of 1X Access Array Harvest Reagent into each of the Sample Inlets on the IFC.
- 5 Load the IFC into the **Post-PCR** IFC Controller AX located in the Post-PCR lab.
- 6 Press **Eject** to move the tray out of the IFC Controller AX.
- 7 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 8 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 9 Select **Harvest (151x)** and **Run Script**.
- 10 Once the script is complete, press **Eject** to remove the IFC.
- 11 Label a 96-well plate with the 48.48 Access Array IFC barcode. Carefully transfer **10 µL** of harvested PCR products from each of the Sample Inlets into columns 1-6 of a 96-well PCR plate, using an 8-channel pipette.



NOTE: Remove PCR products from the IFC in the same order as the IFC was loaded, using an 8-channel pipette.

Step 1	Step 2	Step 3	Step 4	Step 5	Step 6
1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
4 5 6	4 5 6	4 5 6	4 5 6	4 5 6	4 5 6
7 8 9	7 8 9	7 8 9	7 8 9	7 8 9	7 8 9
10 11 12	10 11 12	10 11 12	10 11 12	10 11 12	10 11 12
13 14 15	13 14 15	13 14 15	13 14 15	13 14 15	13 14 15
16 17 18	16 17 18	16 17 18	16 17 18	16 17 18	16 17 18
19 20 21	19 20 21	19 20 21	19 20 21	19 20 21	19 20 21
22 23 24	22 23 24	22 23 24	22 23 24	22 23 24	22 23 24
25 26 27	25 26 27	25 26 27	25 26 27	25 26 27	25 26 27
28 29 30	28 29 30	28 29 30	28 29 30	28 29 30	28 29 30
31 32 33	31 32 33	31 32 33	31 32 33	31 32 33	31 32 33
34 35 36	34 35 36	34 35 36	34 35 36	34 35 36	34 35 36
37 38 39	37 38 39	37 38 39	37 38 39	37 38 39	37 38 39
40 41 42	40 41 42	40 41 42	40 41 42	40 41 42	40 41 42
43 44 45	43 44 45	43 44 45	43 44 45	43 44 45	43 44 45
46 47 48	46 47 48	46 47 48	46 47 48	46 47 48	46 47 48

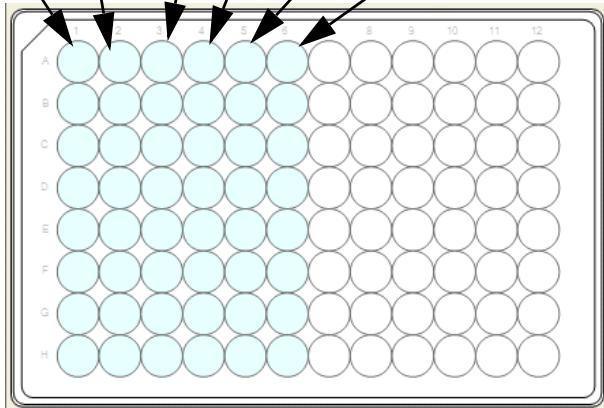


Figure 4 PCR product transfer map from the 48.48 Access Array IFC to a 96-well PCR plate.

Attaching Sequence Tags and Sample Barcodes



NOTE: All subsequent steps need to be carried out in a Post-PCR Lab to avoid contamination.

Preparing the Sample Pre-Mix Solution

- 1 Working in a DNA-free hood, combine the components listed in the table below. This protocol prepares enough Sample Pre-Mix for 60 reactions. This is enough reagent to amplify the 48 PCR product pools harvested from one 48.48 Access Array IFC with 12 additional reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μ L)	Volume for 60 Reactions (μ L)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl ₂ (Roche)	2.0	120.0	1X
25 mM MgCl ₂ (Roche)	3.6	216.0	4.5 mM
DMSO (Roche)	1.0	60.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.4	24.0	200 μ M ea
5 U/ μ L FastStart High Fidelity Enzyme Blend (Roche)	0.2	12.0	0.05 U/ μ L
PCR Certified Water (TEKnova)	7.8	468.0	
Total	15.0	900.0	

Table 5 Sample Pre-Mix Solution

- 2 Vortex the Sample Pre-Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Preparing a 100-Fold Dilution of the Harvested PCR Products

- 1 In a 96-well plate pipette **99 μ L** PCR certified water into 48 wells.
- 2 Add **1 μ L** of PCR product from each sample harvested from the 48.48 Access Array IFC to a separate well in the 96-well plate as described in the section “Harvesting PCR Products from the 48.48 Access Array IFC”.

- 3 Vortex the PCR product dilutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Preparing the Sample Mix Solutions

- 1 Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume (μ L)
Sample Pre-Mix	15.0
Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction)	4.0
Diluted Harvested PCR Product Pool	1.0
Total	20.0

Table 6 Sample Mix Solutions



IMPORTANT: It is essential to vortex all components to ensure complete mixing.



NOTE: The final concentrations of the forward and reverse barcode primers are 400 nM per well.

NOTE: Each well should receive a unique barcode primer pair.

- 7 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Thermal Cycling the 96-Well PCR Plate

- 1 Place the PCR plate on the PCR thermal cycler and run the following PCR protocol:

PCR Stages	Number of Cycles
95°C 10 min	1
95°C 15 s 60°C 30 s 72°C 1 min	15
72°C 3 min	1

Table 7 PCR protocol for attaching sequence tags and sample barcodes

Checking PCR Products on the Agilent 2100 BioAnalyzer

- 1 Use the Agilent DNA 1000 chips from the Agilent DNA 1000 Kit to check 1 μ L of PCR product from each of the PCR reactions described above. Follow the *Agilent DNA 1000 Kit Guide* for details.
- 2 Check the results of the chip to determine if the PCR product pool has the expected size. Depending on the expected sizes of the PCR products, a smear may be visible. Comparison of the electropherogram to a histogram of the expected sizes indicates correctness of the product size range. The PCR products of the barcoding step should exhibit a band shift of +59bp when compared to Harvest Pool Products.
- 3 Store the PCR products at -20°C.

Bidirectional Amplicon Tagging on the 48.48 Access Array IFC

7

Bidirectional Amplicon Tagging using the 48.48 Access Array IFC Introduction	74
Multiplex PCR on the 48.48 Access Array IFC	79
Barcode PCR Products in Two 96-Well Plates for Bidirectional Amplicon Tagging	79

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Bidirectional Amplicon Tagging using the 48.48 Access Array IFC Introduction

This protocol outlines the bidirectional amplicon tagging strategy for PCR products that have been generated on the Access Array™ System. The goal of this protocol is to sequence both ends of PCR products with a single-read sequencing run. This protocol is a derivative of the 4-primer amplicon tagging scheme described in Chapter 5 of this user guide. In the standard 4-primer amplicon tagging approach, tagged target-specific (TS) primer pairs are combined with sample-specific primer pairs containing a barcode sequence (BC) and the adaptor sequences used by the Illumina sequencers (PE1 and PE2, Figure 1A). In the bidirectional amplicon tagging strategy, tagged target-specific primer pairs are combined with two sets of sample-specific primer pairs. The sample-specific primer pairs are comprised of common sequence tags CS1 or CS2, appended with the Illumina adaptor sequences in both permutations (PE1 and PE2, Figure 1B). This approach requires only one set of target-specific primer pairs while the sample-specific barcode primers are universal and can be used in multiple experiments.

Bidirectional amplicon tagging generates two types of PCR products per target region: one PCR product that allows for sequencing of the 5' end of the target region (product A) and one PCR product that allows for sequencing of the 3' end of the target region (product B). Because both PCR products are present on the flow cell at the same time, one single-read sequencing run will yield sequence information for both ends of the target region. The main difference with standard single direction sequencing is that the 5' read and the 3' read are not derived from the same cluster, i.e. from the same template molecule. Instead, an average of the template population will be derived.

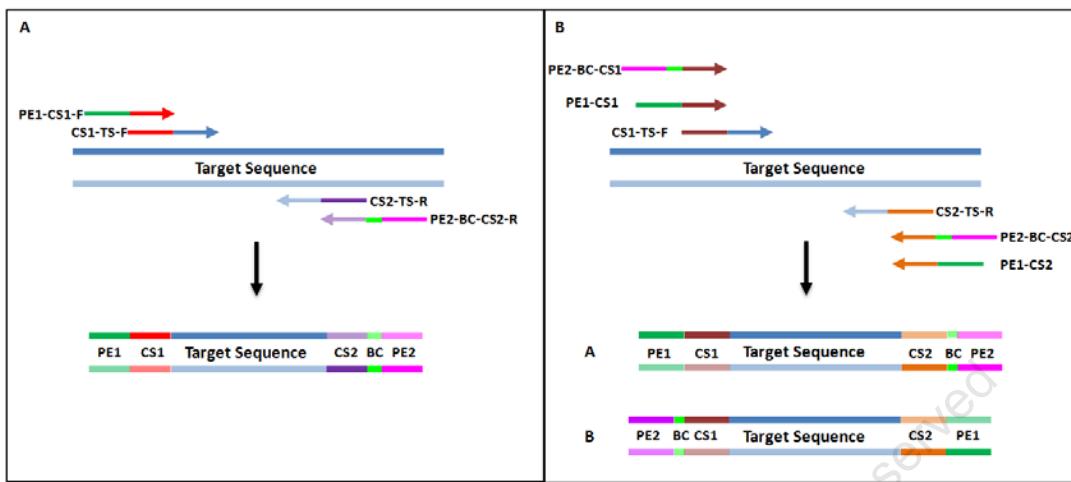


Figure 1 A. Standard 4-primer amplicon tagging versus bidirectional amplicon tagging. The standard 4-primer amplicon tagging approach incorporates the paired-end Illumina sequencing primer annealing sites in Common Sequence tag 1 (CS1) and Common Sequence tag 2 (CS2). Sequencing of both the 5' end and the 3' end of each PCR product requires a paired-end sequencing run. B. Target-specific primers are appended with Common Sequence tags CS1 and CS2. The sample-specific primer pairs are comprised of common sequence tags CS1 or CS2, appended with the adaptor sequences used by the GAI (PE1 and PE2) in both permutations. Two PCR product types are generated from the same target region: Product A allows for sequencing of the 5' end of the target region whereas product B allows for sequencing of the 3' end of the target region during the same sequencing read.

The multiplex amplicon tagging strategy described in Chapter 6 can be done prior to adding the bidirectional sample barcodes. In short, the protocol adopts a two-step approach: the PCR on the Access Array IFC is run in the presence of multiplexed, tagged, target-specific primers only. The harvested PCR product pools are then used as template in a second PCR with the sample-specific barcode primers. The two sets of barcode primers are added in independent PCR reactions as described below.

Sample-specific barcode primer pairs are segregated out into two separate PCR reactions (Figure 2).

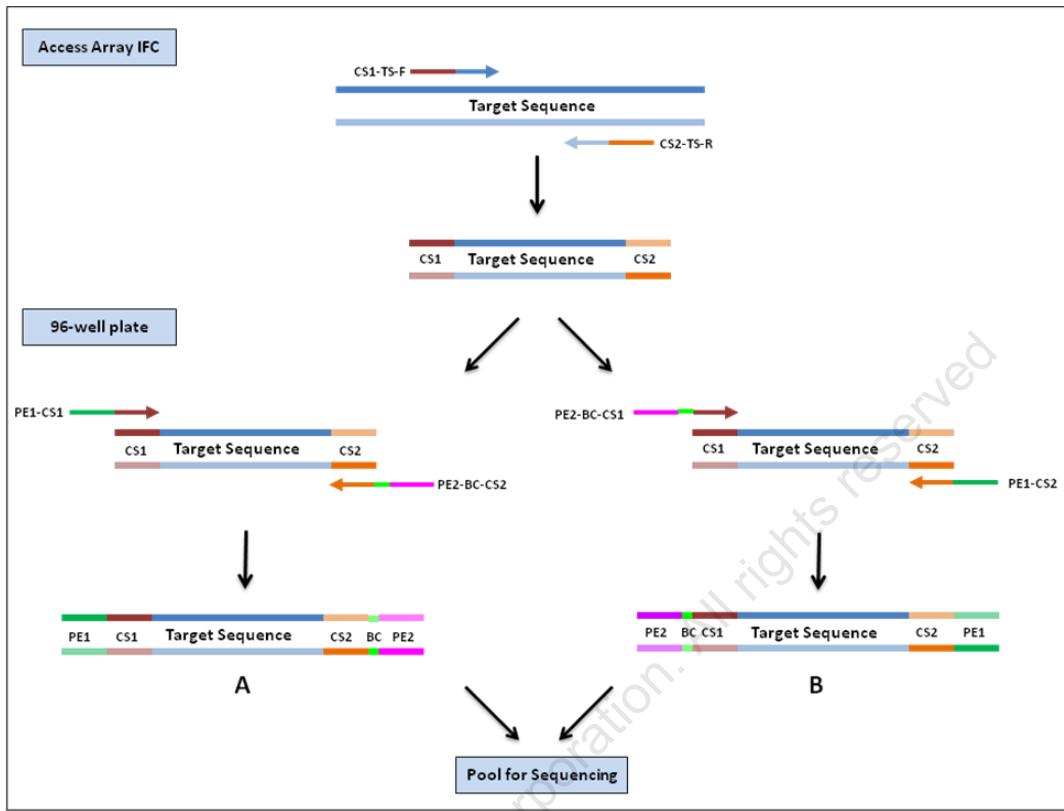


Figure 2 Overview of the segregated-primer PCR strategy. The first PCR with the target-specific primer pairs is carried out in the Access Array IFC. The harvested PCR product pools are split into two subsequent PCR reactions with sample-specific barcode primers. A. The reaction that generates products that will allow for sequencing of the 5' end of the target region utilizes the PE1_CS1 and PE2_BC_CS2 primer combination. B. The reaction that generates products that will allow for sequencing of the 3' end of the target region utilizes the PE1_CS2 and PE2_BC_CS1 primer combination.

After the barcoding PCR, the PCR products of both the 5' reaction and the 3' reaction are combined and used as template for cluster formation on the flow cell. Because both PCR product types will be present and form clusters on the flow cell, an equimolar mixture of the CS1 and CS2 sequencing primers allows for simultaneous sequencing of both PCR product types (Figure 3). Similarly, the index read with an equimolar mixture of the CS1rc and CS2rc sequencing primers allows for simultaneous sequencing of the barcodes of both PCR product types.

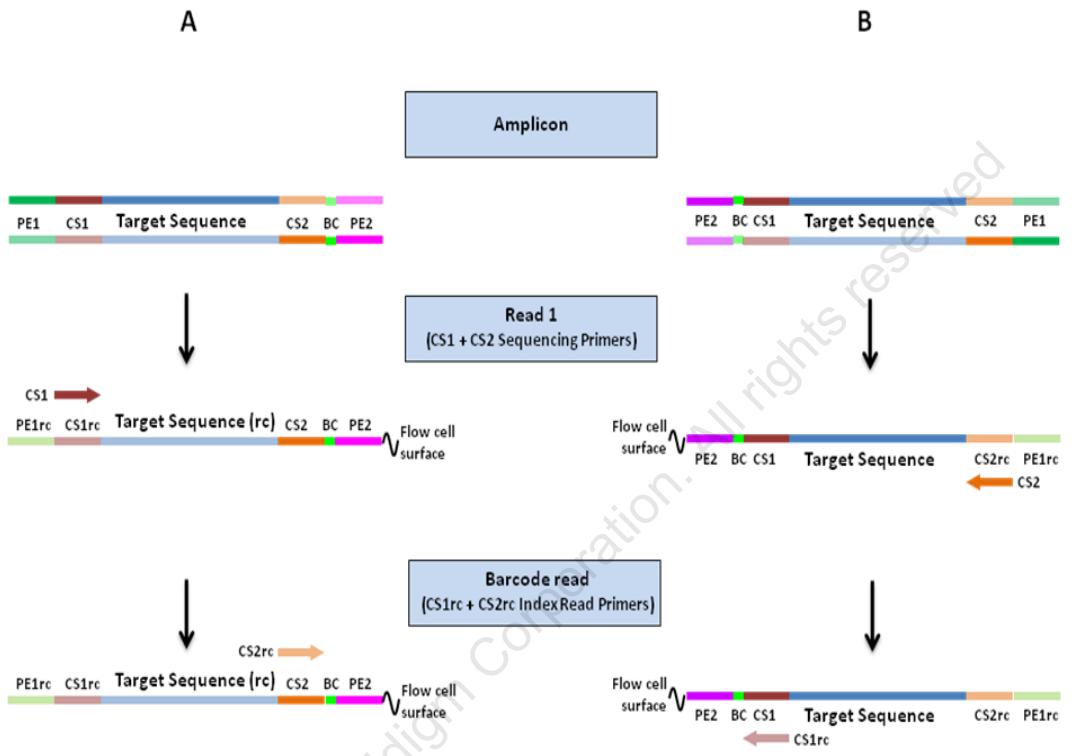


Figure 3 Overview of the sequencing work flow.
Both PCR product types are present on the flow cell. An equimolar mixture of CS1 and CS2 allows for sequencing of both the 5' end and 3' end of the target regions. After stripping and rehybridization of the clusters with an equimolar mixture of CS1rc and CS2rc the barcodes are sequenced. The sequencing primers CS1 and CS2 are provided in the reagent FL1. The indexing primers CS1rc and CS2rc are provided in the reagent FL2.

Reference Documents

- *Fluidigm® IFC Controller for Access Array™ System User Guide* (PN 68000157)
- *Fluidigm® Control Line Fluid Loading Procedure Quick Reference* (PN 68000132)
- *Agilent DNA 1000 Kit Guide*

Required Reagents

Stored at -20°C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)
- Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771)
- Target-specific primer pairs tagged with universal tags (CS1 forward tag, CS2 reverse tag)
 - 50 µM CS1-Tagged TS Forward Primer
 - 50 µM CS2-Tagged TS Reverse Primer
- Template DNA at 50ng/µL



NOTE: 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) is not packaged for individual sale. It can be purchased in units of 10, under the name Access Array Harvest Pack, PN 100-3155, or as a component in the 48.48 Access Array Loading Reagent Kit, PN 100-1032.

Stored at 4°C

- Agilent DNA 1000 Kit Reagents (Agilent, PN 5067-1504)

Stored at Room Temperature

- PCR Certified Water (TEKnova, PN W330)

Multiplex PCR on the 48.48 Access Array IFC

For a detailed protocol, please follow the instructions as detailed in Chapter 6 - Multiplex Amplicon Tagging on the 48.48 Access Array IFC, then barcode the harvested PCR products following the instructions below.

2-Primer Target-Specific PCR on the 48.48 Access Array IFC

For a detailed protocol for bidirectional amplicon tagging without multiplexing, please follow the instructions as detailed in Appendix C, then barcode the harvested PCR products following the instructions below.

Barcode PCR Products in Two 96-Well Plates for Bidirectional Amplicon Tagging

For a more detailed protocol, please follow the instructions as detailed in Chapter 6 - Attaching Sequence Tags and Sample Barcodes.

- 1 The 100-fold dilution of the harvested PCR product pool serves as template in two (rather than one) barcoding PCR reactions: one reaction will generate PCR product A that allows for sequencing of the 5' end of the target region in one 96-well plate, and the other reaction will generate PCR product B that allows for sequencing of the 3' end of the target region in a second 96-well plate. The setup of the reaction is identical to “[Attaching Sequence Tags and Sample Barcodes](#)” on page 70. However, the quantities in the Sample Pre-Mix Solution should be doubled to compensate for the increase in the number of reactions, and Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771) should be used in the preparation of the Sample Mix Solution.

Component	Volume (μ L)
Sample Pre-Mix	15.0
Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) A	4.0
Diluted Harvested PCR Product Pool	1.0
Total	20.0

Table 1 Sample Mix Solutions - PCR Product A

Component	Volume (μ L)
Sample Pre-Mix	15.0
Access Array Barcode Library for Illumina Sequencer - 384 (Bidirectional) B	4.0
Diluted Harvested PCR Product Pool	1.0
Total	20.0

Table 2 Sample Mix Solutions - PCR Product B

- 1 After the second PCR has finished, combine PCR Product A and PCR Product B pools prior to sequencing. Please refer to [Chapter 8](#) for methods describing post-PCR product library purification and quantitation.



IMPORTANT: It is essential to use Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771) to generate bidirectional amplicons for sequencing.

Post-PCR Amplicon Purification and Quantitation

8

PCR Product Purification and Quantification	82
Reference Documents	82
Required Equipment	82
Required Reagents	82
Pooling Products from Multiple Access Array IFCs	83
Purification of Harvested PCR Products.	83
PCR Product Library Quantification Procedure	85

PCR Product Purification and Quantification

This chapter describes a standard procedure for the analysis of PCR products harvested from a 48.48 Access Array IFC.

The quality of the PCR products prepared on an Access Array IFC is critical for successful amplicon sequencing. Any contamination of primers or primer dimers in the PCR products will be directly reflected in the quality of sequencing reads. Therefore, the PCR products generated on a 48.48 Access Array IFC should be qualified and purified before sequencing.

The PCR products generated on the 48.48 Access Array IFC are first analyzed using an Agilent 2100 Bioanalyzer to check for quality. Next, the PCR products are pooled together in equal volume to create one PCR product library. The PCR product library is then purified using AMPure XP beads, and quantified before proceeding to cluster generation. One technique recommended as an option to quantify the PCR product library is the Quant-iT PicoGreen fluorimetry system.

Reference Documents

- *Agilent DNA 1000 Kit Guide*
- *Quant-iT™ PicoGreen® User Guide*

Required Equipment

- SPRIPlate 96R Magnet Plate (Agencourt, PN 000219) or DynaMag™-2 magnet (Invitrogen, PN 123-21D)
- Agilent 2100 Bioanalyzer and DNA 1000 Kit (Agilent, PN 5067-1504)
- Fluorimeter-compatible 96- or 384-well microtiter plates

Procedure

Required Reagents

Stored at 4 °C

- Agencourt AMPure® XP Reagent beads (Beckman Coulter Genomics, PN A63880)
- Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, P11496)

Stored at Room Temperature

- DNA suspension buffer (10 mM TRIS, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)

- 100% Ethanol
- PCR certified water (TEKnova, PN W330)

Qualification of PCR Products

The PCR products can be qualified using an Agilent 2100 Bioanalyzer with DNA 1000 Chips.

- 1 Run 1 μ L of the 48 pooled PCR products from each sample on a Bioanalyzer DNA 1000 Chip following the manufacturer's instructions.
 - a Ensure that amplicon sizes and distribution are within the expected range (+/- 5% for amplicons in the range of 200-400 bp including tags).
 - b Primer dimer contamination in the PCR product pool (in the range of 50-130 bp) should be less than 25% based on the Bioanalyzer quantification ([Appendix D, “Electropherogram Examples.”](#))
- 2 Continue with the purification procedure below for all of the pooled PCR products that contain less than 25% primer dimers.

Pooling Products from Multiple Access Array IFCs

To pool amplicons from more than one Access Array IFC, pool and purify each Access Array IFC separately.

- 1 Follow the steps outlined in this chapter under “Purification of Harvested PCR Products” for each Access Array IFC.
- 2 For bidirectional amplicon tagged libraries (generated in Chapter 7), pool products A and B from the same Access Array IFC (up to 48 samples) together for purification.
- 3 Continue to quantify the pooled, purified library by calculating the PCR Product Library Concentration outlined later in this chapter.
- 4 Once each IFC Library has been purified and quantified, normalize the concentration of each IFC Library and pool libraries volumetrically by adding an equal volume of each library to a new microcentrifuge tube.
- 5 The pooled Access Array IFC library is now ready for sequencing.

Purification of Harvested PCR Products

- 1 Remove Ampure XP beads from refrigerator and warm up at room temperature for 30 minutes.
- 2 Prepare 70% ethanol solution:
 - a To a 15 mL tube, add 3 mL of PCR-certified water and 7 mL of 100% ethanol.
 - b Vortex for 5 seconds.

-
- 3 Pool 1 μ L of each sample pool from the Access Array IFC Harvest (up to 48 total) into a new microcentrifuge tube. This is the **Harvest Sample Pool**.
 - 4 Vortex Ampure XP beads for 10 seconds to resuspend. Bead solution should appear homogeneous and consistent in color.
 - 5 Pipette the Harvest Sample Pool, DNA suspension buffer, and Ampure XP beads into a 1.5 mL microtube according to the table below:

Component	Volume
Harvest Sample Pool	12.0 μ L
TE	24.0 μ L
Ampure XP Beads	36.0 μ L

- 6 Vortex the tube and incubate at room temperature for 10 minutes.
- 7 Place the microtube onto a magnetic separator and allow it to sit for 1 minute.
- 8 Carefully pipette out the supernatant without disturbing the beads (remove as much liquid as possible).
- 9 Add 180 μ L of 70% ethanol and vortex for 10 seconds.
- 10 Place the microtube onto a magnetic separator and allow it to set for 1 minute.
- 11 Carefully pipette out the supernatant without disturbing the beads.
- 12 Add 180 μ L of 70% ethanol and vortex for 10 seconds.
- 13 Place the microtube onto a magnetic separator and allow it to set for 1 minute.
- 14 Carefully pipette out the supernatant without disturbing the beads.
- 15 Allow the beads to air dry for approximately 10 minutes by leaving the tube on the bench. Make sure the tube is completely dry before proceeding.
- 16 Add 40 μ L of DNA suspension buffer to the microtube and vortex for 5 seconds.
- 17 Place the microtube onto a magnetic separator and allow it to set for one minute.
- 18 Carefully transfer the supernatant to a new 1.5 mL microtube.

Agilent 2100 Bioanalyzer Qualification:

- 1 Run 1 μ L of the PCR product library on a Bioanalyzer DNA 1000 Chip following the manufacturer's instructions.
- 2 Define a region of interest in the electropherogram to determine the PCR product library concentration.
 - a Select the Region Table subtab on the bottom panel of the Electropherogram tab.

- b Right-click the electropherogram and select Add region. Define the region to cover all of the PCR product library peaks.

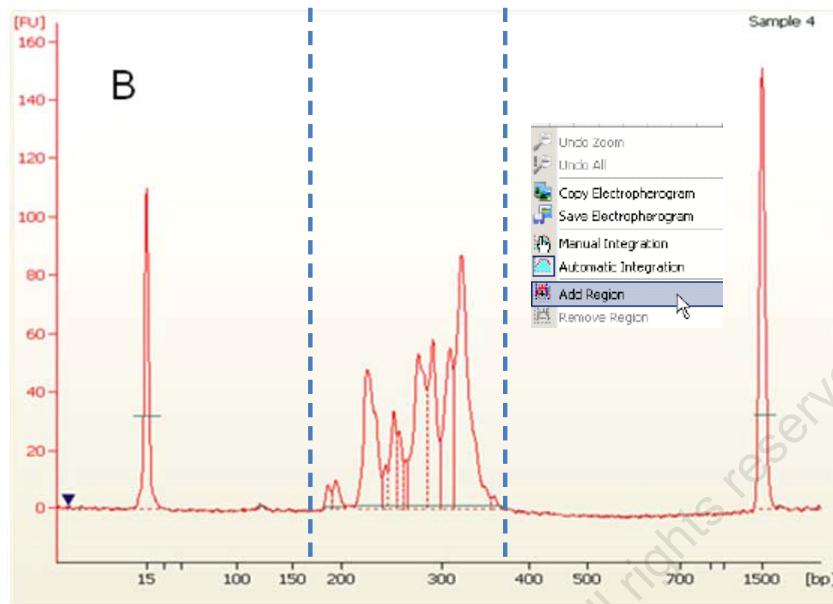


Figure 1. Define a region that includes all of the PCR product library peaks.

- c The Region Table listed below the electropherogram will show the concentration of the Region containing the PCR product library. Refer to the Agilent 2100 Bioanalyzer User Guide for additional information on Regions.

PCR Product Library Quantification Procedure

Use the following Quantification method to quantify the PCR product library.

Picogreen Fluorimetry Quantification:

Quantitate the PCR product library by fluorimetry, using the Quant-iT PicoGreen dsDNA Assay Kit, following the manufacturer's instructions.

PCR Product Library Concentration

Given the PCR product library concentration (in ng/µL) calculate the concentration in molecules/µL using the following equation:

$$\text{Molecules/}\mu\text{L} = \frac{(\text{PCR product conc.; ng/}\mu\text{L}) \times (6.022 \times 10^{23})}{(656.6 \times 10^9) \times (\text{average amplicon length; bp})}$$

Where 6.022×10^{23} is Avogadro's number (molecules/mole), 656.6 is the average molecular weight of nucleotide pairs (g/mole), and average amplicon length (bp) is the average length of the 48 amplicons generated in the Access Array experiment.

See [Appendix D, Electropherogram Examples](#) for examples of an Agilent 2100 BioAnalyzer Electropherogram of a harvested PCR product pool.

See [Appendix H, Access Array Barcodes for the Illumina Sequencing Systems](#) for the list of barcode sequences.

Using the Fluidigm FC1 Cycler

A

Using the Fluidigm FC1 Cycler88
Powering On the FC1 Cycler89
Login89
Prepare Chip for Thermal Cycling90
Running a Protocol90
Cleaning Protocol91
Troubleshooting92

Using the Fluidigm FC1 Cycler



Powering On the FC1 Cycler



NOTE: The FC1 Cycler uses a touch-screen interface, therefore all interactions with the application occur by touching the screen.

- 1 If you are using a powerstrip, turn it ON.
- 2 Press the switch at the back of the FC1 Cycler to the ON position.



NOTE: Initialization may take up to 1 minute to complete.

- 3 At completion of power up and instrument initialization, the Fluidigm FC1 Cycler home screen appears.

Login

- 1 Press Login button to log in.



- 2 Default password for the following users are:

Admin: use **123456**

User: no password

Enter password for Admin

1	2	3
4	5	6
7	8	9
0	◀ back	

Cancel **LOGIN**



NOTE: For information on setting passwords, see the Fluidigm FC1 Cycler User Guide, PN 100-1279.

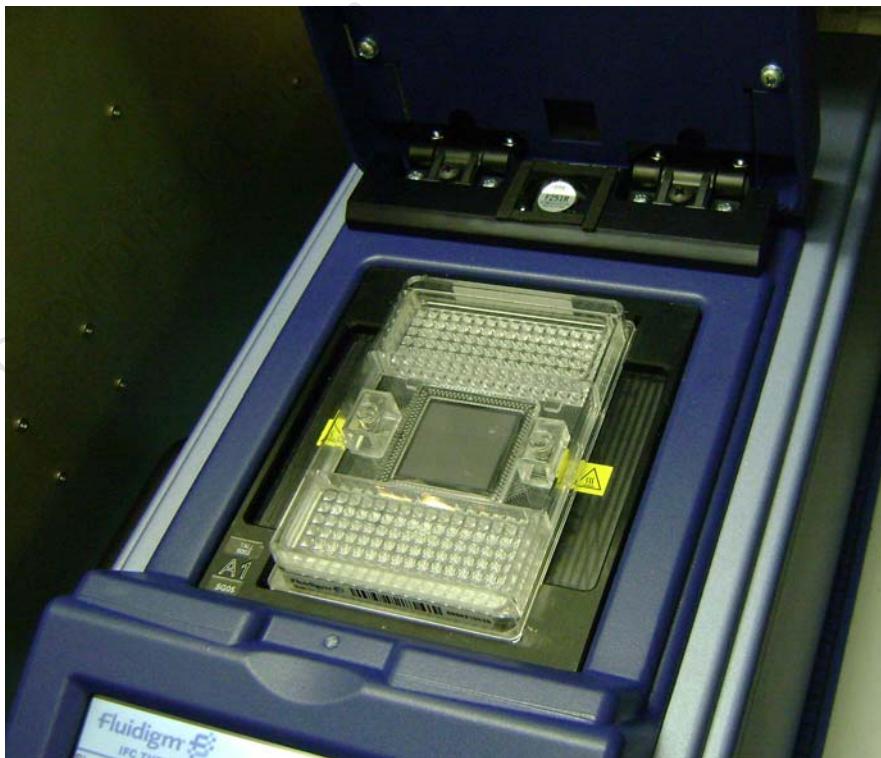
Prepare Chip for Thermal Cycling

- 1 Remove the protective film from the bottom of the chip.



Running a Protocol

- 1 Press the Start button.
- 2 Open the lid.
- 3 Place the chip onto the thermal cycling block (chuck) on the top of the instrument by aligning the notched corner of the IFC chip to the A1 mark.



- 4 Close the lid.
- 5 Press Continue to display available thermal protocols.
- 6 Choose a protocol to run from the protocol selection window.



- 7 Press Run.



WARNING! Never press down on the chip when it is on the FC1 Cycler.



NOTE: A status screen appears with a time estimate for completion.

- 8 Once the protocol is finished, a confirmation screen appears. (During an active protocol, **Abort** will cancel the chip run.)

Cleaning Protocol

It is important to keep the thermal chuck surface clean. Any grease or debris will impact the thermal contact between the chuck and chip. Turn off system prior to cleaning the chuck.

- 1 Turn system Off.
Let system cool down.
- 2 Use a lint-free cloth and 70% isopropyl alcohol to gently wipe the chuck.



CAUTION! Make sure chuck has had time to cool. It can get very hot and cause burn injury.

Troubleshooting

Error Message	Possible Solutions
The screen is blank	Check power connection at the wall outlet, power strip and instrument inlet.
Error while loading the chip, no vacuum	Verify the blue protective film has been removed from the bottom of the chip. Verify the chip is properly seated. Make sure the chuck surface and bottom of the chip are clean. Follow cleaning protocol above.

4-Primer Amplicon Tagging on the BioMark System

B

Running the 48.48 Access Array IFC on the BioMark System	94
Preparing the 20X Primer Solutions	96
Data Acquisition	99
Harvesting the 48.48 Access Array IFC	100
PCR Data Analysis	101

Running the 48.48 Access Array IFC on the BioMark System

This real-time PCR protocol provides a method to evaluate PCR amplification and PCR products.



NOTE: Evagreen and other DNA-binding dyes have been shown to inhibit PCR, particularly for amplicons with a percentage of GC greater than 60%.

Required Reagents

Stored at -20°C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)



NOTE: 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) is not packaged for individual sale. It can be purchased in units of 10, under the name Access Array Harvest Pack, PN 100-3155, or as a component in the 48.48 Access Array Loading Reagent Kit, PN 100-1032.

- Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, PN 100-4876)
- ROX reference dye, 50X (Invitrogen, PN 12223-012)
- Template DNA at 50 ng/µL

Stored at 4°C

- 20X EvaGreen® dye (Biotium, PN 31000)

Stored at Room Temperature

- PCR certified water (TEKnova, PN W3330)

Required Equipment

- BioMark™ HD System (Fluidigm)

Priming the 48.48 Access Array IFC



CAUTION! Use the 48.48 Access Array IFC within 24 hours of opening the package.

CAUTION! Control Line Fluid on the IFC or in the inlets makes the IFC unusable. Use only 48.48 syringes with 300 µL of Control Line Fluid (Fluidigm, PN 89000020).

CAUTION! Load the IFC into the **Pre-PCR** IFC Controller AX in the Pre-PCR lab within 60 minutes of priming.

- 1 Inject Control Line Fluid into each accumulator on the IFC.
- 2 Add 500 µL of 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) into the H1-H4 wells on the IFC.
- 3 Remove and discard the blue protective film from the bottom of the AA IFC.
- 4 Load the IFC into the **Pre-PCR** IFC Controller AX located in the Pre-PCR lab.
- 5 Press **Eject** to move the tray out of the IFC Controller AX.
- 6 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 7 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 8 Select **Prime (151x)** and **Run Script** to prime the IFC.
- 9 Once the script is complete, press **Eject** to remove the IFC.

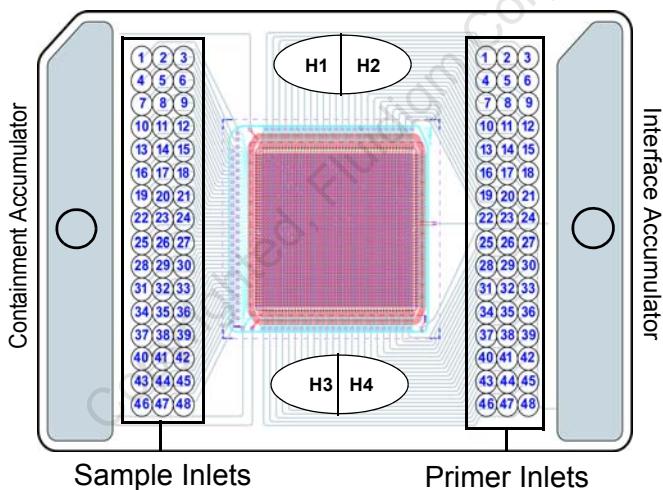


Figure 1 48.48 Access Array IFC overview

Preparing the 20X Primer Solutions



IMPORTANT: Warm up the 20X Access Array Loading Reagent to room temperature before use.

- 1 Prepare the 20X Primer Solutions for 48 individual primer pairs as shown in the table below. These will be loaded into the Primer Inlets of a 48.48 Access Array IFC.

Component	Volume (μL)	Final Concentration
50 μM CS1-Tagged TS Forward Primer	2.0	1 μM
50 μM CS2-Tagged TS Reverse Primer	2.0	1 μM
20X Access Array Loading Reagent (Fluidigm)	5.0	1X
PCR Certified Water (TEKnova)	91.0	
Total	100.0	

Table 1 20X Primer Solution

- 2 Vortex the 20X Primer Solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final Tagged TS Forward and Reverse Primer concentrations are 1 μM in the 20X Primer Solution. The final TS Forward and Reverse Primer concentrations in the Access Array reaction chamber are 50 nM.

Preparing Sample Master Mix Solutions

All DNA samples and the barcode primers need to be added into the Sample Pre-Mix individually, prior to loading the Sample Mix solutions into the Sample Inlets of an 48.48 Access Array IFC.

Prepare the Sample Pre-Mix Solutions

- 1 Working in a DNA-free hood, combine the components listed in the table below.

This protocol prepares enough Sample Pre-Mix for 60 reactions. This is enough reagent to load one 48.48 Access Array IFC with 16 additional reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 60 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer <u>with</u> 18 mM MgCl ₂ (Roche)	0.50	30.0	1X
25 mM MgCl ₂ (Roche)	0.54	32.4	2.7 mM
DMSO (Roche)	0.25	15.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.10	6.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/ μL
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
50X ROX Reference Dye (Invitrogen)	0.05	3.0	0.5X
20X EvaGreen® Dye (Biotium)	0.25	15.0	1X
PCR-Certified Water (TEKnova)	1.01	60.6	
Total	3.0	180.0	

Table 2 Sample Pre-Mix Solution

- 2 Vortex the Sample Pre-Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: Due to restrictions in volume, it is important to use the 10X FastStart High Fidelity Reaction Buffer with 18mM MgCl₂ when using ROX and EvaGreen dye in the reaction. The final MgCl₂ concentration of reaction buffer and the extra MgCl₂ combined will be 4.5 mM.

Preparing the Sample Mix Solutions

- 1 Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume per Reaction (μ L)	Final Concentration
Sample Pre-Mix	3.0	
50 ng/ μ L Genomic DNA	1.0	10 ng/ μ L
Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction)	1.0	400 nM
Total	5.0	

Table 3 Pre-Sample Master Mix Solution

- 2 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



IMPORTANT: It is essential to vortex all components to ensure complete mixing.



NOTE: The final Access Array Barcode Library Forward and Reverse Primers concentrations are 400 nM in the Sample Mix solutions.

Loading the 48.48 Access Array IFC

- 1 Pipette 4 μ L of 20X Primer Solution into each of the Primer Inlets.
- 2 Pipette 4 μ L of Sample Mix solution into each of the Sample Inlets.



CAUTION! While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.



NOTE: An 8-channel pipette is recommended to load the Sample Mix and 20X Primer Solutions. The recommended pipetting order is shown below.

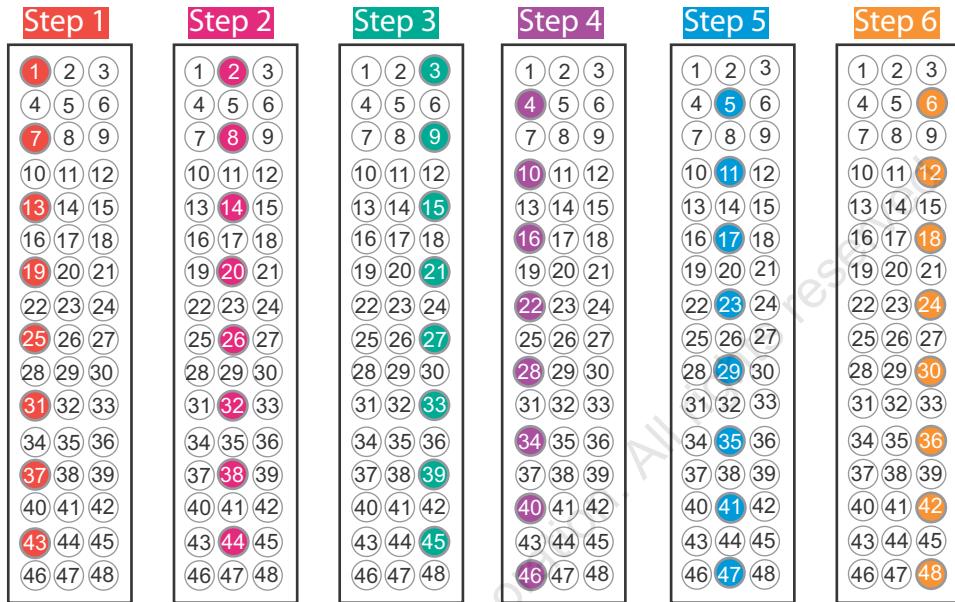


Figure 2 Pipetting order

- 3 Load the 48.48 Access Array IFC into the Pre-PCR IFC Controller AX in the Pre-PCR lab.
- 4 Press **Eject** to move the tray out of the IFC Controller AX.
- 5 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 6 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 7 Select **Load Mix (151x)** and **Run Script**.
- 8 Once the script is complete, press **Eject** to remove the IFC.

Data Acquisition

Running the Access Array IFC with Detection Chemistry

- 1 Open BioMark Data Collection software. Refer to the *Fluidigm Data Collection User Guide* (PN 68000127) for details on using the BioMark System.
- 2 Check the top surface of the chip for any large particles or debris, remove gently with scotch tape.

-
- 3 Remove the blue IHS protector from the back of the chip.
 - 4 Place the chip in the loading position of the BioMark instrument.
 - 5 Select **Start** and **Load Chip**, and the chip will be loaded into the instrument.
 - 6 Select: Application Type: **Gene Expression**.
 - 7 Select: Assay: **Single Probe Type**.
 - 8 Select: Probe Type: **EvaGreen**.
 - 9 Select: Protocol: **AA 48x48 Standard v1.pcl**.
 - 10 Make sure “Auto Exposure” box is checked.
 - 11 Set to capture image at the end of slice.
 - 12 Select the hard drive location where you would like to store the experiment data.
 - 13 Select **Run** to start PCR.

Running the Access Array IFC without Detection Chemistry

- 1 Open BioMark Data Collection software. Refer to *Fluidigm Data Collection User Guide* (PN 68000127) for details on using the BioMark System.
- 2 Check the top surface of the chip for any large particles or debris, remove gently with scotch tape.
- 3 Remove the blue IHS protector from the back of the chip.
- 4 Place the chip in the loading position of the BioMark instrument.
- 5 Select **Start** and **Load Chip**, and the chip will be loaded into the instrument.
- 6 Select: Application Type: **Gene Expression**.
- 7 Select: Assay: **Single Probe Type**.
- 8 Select: Probe Type: **EvaGreen**.
- 9 Select protocol: **AA 48x48 Standard v1.pcl**.
- 10 Uncheck “Auto Exposure” box.
- 11 Under Exposure Times, enter 1.0 second for ROX and 1.0 second for FAM.
- 12 Select the hard drive location where you would like to store the experiment data.
- 13 Select **Run** to start PCR.

Harvesting the 48.48 Access Array IFC

- 1 After the PCR has finished, move the 48.48 Access Array IFC from the BioMark into the Post-PCR lab for harvesting.
- 2 Remove the remaining 1X Access Array Harvest Reagent from the H1-H4 wells.
- 3 Pipette 600 μ L of fresh 1X Access Array Harvest Reagent into the H1-H4 wells.
- 4 Pipette 2 μ L of 1X Access Array Harvest Reagent into each of the Sample Inlets on the IFC.

- 5 Load the IFC into the **Post-PCR** IFC Controller AX located in the Post-PCR lab.
- 6 Press Eject to move the tray out of the IFC Controller AX.
- 7 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 8 Press Load Chip to register the barcode of the IFC and activate the script selection.
- 9 Select **Harvest (151x)** and **Run Script**.
- 10 Once the script is complete, press **Eject** to remove the IFC.
- 11 Label a 96-well plate with the 48.48 Access Array IFC barcode. Carefully transfer **10 µL** of harvested PCR products from each of the Sample Inlets into columns 1-6 of the 96-well PCR plate, using an 8-channel pipette.

PCR Data Analysis

- 1 Launch the Fluidigm BioMark Real-time PCR Analysis Software.
- 2 Click **Open a Chip Run** and then double-click the chip run.bml file to open it in the software.
- 3 Click **Analysis Views** under chip explorer.
- 4 Set the “Quality Threshold” to **0.3** in the Analysis Setting pane.
- 5 Select **Constant** for Baseline Correction in the Analysis Settings pane.
- 6 Select **Auto (Global)** for C_t Threshold Method in the Analysis Setting pane.
- 7 Click **Analyze** to process the data.
- 8 Save data and export as a .csv file. The file can be opened in Microsoft Excel® or Word®.

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2-Primer Target-Specific PCR Amplification

C

2-Primer Target-Specific PCR Amplification Overview	104
Target-Specific Primer Validation for 2-Primer Reactions on the 48.48 Access Array IFC107	
Stored at -20°C	107
Stored at 4°C	107
Stored at Room Temperature	107
Setting up the PCR Reactions in a 384-well PCR Plate	110
Running the PCR Reactions	111
2-Primer Target-Specific PCR Amplification on the 48.48 Access Array IFC.	114
Reference Documents	114

2-Primer Target-Specific PCR Amplification Overview

The Access Array Target-Specific PCR amplification scheme combines 48 target-specific (TS) forward and reverse primers with 48 samples to generate 2,304 PCR reactions.

In Figure 1 below, a 3 x 3 grid of reaction wells is shown before any reagents are loaded into the 48.48 Access Array IFC.

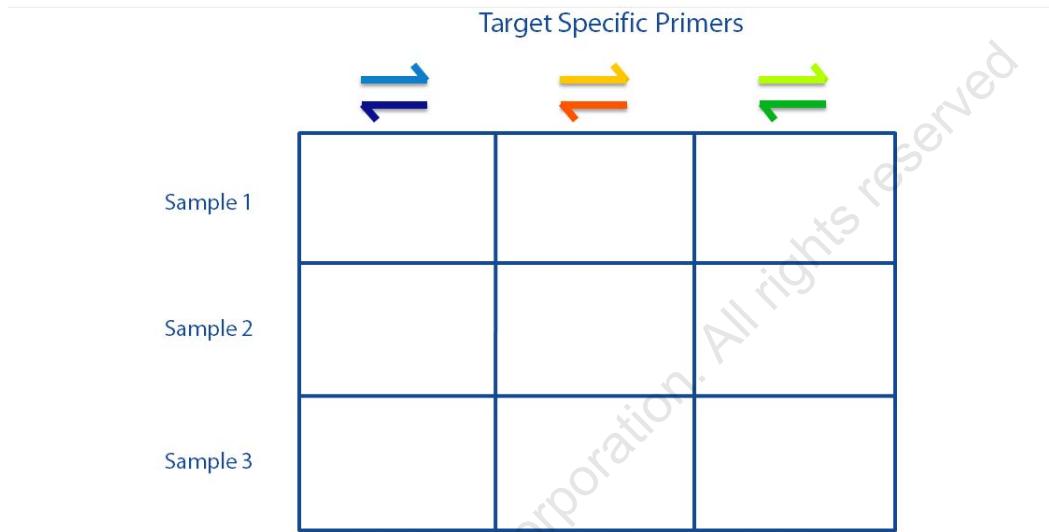


Figure 1

In Figure 2 below, TS Forward and Reverse Primers are loaded into each column of reaction wells on the 48.48 Access Array IFC.

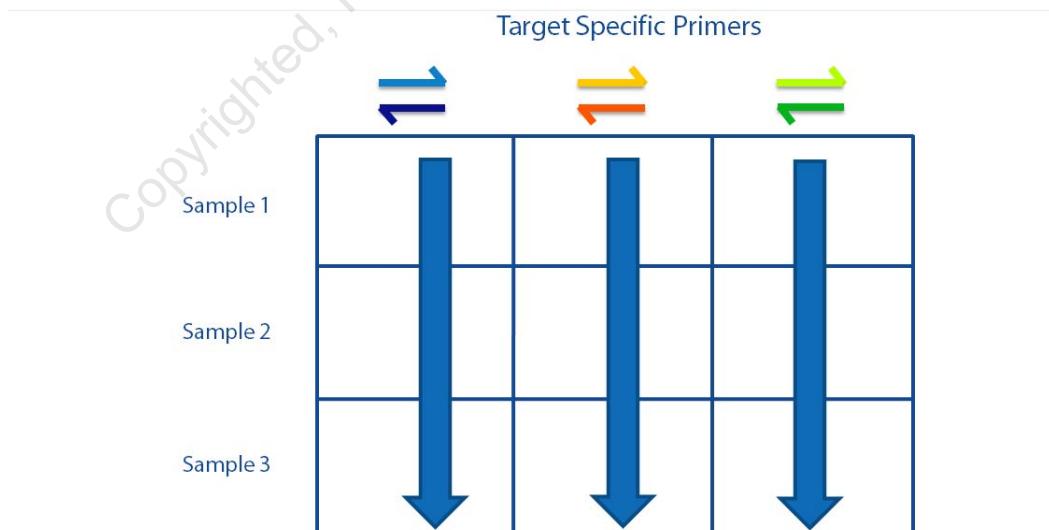


Figure 2

In Figure 3 below, samples are loaded into each row of reaction wells on the 48.48 Access Array IFC.



Figure 3

In Figure 4 below, each reaction well contains a unique combination of TS primer pairs and samples.

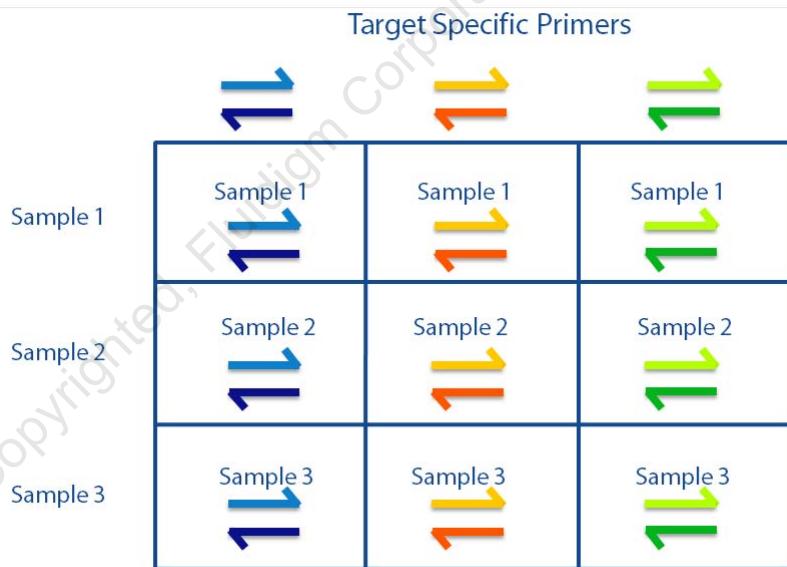


Figure 4

In Figure 5 below, the resultant PCR products are shown.

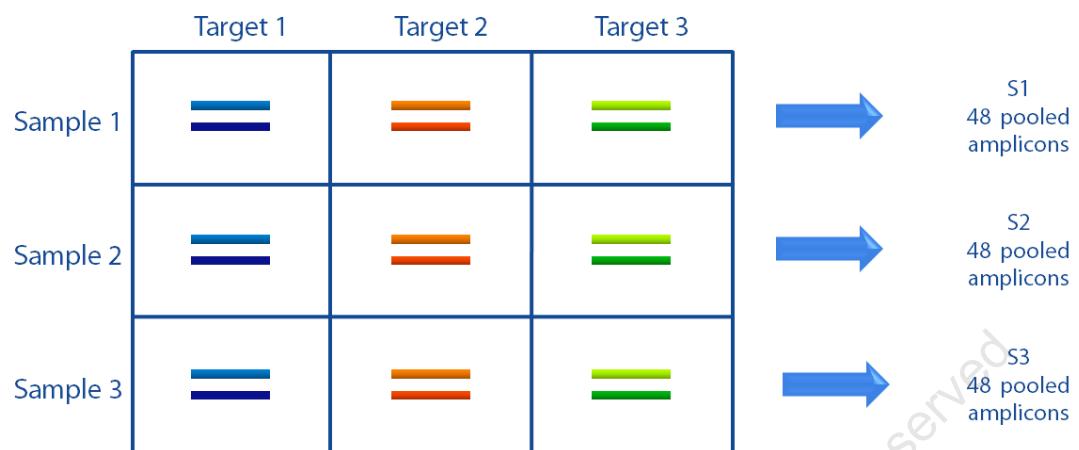


Figure 5

Target-Specific Primer Validation for 2-Primer Reactions on the 48.48 Access Array IFC

This section describes the validation procedure for the TS primers designed in Chapter 2.

Reference Documents

- *Agilent® DNA 1000 Kit Guide*

Required Reagents

Stored at -20 °C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- Target-specific (TS) forward and reverse primer pairs
 - 50 µM TS Forward Primer
 - 50 µM TS Reverse Primer
- 60 ng/µL Genomic DNA (Coriell, PN NA17317, optional)

Stored at 4 °C

- Agilent DNA 1000 Kit Reagents (PN 5067-1504)

Stored at Room Temperature

- PCR certified water (TEKnova, PN W3330)

Preparing the Primer Validation Reaction

The Primer Validation protocol prepares enough reagents to perform 48 primer validation reactions and 48 non-template-control (NTC) primer validation reactions.

-
- 1 Prepare the 5X Target-Specific Primer Solutions for 48 individual primer pairs as shown in the table below.

Component	Volume (μ L)	Final Concentration
50 μ M TS Forward Primer	2	1 μ M
50 μ M TS Reverse Primer	2	1 μ M
PCR Certified Water (TEKnova)	96	
Total	100	

Table 1 5X Target-Specific Primer Solution Preparation

- 2 Vortex the 5X Primer Solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final TS Forward and Reverse Primer concentrations are 1 μ M in the 5X Primer Solution. The final TS Forward and Reverse Primer concentrations in the PCR reaction are 200 nM.

3 Prepare the primer validation reaction components.



IMPORTANT: Warm up the 20X Access Array Loading Reagent to room temperature before use.

Component	Volume per reaction (μL)	Volume for 60 reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl ₂ (Roche)	0.5	30.0	1X
25 mM MgCl ₂ (Roche)	0.9	54.0	4.5 mM
DMSO (Roche)	0.25	15.0	5%
10mM PCR Grade Nucleotide Mix (Roche)	0.10	6.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/ μL
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
60 ng/ μL Genomic DNA (Coriell)	0.83	49.8	10 ng/ μL
PCR Certified Water (TEKnova)	1.12	67.2	
Total	4.0	240.0	

Table 2 Target-Specific Primer Validation Reaction Preparation

4 Vortex the Primer Validation Reaction Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The volumes provided in the table above are sufficient for 60 5 μL reactions on a 384-well plate. We recommend preparing this amount to provide sufficient reagent to minimize errors due to pipetting.

NOTE: For target-specific PCR reaction validation experiment, the 2 μM Access Array Barcode 1 Primers for Illumina solution is not used.

5 Prepare the NTC primer validation reaction components.

Component	Volume per reaction (μ L)	Volume for 60 reactions (μ L)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl ₂ (Roche)	0.5	30.0	1X
25 mM MgCl ₂ (Roche)	0.9	54.0	4.5 mM
DMSO (Roche)	0.25	15.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.1	6.0	200 μ M ea
5 U/ μ L FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/ μ L
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
PCR Certified Water (TEKnova)	1.95	117.0	
Total	4.0	240.0	

Table 3 NTC Target-Specific Primer Validation Reaction Preparation

- 6 Vortex the NTC Reaction Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.**



NOTE: The volumes provided in the table above are sufficient for 60 5 μ L reactions on a 384-well plate. We recommend preparing this amount to provide sufficient reagent to minimize errors due to pipetting.

Setting up the PCR Reactions in a 384-well PCR Plate

- 1 Prepare the Primer Validation PCR reactions:**
 - a Add 4 μ L of Primer Validation Reaction Mix to each well.**
The total PCR reaction volume is 5 μ L.
 - b Add 1 μ L of the 5X Target-Specific Primer Solution.**
- 2 Next prepare the NTC Primer Validation Reactions:**
 - a Add 4 μ L of NTC Primer Validation Reaction Mix to 48 wells.**
The total PCR reaction volume is 5 μ L.
 - b Add 1 μ L of the 5X Target-Specific Primer Solution.**
- 3 Vortex the 384-well PCR reaction plate for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.**

Running the PCR Reactions

- Load the 384-well plate onto the PCR thermal cycler.
Run 35 cycles of PCR using the protocol described below:

1

PCR Stages	Number of Cycles
50°C 2 minutes	1
70°C 20 minutes	1
95°C 10 minutes	1
95°C 15 seconds 60°C 30 seconds 72°C 1 minute	10
95°C 15 seconds 80°C 30 seconds 60°C 30 seconds 72°C 1 minute	2
95°C 15 seconds 60°C 30 seconds 72°C 1 minute	8
95°C 15 seconds 80°C 30 seconds 60°C 30 seconds 72°C 1 minute	2
95°C 15 seconds 60°C 30 seconds 72°C 1 minute	8
95°C 15 seconds 80°C 30 seconds 60°C 30 seconds 72°C 1 minute	5

Table 4 PCR Protocol



NOTE: The thermal protocol has been optimized for tagged primers. For validation of untagged primers, alternative PCR protocols can be used as long as PCR Stages 1 and 2 are included and the total duration of the protocol does not exceed that of the protocol. Please contact Technical Support with questions.

Checking PCR Products on the Agilent 2100 BioAnalyzer

- Use Agilent DNA 1000 chips from the Agilent DNA 1000 Kit to check 1 μ L of PCR product from each of the PCR reactions, including NTC reactions, described above. Follow the *Agilent DNA 1000 Kit Guide* for details.
 - Run and compare the NTC and DNA reactions side by side on the Agilent DNA1000 chips.

-
- 2 Check the results of the chip to determine if the PCR product in the DNA reaction has the expected size.



NOTE: The product size should match the length of the target region.

- 3 As shown in Figure 6, Gene 1 did not generate the specific product since the PCR product in the DNA reaction has the same size as in the NTC reaction. Gene 2 did generate the specific product.

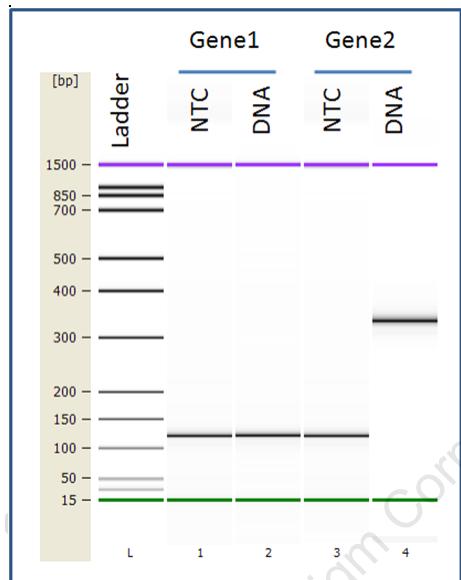


Figure 6

- 4 The primer pair for Gene 2 can be used for PCR on a 48.48 Access Array IFC.
5 The Gene 1 primer pair needs to be redesigned following the instructions in Chapter 2 and validated as described above before continuing.

- 6 Figure 7 shows another example of 48 primer pairs checked on an Agilent chip. The red labeled reactions did not generate products with the correct size or generated multiple products in the PCR reactions. Therefore these primers will need to be redesigned following the instruction in Chapter 2 and validated before continuing.

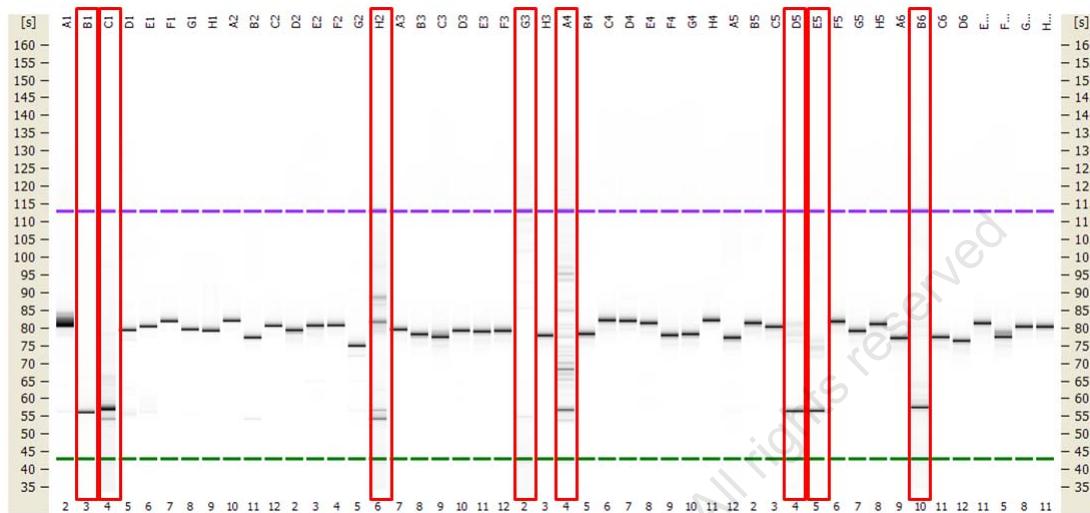


Figure 7

2-Primer Target-Specific PCR Amplification on the 48.48 Access Array IFC

Reference Documents

- *Fluidigm® IFC Controller for Access Array™ System User Guide* (PN 68000157)
- *Fluidigm® Control Line Fluid Loading Procedure Quick Reference* (PN 68000132)
- *Agilent DNA 1000 Kit Guide*

Required Supplies

Stored at -20°C

- FastStart High Fidelity PCR System, dNTPack (Roche, PN 04-738-292-001)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)
- Target-specific (TS) Forward and Reverse Primer Pairs
 - 50 µM TS Forward Primer
 - 50 µM TS Reverse Primer
- Template DNA at 50ng/µL



NOTE: 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) is not packaged for individual sale. It can be purchased in units of 10, under the name Access Array Harvest Pack, PN 100-3155, or as a component in the 48.48 Access Array Loading Reagent Kit, PN 100-1032.

Stored at 4°C

- Agilent DNA 1000 Kit Reagents (Agilent, PN 5067-1504)

Stored at Room Temperature

- PCR Certified Water (TEKnova, PN W330)

48.48 Access Array IFC Workflow

Priming the 48.48 Access Array IFC



CAUTION! Use the 48.48 Access Array IFC within 24 hours of opening the package.

CAUTION! Control Line Fluid on the IFC or in the inlets makes the IFC unusable. Use only 48.48 syringes with 300 µL of Control Line Fluid (Fluidigm, PN 89000020).

CAUTION! Load the IFC into the **Pre-PCR** IFC Controller AX in the Pre-PCR lab within 60 minutes of priming.

- 1 Inject Control Line Fluid into each accumulator on the IFC.
- 2 Add 500 µL of 1X Access Array Harvest Solution (Fluidigm, PN 100-1031) into the H1-H4 wells on the IFC.
- 3 Remove and discard the blue protective film from the bottom of the AA IFC.
- 4 Load the IFC into the **Pre-PCR** IFC Controller AX located in the Pre-PCR lab.
- 5 Press **Eject** to move the tray out of the IFC Controller AX.
- 6 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 7 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 8 Select **Prime (151x)** and **Run Script** to prime the IFC.
Once the script is complete, press **Eject** to remove the IFC.

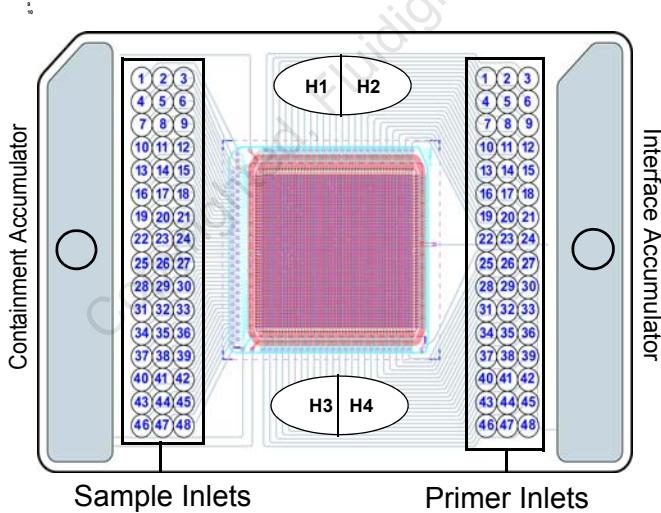


Figure 8 48.48 Access Array IFC overview

Preparing the 20X Primer Solutions



IMPORTANT: Warm up 20X Access Array Loading Reagent to room temperature before use.

Prepare the 20X Primer Solutions for 48 individual primer pairs as shown in the table below. These will be loaded into the Primer Inlets of an 48.48 Access Array IFC.

Component	Volume (μL)	Final Concentration
50 μM TS Forward Primer	8.0	4 μM
50 μM TS Reverse Primer	8.0	4 μM
20X Access Array Loading Reagent (Fluidigm)	5.0	1X
PCR Certified Water (TEKnova)	79.0	
Total Volume	100.0	

Table 5 20X Target-Specific Primer Solution

- 1 Vortex the 20X Primer Solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final TS forward and reverse primer concentrations are 4 μM in the 20X Primer Solution. The final TS forward and reverse primer concentrations in the Access Array IFC reaction chamber are 200 nM.

Preparing Sample Mix Solutions

All 48 DNA samples need to be added to the Sample Pre-Mix individually, prior to the loading the Sample Mix solutions into the Sample Inlets of a 48.48 Access Array IFC.

Prepare the Sample Pre-Mix

- Working in a DNA-free hood, combine the components listed in the table below.

This protocol prepares enough Sample Pre-Mix for 60 reactions. This is enough reagent to load one 48.48 Access Array IFC with 16 additional reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 60 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer without MgCl_2 (Roche)	0.50	30.0	1X
25 mM MgCl_2 (Roche)	0.90	54.0	4.5 mM
DMSO (Roche)	0.25	15.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.10	6.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.0	0.05 U/ μL
20X Access Array Loading Reagent (Fluidigm)	0.25	15.0	1X
PCR-Certified Water (TEKnova)	1.95	117.0	
Total	4.0	240.0	

Table 6 Sample Pre-Mix Solution

- Vortex the Sample Pre-Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare the Sample Mix Solutions

- Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume per Reaction (μL)	Final Concentration
Sample Pre-Mix	4.0	
50 ng/ μL Genomic DNA	1.0	10 ng/ μL
Total	5.0	

Table 7 Sample Mix Solutions

-
- 2 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



IMPORTANT: It is essential to vortex all components to ensure complete mixing.

Loading the 48.48 Access Array IFC

- 1 Pipette 4 μ L of 20X Primer Solution into each of the Primer Inlets.
- 2 Pipette 4 μ L of Sample Mix solution into each of the Sample Inlets.



CAUTION! While pipetting, do not go past the first stop on the pipette. Doing so may introduce air bubbles into the inlets.



NOTE: An 8-channel pipette is recommended to load the Sample Mix and 20X Primer Solutions. The recommended pipetting order is shown below.

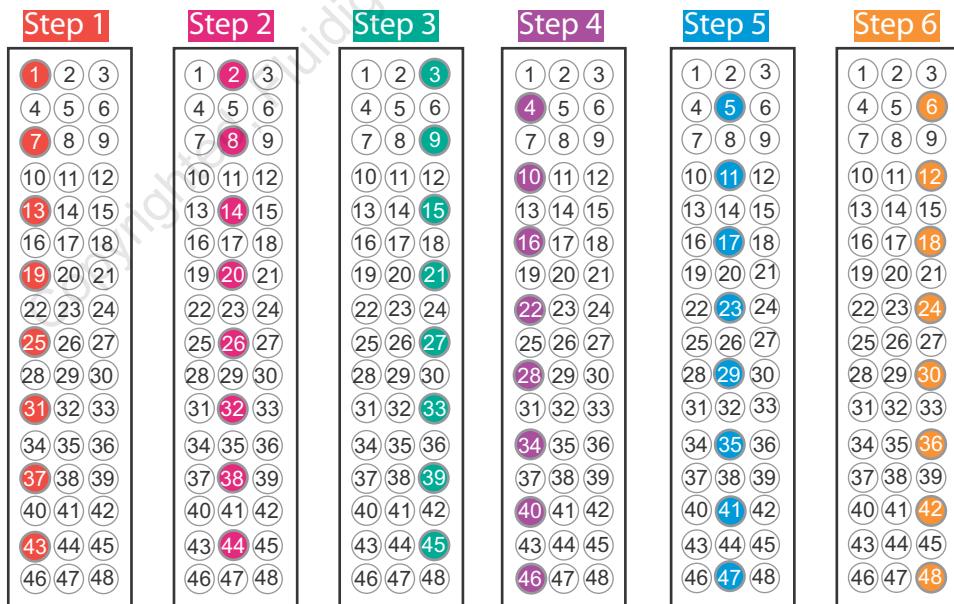


Figure 9 Pipetting order

- 3 Load the 48.48 Access Array IFC into the **Pre-PCR** IFC Controller AX in the Pre-PCR lab.
- 4 Press **Eject** to move the tray out of the IFC Controller AX.
- 5 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 6 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 7 Select **Load Mix (151x)** and **Run Script**.
- 8 Once the script is complete, press **Eject** to remove the IFC.

Thermal Cycling the 48.48 Access Array IFC

- 1 Place the 48.48 Access Array IFC onto the FC1 Cycler and start PCR by selecting the protocol **AA 48x48 Standard v1**.

See the FC1 Cycler appendix at the end of this manual for more detailed instructions about using the cycler.

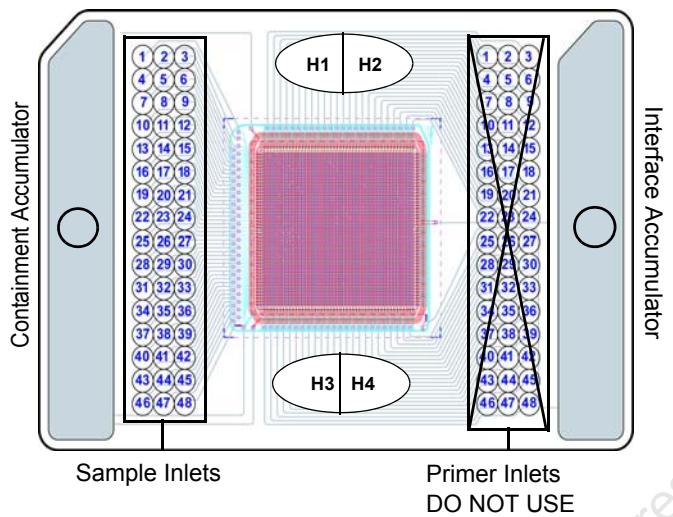
For Stand-Alone Thermal Cycler, select protocol **AA48v1**.



NOTE: The protocol as programmed into the Fluidigm Stand-Alone Thermal Cycler takes undershooting and overshooting of target temperatures into consideration and is therefore not identical to the PCR protocol as written out in tables 3 and 6. Contact Fluidigm Technical Support (1-866-358-4353 or techsupport@fluidigm.com) if you need assistance in programming your Fluidigm FC1 Cycler or Stand-Alone Thermal Cycler.

Harvesting the 48.48 Access Array IFC

- 1 After the PCR has finished, move the 48.48 Access Array IFC into the Post-PCR lab for harvesting.
- 2 Remove the remaining 1X Access Array Harvest Reagent from the H1-H4 wells.
- 3 Pipette 600 μ L of fresh 1X Access Array Harvest Reagent into the H1-H4 wells.
- 4 Pipette 2 μ L of 1X Access Array Harvest Reagent into each of the Sample Inlets on the IFC.
- 5 Load the IFC into the **Post-PCR** IFC Controller AX located in the Post-PCR lab.
- 6 Press **Eject** to move the tray out of the IFC Controller AX.
- 7 Place the IFC onto the tray by aligning the notched corner of the IFC to the A1 mark.
- 8 Press **Load Chip** to register the barcode of the IFC and activate the script selection.
- 9 Select **Harvest (151x)** and **Run Script**.
- 10 Once the script is complete, press **Eject** to remove the IFC.
- 11 Label a 96-well plate with the 48.48 Access Array IFC barcode. Carefully transfer 10 μ L of harvested PCR products from each of the Sample Inlets into columns 1-6 of a 96-well PCR plate, using an 8-channel pipette.



NOTE: Remove PCR products from the IFC in the same order as the IFC was loaded, using an 8-channel pipette.

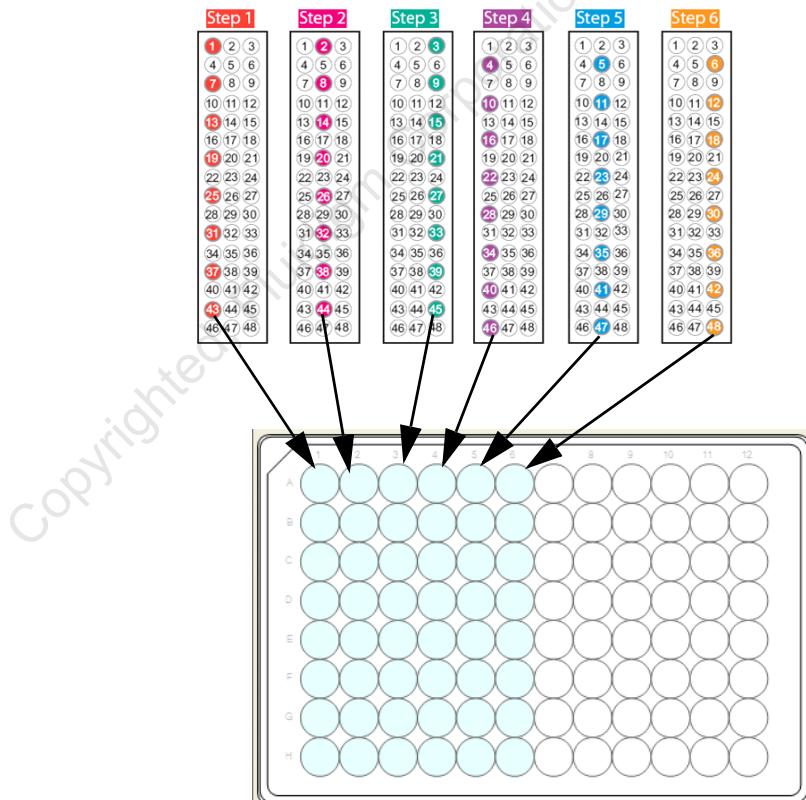


Figure 10 PCR product transfer map from the 48.48 Access Array IFC to a 96-well PCR plate.

Electropherogram Examples

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Examples of Electropherograms 122

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Examples of Electropherograms

Examples of an Agilent 2100 BioAnalyzer Electropherogram of a harvested PCR product pool

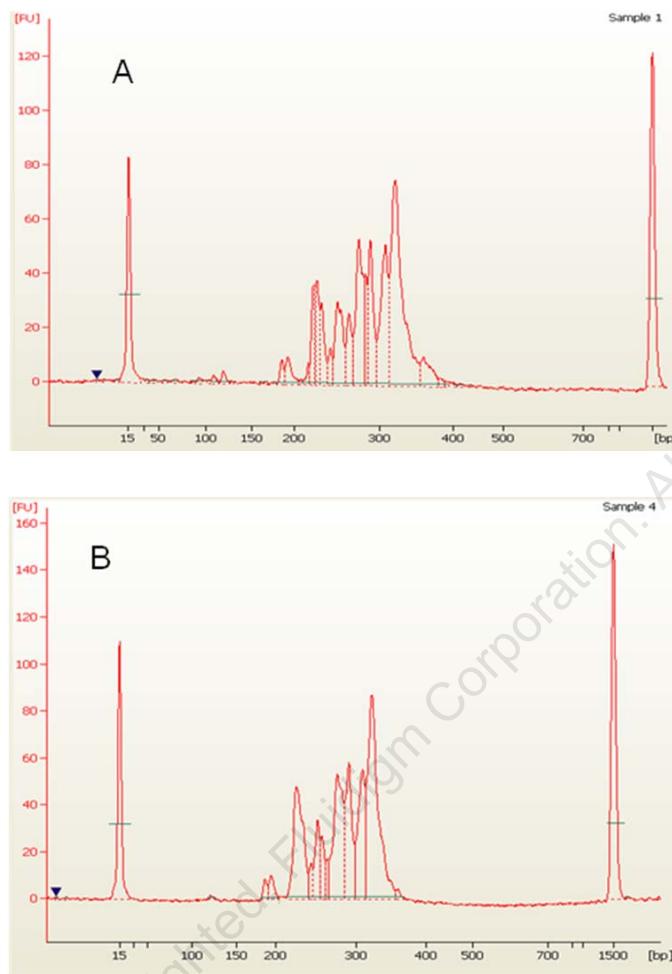


Figure 1 DNA 1000 Chip Electropherogram of a pooled PCR product with 48 amplicons ranging between 180-350 bp. A. Harvested PCR product pool from a 48.48 Access Array IFC; B. The same PCR product pool after SPRI cleanup.

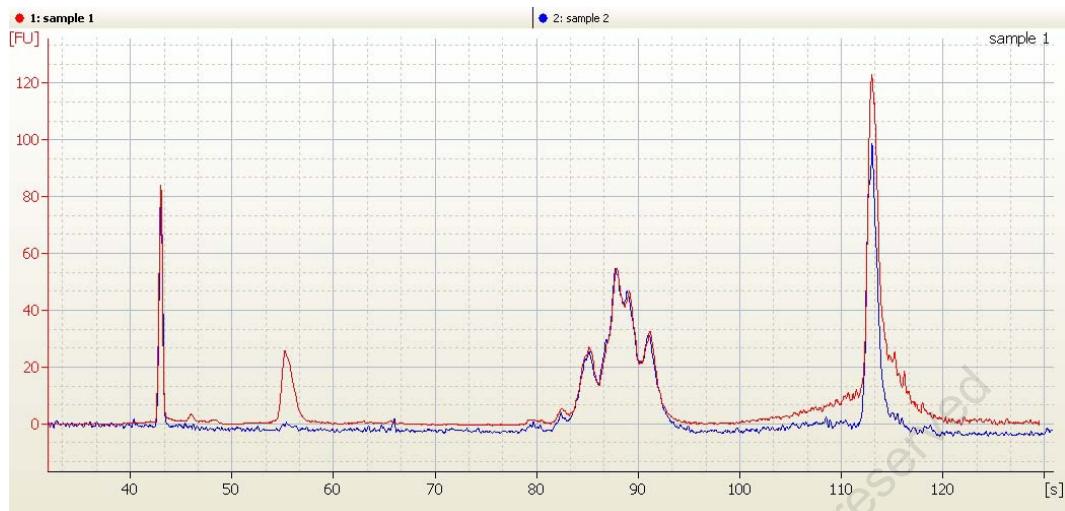


Figure 2 DNA 1000 Chip Electropherogram overlay of an unpurified pooled PCR product library and the same purified pooled PCR product library after Ampure XP cleanup.

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Ordering Instructions for FL1 and FL2 Sequencing Primers

E

Ordering Oligos for FL1 and FL2 Primers	126
Preparation of FL1 and FL2 Primer Mixes	128

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Ordering and Preparing FL1 and FL2 Sequencing Primers for the Illumina Sequencing Systems

Intended Use

This appendix describes the procedures for ordering and preparing FL1 and FL2 sequencing primers used for single-read or paired-end sequencing on the Illumina sequencers. The FL1 and FL2 sequencing primers are essential for sequencing amplicon libraries that have been generated using the Access Array Barcode Libraries.

Ordering Oligos for FL1 and FL2 Primers

- 1 Go to Exiqon's website order page for Custom LNA™ Oligos (<http://www.exiqon.com/order-lna-oligos>).
- 2 Under Enter an oligonucleotide sequence below, copy Oligo Name "CS1" from Table 1 for Oligo name.
- 3 Select DNA oligo from Oligo type drop-down menu.
- 4 Select 100 nmole from Synthesis scale drop-down menu. A larger synthesis scale can be selected if multiple sequencing runs are anticipated.
- 5 Select HPLC Purification from Purification drop-down menu.
- 6 Copy the sequence for the oligo CS1 from table 1 for Sequence (5'-3').

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

LNA nucleotides preceded by a “+”

Table 1 Oligo Sequences

7 Click Add to basket.

Enter an oligonucleotide sequence below

Oligo name CS1	Oligo type DNA oligo	Synthesis scale 100 nmole	Purification HPLC Purification
-------------------	-------------------------	------------------------------	-----------------------------------

A design ID will be added to the name of your oligo to ensure traceability

Sequence (5'-3')
A+CA+CTG+ACGACATGGTTCTACG

Analyze oligo
Add to basket
View basket
Clear data

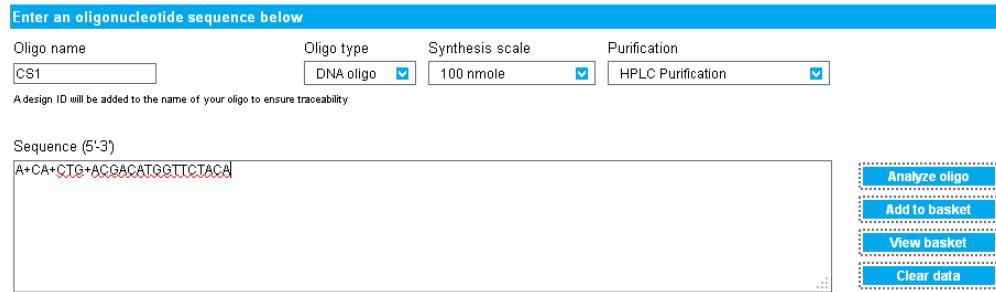


Figure 1 Example of CS1 oligo

8 Repeat steps 2-7 to order CS2, CS1rc and CS2rc oligos.

9 Click View basket.

10 Review the oligo sequence information for accuracy.

Basket

C\$1 Design ID: 130227 Product number: 500100 Description: A+G+A+CTG+ACG+ACATGGTTCTACA - custom 100 nmole DNA oligo, HPLC Purification, RNA Tm: 72 °C, DNA Tm: 69 °C, Purity: 82.5%, Yield: 1.00 D / 4.6 nmole / 31.5 µg Note: Price: USD 149.62	Quantity: <input type="button" value="1"/> Total: USD 149.62	<input type="button" value="Edit"/> <input type="button" value="Remove"/>
C\$2 Design ID: 130240 Product number: 500100 Description: T+A+C+G+GT+AGC+AGACTTGGTCT - custom 100 nmole DNA oligo, HPLC Purification, RNA Tm: 79 °C, DNA Tm: 71 °C, Purity: 82.5%, Yield: 1.00 D / 4.6 nmole / 32.0 µg Note: Price: USD 149.62	Quantity: <input type="button" value="1"/> Total: USD 149.62	<input type="button" value="Edit"/> <input type="button" value="Remove"/>
C\$3rc Design ID: 130241 Product number: 500100 Description: T+GT+AG+AACC+ATG+TCGTCAGTGT - custom 100 nmole DNA oligo, HPLC Purification, RNA Tm: 71 °C, DNA Tm: 69 °C, Purity: 82.5%, Yield: 1.00 D / 4.6 nmole / 31.8 µg Note: Price: USD 149.62	Quantity: <input type="button" value="1"/> Total: USD 149.62	<input type="button" value="Edit"/> <input type="button" value="Remove"/>
C\$2rc Design ID: 130242 Product number: 500100 Description: A+G+A+C+A+AGT+CTCTGCTACCGTA - custom 100 nmole DNA oligo, HPLC Purification, RNA Tm: 77 °C, DNA Tm: 71 °C, Purity: 82.5%, Yield: 1.00 D / 4.7 nmole / 32.0 µg Note: Price: USD 149.62	Quantity: <input type="button" value="1"/> Total: USD 149.62	<input type="button" value="Edit"/> <input type="button" value="Remove"/>

Price for products: USD 598.48
Shipping price: USD 0.00
Tax: USD 0.00
Total: USD 598.48

Figure 2 Example of oligo order

11 Click Check out.

12 Continue through the shipping and payment information to complete the ordering process.

Preparation of FL1 and FL2 Primer Mixes

- 1 Spin tubes briefly before opening.
- 2 Calculate the resuspension volume required for each oligo, use the following formula: $(X \text{ nmol oligo}) * 10 = \mu\text{L volume needed to resuspend oligo}$.
- 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 in Table 2 to a final concentration of 50 µM for each oligo.
- 6 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	

Table 2 Instructions for preparing FL1 stock

- 7 Prepare FL2 primer mix. Mix CS1rc oligo and CS2rc oligo as described in Table 3 to a final concentration of 50 µM for each oligo. Vortex after mixing to ensure complete mixing. Spin down.
- 8 Store FL1 and FL2 stock at -20°C.

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

Table 3 Instructions for preparing FL2 stock

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Sequencing Workflow Using Fluidigm FL1 and FL2 Sequencing Primers

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Sequencing Workflow Using Fluidigm FL1 and FL2 Sequencing Primers	132
Preparing Reagents for Sequencing on the Illumina GAII and HiSeq Sequencing Systems	133
Performing a Sequencing Run.	136

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Sequencing Workflow Using Fluidigm FL1 and FL2 Sequencing Primers

The following instructions are intended for use with Illumina TruSeq sequencing reagents on the Illumina MiSeq, GAIi and HiSeq systems. The Fluidigm sequencing reagents FL1 and FL2, contain equimolar mixtures of the CS1 and CS2 sequencing and indexing primers, respectively. Results from our PCR experiments to test for cross talk between Fluidigm Sequencing Primers and TruSeq Sequencing Primers can be found at the end of this Appendix.

- FL1 is the custom sequencing primer and contains 50 µM each of the CS1 and CS2 primers (prepared from [Appendix E](#)).
- FL2 is the custom indexing primer and contains 50 µM each of the CS1rc and CS2rc primers (also prepared from [Appendix E](#)).
- For **single-read sequencing**, prepare reagents for Read 1 and the Indexing primers.
- For **paired-end sequencing**, prepare reagents for Read 1, the Indexing, and Read 2 primers.

Reference Documents

- *Illumina MiSeq™ User Guide*
- *Illumina cBot™ User Guide*
- *Illumina Genome Analyzer II™ User Guide*
- *Illumina HiSeq™ User Guide*



IMPORTANT: Please refer to the MiSeq, Illumina GAIi, or HiSeq User Guide for instructions on how to perform a sequencing run, or contact Technical Support at Illumina.

Preparing Reagents for Sequencing on the Illumina GAIi and HiSeq Sequencing Systems

Preparing Read 1 Sequencing Primer HT1/FL1 for the cBot

Dilute the FL1 stock to a final concentration of 500 nM with Hybridization Buffer (HT1) in a DNase-, RNase-free 1.5 mL microcentrifuge tube. Vortex the tube for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components. The following instructions outline preparation of the HT1/FL1 sequencing primer mix for Read 1 (per mL). Approximately 300 μ L is required per lane, using the cBot Custom Primers Reagent Stage. Align the custom primer orientation in the tube strip with the lanes of the GAIi or HiSeq flow cell.

Reagent	Volume
HT1 Buffer	990 μ L
FL1 Stock at 50 μ M per primer (from Appendix E)	10 μ L
Total	1.0 mL

Table 1 Instructions for Preparing HT1/FL1 (per mL)

Adding Read 2 Indexing Primer FL2 to HP8

The TruSeq Reagent Rack is filled with approximately 3.15 mL of HP8. Add 31.5 μ L of FL2 Stock at 50 μ M per primer (from Appendix E) to the HP8 reagent position. Make sure to thoroughly mix by pipetting. The final concentration of FL2 is now 500 nM.

Adding Read 3 Sequencing Primer FL1 to HP7 (for Paired-End Sequencing)

The TruSeq Reagent Rack is filled with approximately 3.15 mL of HP7. Add 31.5 μ L of FL1 stock at 50 μ M per primer (from Appendix E) to the HP7 reagent position. Make sure to thoroughly mix by pipetting. The final concentration of FL1 is now 500 nM.

Preparing Reagents for Sequencing on the Illumina MiSeq Sequencer

The following instructions are intended for use with the MiSeq custom ports. Please refer to the Illumina MiSeq User Guide for sample sheet setup and for instructions on how to perform a sequencing run. For guidance on using PhiX control in a sequencing run using custom ports, please contact Fluidigm Technical Support.

Preparing Read 1 Sequencing Primer FL1

Dilute Fluidigm sequencing reagent FL1 (which contains the custom sequencing primers) to a final concentration of 500 nM with HT1 buffer in a DNase-, RNase-free 1.5 mL microcentrifuge tube. Vortex the tube for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Reagent	Volume
HT1 Buffer	693 µL
FL1 Stock at 50 µM per primer (from Appendix E)	7 µL
Total	700 µL

Table 2 Instructions for diluting FL1 with HT1 Buffer

Pierce the foil seal covering reservoir 18 and load 680 µL of diluted FL1 for Read 1.

Preparing Read 2 Indexing Primer FL2

Dilute Fluidigm sequencing reagent FL2 (which contains the custom barcode sequencing primers) to a final concentration of 500 nM with HT1 buffer in a DNase-, RNase-free 1.5 mL microcentrifuge tube. Vortex the tube for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Reagent	Volume
HT1 Buffer	693 µL
FL2 Stock at 50 µM per primer (from Appendix E)	7 µL
Total	700 µL

Table 3 Instructions for diluting FL2 with HT1 Buffer

Pierce the foil seal covering reservoir 19 and load 680 µL of diluted FL2 for Read 2.

Preparing Read 3 Sequencing Primer FL1 (for Paired-End Sequencing only)

Dilute Fluidigm sequencing reagent FL1 (which contains the custom sequencing primers) to a final concentration of 500nM with HT1 buffer in a DNase-, RNase-free 1.5mL microcentrifuge tube. Vortex the tube for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Reagent	Volume
HT1 Buffer	693 µL
FL1 Stock at 50 µM per primer (from Appendix E)	7 µL
Total	700 µL

Table 4 Instructions for diluting FL1 with HT1 Buffer

Pierce the foil seal covering reservoir 20 and load 680 µL of diluted FL1 for Read 3.

Tap the reagent cartridge 2-3 times, and inspect the bottom of the cartridge to check for bubbles.

Performing a Sequencing Run

Please refer to the Illumina MiSeq, GAII or HiSeq User Guide for instructions on how to perform a sequencing run, or contact Technical Support at Illumina.

Index Read

The barcode sequences used in the Access Array Barcode Library for Illumina have been designed so that they can be distinguished even when sequencing errors are present. As more samples are run in parallel, the length of the index read required to distinguish the barcode sequences unambiguously increases. The table below describes our recommendations.

Number of samples per lane	1-384	385-1920
Length of index read	8 bases	10 bases

Table 5 Index Read Recommendations

When preparing the sequencing run, the length of the index read should be adjusted according to the guidelines in Table 4. Please ensure that the volumes of the sequencing reagents loaded onto the sequencer are sufficient for the index cycles. For detailed instructions on how to implement these changes, please consult with the Illumina Sequencer User Guide, or contact Technical Support at Illumina.

The figure below illustrates the results from our PCR experiment to test for cross talk between Fluidigm and Illumina TruSeq Sequencing Primers on Illumina generated libraries.

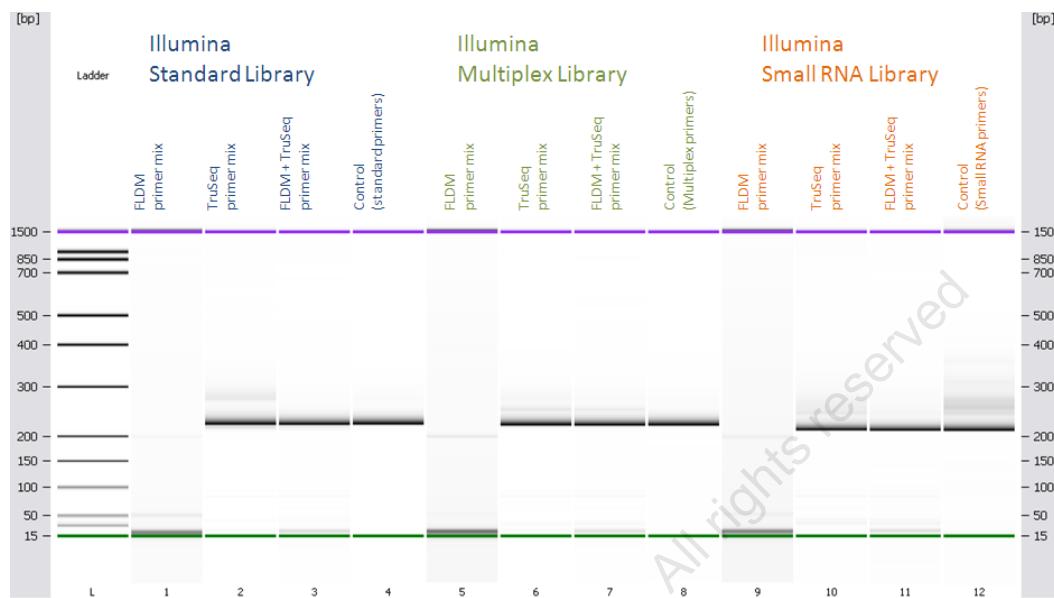


Figure 1 Agilent Bioanalyzer results from a cross talk experiment between Fluidigm and Illumina TruSeq sequencing primers on Illumina generated libraries. The PCR reactions for each lane are as follows:

- 1 Illumina standard library + Fluidigm FL1 sequencing primers
- 2 Illumina standard library + Illumina TruSeq sequencing primers
- 3 Illumina standard library + Fluidigm FL1 and Illumina TruSeq sequencing primers
- 4 Illumina standard library + Illumina standard sequencing primers (control)
- 5 Illumina Multiplex library + Fluidigm FL1 sequencing primers
- 6 Illumina Multiplex library + Illumina TruSeq sequencing primers
- 7 Illumina Multiplex library + Fluidigm FL1 and Illumina TruSeq sequencing primers
- 8 Illumina Multiplex library + Illumina Multiplex sequencing primers (control)
- 9 Illumina Small RNA library + Fluidigm FL1 sequencing primers
- 10 Illumina Small RNA library + Illumina TruSeq sequencing primers
- 11 Illumina Small RNA library + Fluidigm FL1 and Illumina TruSeq sequencing primers
- 12 Illumina Small RNA library + Illumina Small RNA sequencing primers (control)

The figure below illustrates the results from our PCR experiment to test for cross talk between Fluidigm and Illumina TruSeq Sequencing Primers on an Access Array generated library.

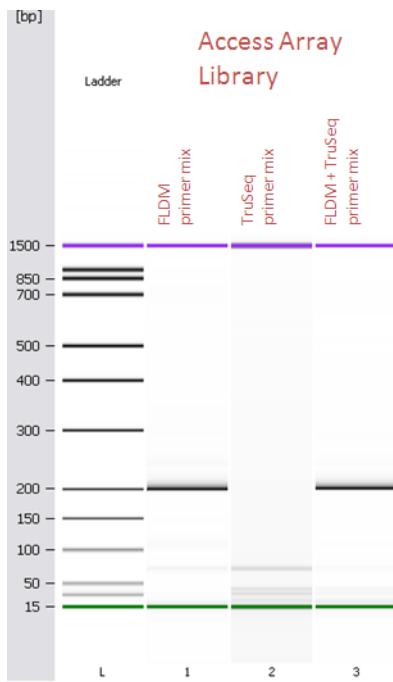


Figure 2 Agilent Bioanalyzer results from a cross talk experiment between Fluidigm and Illumina TruSeq sequencing primers on an Access Array generated library. The PCR reactions for each lane are as follows:

- 1 Fluidigm Access Array library + Fluidigm FL1 sequencing primers
- 2 Fluidigm Access Array library + Illumina TruSeq sequencing primers
- 3 Fluidigm Access Array library + Fluidigm FL1 and Illumina TruSeq sequencing primers

Guidelines for Using FFPE Genomic DNA on the 48.48 IFC

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In this chapter:

Section 1: Introduction	140
Section 2: Extraction of Genomic DNA from FFPE tissue Samples	141
Guidelines	141
Section 3: Sample Quality Assessment.	143
Absolute copy number determination with a 180 bp TaqMan assay.	145
Prepare Standard Curve DNA.	146
Prepare the Sample Mix Solutions	146
Thermal Cycling of the 384-Well Plate	147
Section 4: Amplicon Tagging on the Access Array IFC.	148
General Considerations	149
Enrichment PCR with Target-Specific Primers on the 48.48 Access Array IFC	156
Attaching Sequence Tags and Sample Barcodes	161
Section 5: BioAnalyzer Gel Image Example.	165

Section 1: Introduction

Extraction of functional DNA from formalin-fixed, paraffin-embedded (FFPE) archival tissue samples can present a number of challenges due to damage caused during the fixing process. Fragmentation, base modifications, protein-protein and protein-nucleic acid crosslinks all contribute to the damage, and may reduce the functionality of DNA as a template in PCR reactions. A number of factors affect the DNA quality, including years of storage, temperature of storage, length of time between tissue harvesting and fixation, and the fixation process itself. Some of the chemical modifications can be reversed through the extraction process; however, DNA fragmentation and DNA damage is permanent. In most cases, the fixation and embedding process is outside the control of the laboratory where the genetic analysis takes place. We have prepared this set of guidelines as best practices for amplifying genomic DNA extracted from FFPE samples with the Access Array™ System. These guidelines describe a set of recommendations to be used during DNA extraction, DNA quality assessment, along with guidelines for primer design and protocols for running experiments on the Access Array System.

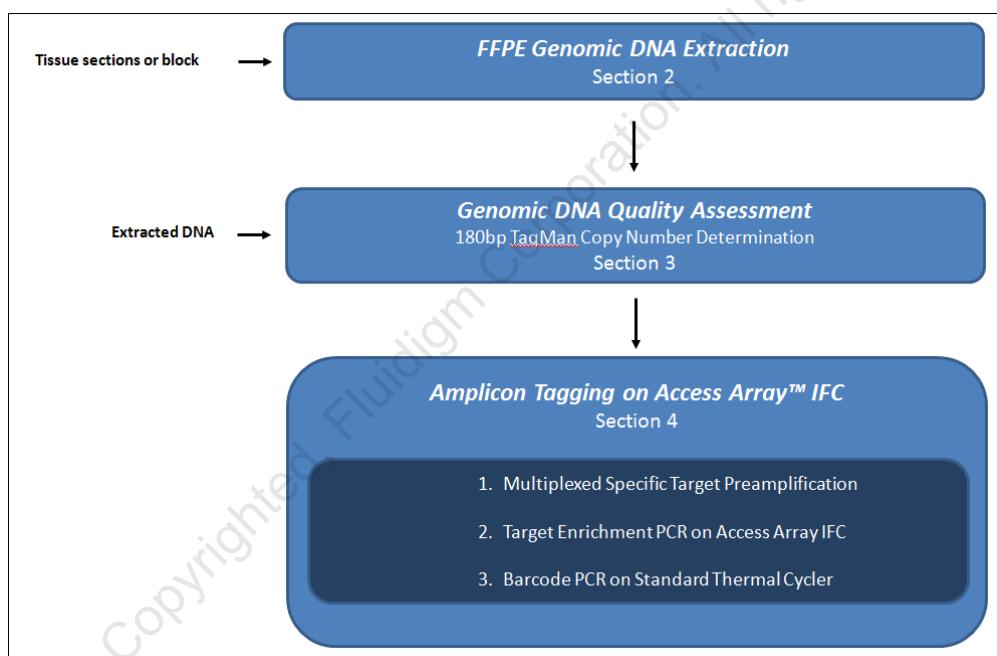


Figure 1 Protocol Outline

Section 2: Extraction of Genomic DNA from FFPE tissue Samples

Reference Documents

- *QIAamp DNA FFPE Tissue Handbook* (Qiagen)

Required Reagents

Stored at -20 °C

- RNase A (Sigma-Aldrich, PN R6513-10MG)

Stored at 4 °C

- MinElute Columns QIAamp DNA FFPE Tissue Kit

Stored at Room Temperature

- Xylene
- 100% ethanol
- QIAamp DNA FFPE Tissue Kit (Qiagen, PN 56404)

Required Equipment

- Surgical Scalpel Handle (Roboz, RS09843)
- Surgical Scalpel Blades (Roboz, RS-9801-11)
- 1.5 mL or 2 mL microcentrifuge tubes
- Microcentrifuge with rotor for 2 mL tubes
- Thermomixer or 56 °C heating block or water bath
- 90 °C heating block

Guidelines

FFPE tissue samples are typically available in two formats-tissue blocks and sections mounted either on microscope slides or in tubes.

- 1 If you have access to the sample block, cut sections 8-10 µm thick and place 4-8 sections directly in a microcentrifuge tube containing 1 mL xylene. Proceed to step 7. If tissue sections are already adhered to histology slides, continue to step 2.
- 2 Attach a new blade to the scalpel handle.
- 3 Working in a fume hood, aliquot 1 mL xylene for each sample in a labeled microcentrifuge tube.
- 4 For best results, use 4-8 sections with a thickness of 8-10 µm.

-
- 5 Dip the scalpel in the xylene and let most of the liquid drip off, leaving behind a thin layer. This prevents the brittle sample from scattering or falling apart.
 - 6 Hold the histology slide at an angle facing you so you can easily see the tissue. Firmly press the entire scalpel blade on the slide at about a 45° angle and slowly draw it toward you, getting as much of the tissue off as possible. Before the blade dries completely, dip it into the microcentrifuge tube containing the 1 mL of xylene. Aim to remove most of the tissue from the slide in one or two passes. Use the tip of the blade to remove the remaining tissue.



NOTE: Be careful not to get too much xylene on the blade as it impedes removal of the tissue from the blade.

- 7 Once all the samples have been transferred to xylene, proceed as directed in the Qiagen QIAamp FFPE tissue kit, step 3. However, take note of the following exceptions:
 - a *QIAamp DNA FFPE Tissue Handbook* steps 5-7 Because the density of the tissue is very similar to that of xylene and the pellet readily dislodges from the bottom of the centrifuge tube, do not attempt to remove the entire supernatant. Rather, remove 700 µL of xylene and add 1 mL of 100% ethanol. Mix by vortexing and spin the contents of the tube down at maximum speed for 2 minutes. Remove the ethanol/xylene mixture and repeat the wash step with 1 mL of ethanol. Continue with step 8 of the *QIAamp DNA FFPE Tissue Handbook*.
 - b *QIAamp DNA FFPE Tissue Handbook* step 10 If the starting material has a surface area that exceeds 250 mm² and you are using more than three slides, you may consider increasing the buffer ATL and proteinase K volumes.
 - c *QIAamp DNA FFPE Tissue Handbook* step 11 During the Proteinase K lysis step, leave the tube at 56 °C for a minimum of 1.5 hours. If available, use a thermomixer, otherwise vortex the tube every 30 minutes. Digestion is complete when there are no more pieces of tissue visible.

Section 3: Sample Quality Assessment

Reference Documents

- TaqMan® Copy Number Assays Protocol (Life Technologies, PN 4397425 Rev. C)

Required Reagents

Stored at -20 °C

- TaqMan Gene Expression Assay (Life Technologies, PN 4331182)
- 20X FTH1 (180 bp) labeled with FAM (Hs01694011_s1)

Stored at 4 °C

- TaqMan Gene Expression Master Mix (Life Technologies, PN 4369016)
- Positive control genomic DNA at 50 ng/µL

Stored at Room Temperature

- DNA Suspension Buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA) (TEKnova, PN T0221)
- PCR Certified Water (TEKnova, PN W3330)

Required Equipment

- 1.5 mL or 2 mL microcentrifuge tubes
- Microcentrifuge with rotor for 2 mL tubes
- Microcentrifuge with rotor for 0.2 mL PCR tube strips
- Centrifuge with plate carriers
- Real-Time PCR system with 384-well block (e.g., Applied Biosystems 7900HT)
- MicroAmp Optical 384-Well Reaction Plate with Barcode (Applied Biosystems, PN 4309849)
- MicroAmp Optical Adhesive Film (Applied Biosystems, PN 4311971)

Fragmentation, base modifications, protein-protein and protein-nucleic acid crosslinks all contribute to the DNA damage caused by the FFPE fixation process. As a result, the functionality of DNA as a template in PCR reactions can be greatly reduced. Quantitation of the total DNA concentration with standard measurements such as picogreen or spectrophotometry therefore does not correlate with amplification efficiency as it does not assess the functionality of the DNA. Similarly, whereas a BioAnalyzer trace yields useful information with regards to the level of DNA fragmentation, at best it can only serve as a rough indicator of amplification success. For example, it is obvious why the samples shown in Figure 2A are good performers in subsequent PCR amplification reactions: The mode of the fragment size distribution peak is greater than 1 kb and there is no peak of degraded DNA visible. However, predicting functionality of the samples shown in Figure 2B and 2C poses a greater challenge. For example, both left hand panels show a peak with a mode around 300 bp. However, the sample in Figure 2B outperforms the sample in Figure 2C in subsequent PCR amplification reactions. Similarly, the right hand panels in Figure 2B and 2C show a peak of degraded DNA around 200 bp. The presence of a second, higher molecular weight peak in the right hand panel of Figure 2C would presumably lead to better amplification results for this sample than for the sample shown in the right hand panel of Figure 2B. However, this is not the case. Underlying causes are therefore unlikely to be fragment size alone and may be attributed to other DNA damage events and crosslinks. We therefore recommend assessing DNA quality in a functional test that utilizes an amplification step.

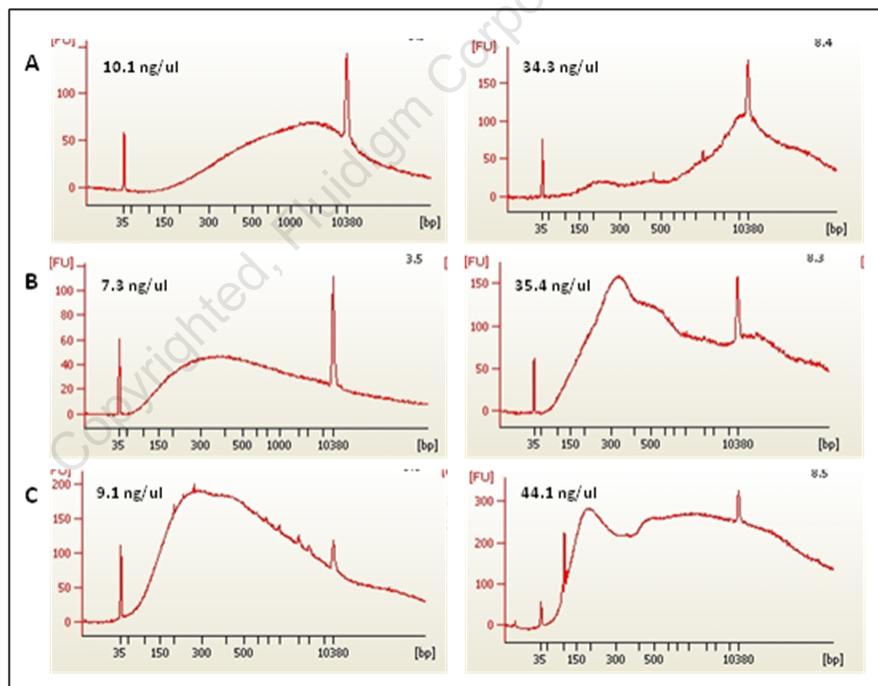


Figure 2 BioAnalyzer traces of FFPE extracted genomic DNA. Good performers are shown in A, intermediate performers in B, and poor performers in C. Total DNA concentrations as determined with picogreen are indicated in each plot.

Absolute copy number determination with a 180 bp TaqMan assay

The 180 bp FTH1 TaqMan amplicon serves as an approximation of the amplifiable 200 bp fraction of the FFPE extracted genomic DNA and can be used to determine the effective DNA concentration. This approach requires the generation of a standard curve with control genomic DNA. The volumes described below are for use with a 384-well plate.



NOTE: If larger amplicons will be targeted on the Access Array (See “General Considerations” on page 149), the 180 bp TaqMan assay should be substituted with a size-appropriate alternative.

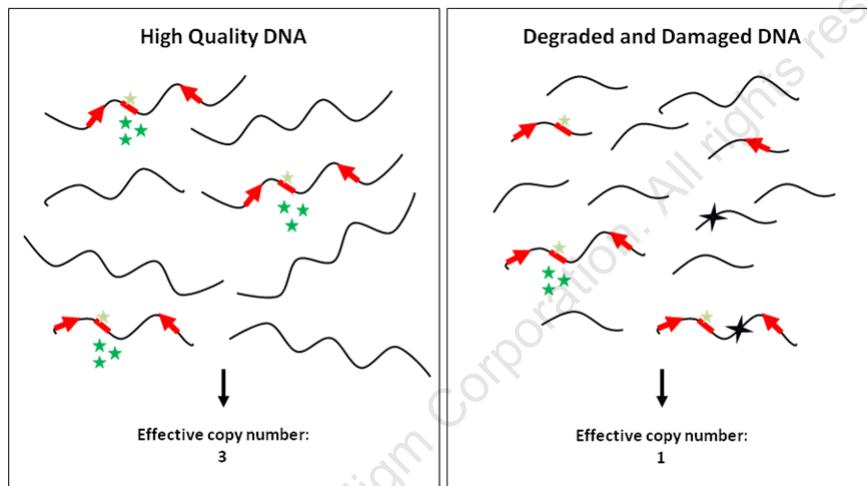


Figure 3 180 bp TaqMan assay performance. In high quality DNA, such as genomic DNA extracted from a cell line, most of the 180 bp target regions are intact and contribute to signal generation. In degraded and damaged genomic DNA, fragmentation may have occurred within the target region or DNA damage may prevent efficient amplification of the target region resulting in reduced signal generation.

Prepare Standard Curve DNA

Prepare a serial dilution of the control DNA as shown in Table 1.

DNA Concentration (ng/ μ L)	Copy Number per μ L
50.0	16667
25.0	8333
12.5	4167
6.25	2083
3.13	1042
1.56	521
0.78	260
0.39	130

Table 1 Serial dilution of control DNA

Prepare the PCR Master Mix

In a 1.5 mL microcentrifuge tube combine the components listed in Table 2. An extra 1 μ L is included in the Volume for Duplicate Reactions to compensate for pipetting error. To generate a master mix for multiple samples, multiply the volumes with $N(1+10\%)$, where N is the number of samples to be tested (including the standard curve samples and an NTC control). The additional 10 percent is to compensate for pipetting error.

Component	Volume for Duplicate Reactions (μ L)	Master Mix	Final Concentration
2X TaqMan Gene Expression Master Mix	5.5	$5.5 \times N \times 1.1$	1X
20X FTH1 TaqMan Assay	0.55	$0.55 \times N \times 1.1$	1X
Nuclease-free water	3.85	$3.85 \times N \times 1.1$	
Total	9.90	$9.90 \times N \times 1.1$	

Table 2 PCR Master Mix Components

Prepare the Sample Mix Solutions

- 1 In a 96-well plate distribute 9.9 μ L PCR Master Mix to each sample well.

- 2 Add 1.1 µL DNA to each sample well for a final volume of 11 µL.
- 3 Cover the plate with PCR film, vortex, and centrifuge the plate to spin down all components.
- 4 With a multichannel pipette, transfer 5 µL to the sample wells of a 384-well plate. Repeat the pipetting step for duplicate wells.
- 5 Seal the plate with optical adhesive film.
- 6 Briefly centrifuge the reaction plate to collect the reagents at the bottom of the wells.

Thermal Cycling of the 384-Well Plate

- 1 Load the plate into a real-time PCR system.
- 2 Select ROX as the passive reference dye. Collect data for FAM-MGB.
- 3 Run the following PCR protocol:

PCR Stages	Number of Cycles
95 °C 10 min	1
95 °C 15 sec	40
60 °C 60 sec	
4 °C	hold

Table 3 PCR protocol

- 4 Analyze the data according to the software manufacturer's instructions. Enter the copy number per µL values from the last column of Table 1 in the corresponding quantity cells of the standard curve samples. The software should automatically generate a standard curve and return copy numbers per µL of the 180 bp amplicon in the test samples.

Calculation of expected 180 bp copy number in Access Array IFC sample chamber

Use the following formula to calculate the 180 bp copy number expected in each Access Array IFC reaction chamber:

$$Y = V \times X \times 33/5000$$

Where Y is the copy number in the Access Array IFC sample chamber; X is the 180 bp copy number per µL as determined with the TaqMan assay; V is the volume of DNA (in µL) to be added to the Access Array Sample Mix (1 or 2 µL); and 33/5000 is the volumetric conversion to the 33 nL sample chamber on the Access Array IFC.

Samples with less than one 180 bp copy predicted per Access Array sample chamber are unlikely to yield usable PCR products and should be excluded from further analysis (see Table 5 on page 152). For samples with greater than one 180 bp copies predicted per Access Array sample chamber, the preamplification strategy should be adopted (see Samples on page 150).

Estimated 180 bp per Sample Chamber (Y)	Access Array Adaptation
Y < 1	Exclude from Experiment PreAmp Strategy
Y > 1	

Table 4 Adaptation Access Array Input

Section 4: Amplicon Tagging on the Access Array IFC

Reference Documents

- *Fluidigm® Access Array™ System for 454 Sequencing Platform User Guide* (PN 68000158)
- *Fluidigm® IFC Controller for Access Array™ System User Guide* (PN 68000157)
- Agilent DNA 1000 Kit Guide

Required Reagents

Stored at -20 °C

- FastStart High Fidelity PCR system, dNTPack (Roche, PN 04-738-292-001)
- ExoSAP-IT (Affymetrix, PN 78200 200 UL)
- 20X Access Array Loading Reagent (Fluidigm, PN 100-0883)
- Target-specific primer pairs tagged with universal tags (CS1 forward tag, CS2 reverse tag)
 - 50 µM CS1-Tagged TS Forward Primer
 - 50 µM CS2-Tagged TS Reverse Primer
- Barcode Library:
 - 96 Access Array Barcode Primers for the 454 FLX Titanium Sequencer (Fluidigm, PN 100-3347)

Or

- Access Array Barcode Library for Illumina Sequencers-384 (Single Direction) (Fluidigm, PN 100-4876)

Or

- Access Array Barcode Library for Illumina Sequencers-384 (Bidirectional) (Fluidigm, PN 100-3771)

Stored at 4 °C

- Agilent DNA 1000 Kit Reagents (Agilent, PN 5067-1504)
- 1X Access Array Harvest Solution (Fluidigm, PN 100-1031)

Stored at Room Temperature

- DNAZap Solution (Ambion, PN AM9890)
- PCR Certified Water (TEKnova, PN W330)
- DNA Suspension Buffer (10 mM Tris HCl, 0.1mM EDTA, pH8.0) (TEKnova, PN T0221)
- Agilent DNA 1000 Chips (included in the Agilent DNA 1000 DNA kit)(Agilent)

Required Equipment and Consumables

- 1.5 mL or 2 mL microcentrifuge tubes
- Microcentrifuge with rotor for 2 mL tubes
- Microcentrifuge with rotor for 0.2 mL PCR tube strips
- Centrifuge with plate carriers
- Agilent 2100 BioAnalyzer(Agilent)
- Thermal cycler with 96-well block (e.g., Applied Biosystems GeneAmp 9700)
- 96-well reaction plate
- MicroAmp Clear Adhesive Film (Applied Biosystems, PN 4306311)
- IFC Controller AX (2 quantity, pre- and post-PCR)(Fluidigm)
- Stand-alone Thermal Cycler (Fluidigm)
- 48.48 Access Array IFCs (Fluidigm)
- Control Line Fluid Syringes (Fluidigm, PN 89000020)

General Considerations

Primer Design

Due to the fragmented state of FFPE extracted genomic DNA, it is recommended to design primers that target regions less than 210 bp in size for successful amplification. Figure 4 shows the effect of target region size on the PCR product yield. Primer sets with average target region sizes of 200 bp and 250 bp were run against a selection of FFPE samples exhibiting varying degrees of degradation on two Access Array IFCs. The PCR product yield was determined with a BioAnalyzer and normalized to the yield of PCR products generated from cell line DNA template.

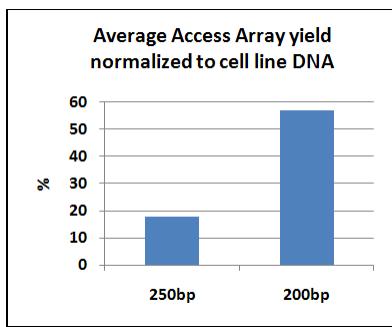


Figure 4 Effect of amplicon size on PCR efficiency

Primer Validation

Prior to running experiments on the Access Array System, primers should be validated individually with genomic DNA derived from cell line DNA. Please refer to the instructions in the *Fluidigm® Access Array™ System for 454 Sequencing Platform User Guide* (PN 68000158).

Samples

- 1 Due to the DNA damage present in FFPE-extracted genomic DNA, a higher false positive rate can be expected. Because of the random nature of these mutations, it is important to add sufficient template copies to the PCR. Random mutations will be amplified clonally, i.e. only reads derived from the same template molecule will exhibit that particular mutation. True mutations should occur in multiple template molecules, and will therefore appear repeatedly in reads derived from different template molecules.
- 2 When the input template copy number is low, the DNA polymerase error rate can make a significant impact on the false positive rate. For example, a reaction that contains 30 template copies and in which 1 error is introduced in the first PCR cycle will result in propagation of this error to a false positive rate of 3%. A PCR reaction that contains 100 template copies on the other hand will yield a 1% false positive rate in the same scenario.
- 3 Tissue is often comprised of a heterogeneous cell population either at the cellular level or for example tumor tissue interspersed with normal tissue or cells; or at the molecular level with the mutation of interest occurring in a subpopulation of cells only. The purity of the tissue sample and the expected mutation frequency both affect the depth of coverage required to detect the mutation of interest with statistical confidence. To increase statistical power, enough starting copies of the template DNA need to be used.

In order to increase the template DNA copy number input, we recommend adopting a preamplification strategy. The workflow consists of three steps and has been outlined in Figure 5. A minimum of 100 effective copies of template DNA as determined with the TaqMan assay (described in the Absolute copy number determination with a 180 bp TaqMan assay on page 145) is added to a preamplification reaction containing a pool of target-specific primer pairs. After the preamplification reaction, unused primers are destroyed with ExoSAP-IT followed by enrichment PCR on the Access Array IFC with target-specific primers. Depending on the number of primer pairs present in the preamplification reaction, multiple Access Arrays can be run. As a final step, the barcodes and sequencer-specific adaptor sequences are added to the PCR products harvested from the Access Array IFC in the final barcoding PCR. The barcoding PCR can be done separately for each individual Access Array IFC or the user can opt to combine PCR product pools from multiple Access Array IFCs derived from the same sample and run one barcoding PCR.

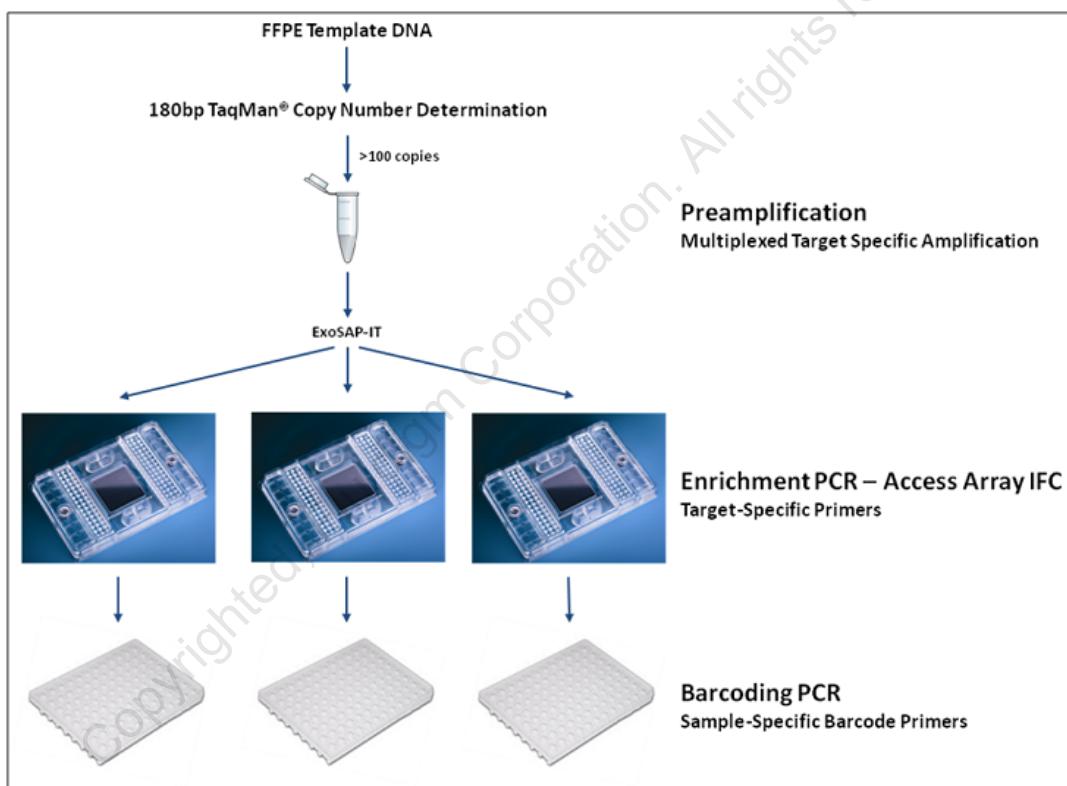


Figure 5 Amplicon tagging workflow

Specific Target Amplification (Preamplification)

Preamplification enriches template DNA for the selected targets, providing more material for subsequent PCR. This is beneficial when template DNA quantity is limited and/or when a single sample will be used for many downstream PCRs. Additionally, in the case of amplifying FFPE samples on the Access Array IFC the volume adjustment required to obtain a sufficient quantity of template input copy numbers often exceeds the 2 µL sample volume limit one can add to the Access Array Sample Mix. The preamplification reaction allows for greater freedom in the volumetric input. By using the same assays in the preamplification reaction as in the PCR reaction on the Access Array, only the targets of interest are amplified and no additional primers are required. The 50 nM concentration of primers creates a primer-limited environment that is further limited by the recommended 15 cycles. This results in small amounts of DNA being amplified equally without introducing bias.

Prepare the Preamplification Primer Pool

In a 1.5 mL microcentrifuge tube combine 1 µL of each tagged target-specific primer pair, up to a total of 100 primer pairs. Dilute the pooled primers using DNA Suspension Buffer so that each primer is at a final concentration of 500 nM. The table below provides an example using 48 primer pairs:

48 Primer Pairs (50 µM each)	DNA Suspension Buffer	Total Volume
1 µL each tagged target-specific primer pair	52 µL	100 µL

Table 5 48 Primer Pool

Prepare the Preamplification Master Mix

- In a 1.5 mL microcentrifuge tube, combine the components listed in the table below. This protocol prepares enough Preamplification Master Mix for 48 samples with extra reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 60 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer <u>with</u> MgCl ₂ (Roche)	1.0	60.0	1X
25 mM MgCl ₂ (Roche)	1.08	64.8	4.5 mM total
DMSO (Roche)	0.5	30.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.2	12.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.2	12.0	0.1 U/ μL
Total	2.98	178.8	

Table 6 PreAmp Master Mix

- Vortex the Preamplification Master Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare the Preamplification Sample Mix Solutions

- Combine the components listed below in a 96-well plate to prepare each individual Preamplification Sample Mix solution.

Component	Volume (μL)
Preamplification Master Mix	2.98
Primer Pool	1.0
FFPE DNA	Corresponding to 100 Copies*
PCR Certified Water (TEKnova)	to 10.0
Total	10.0

* If the effective DNA concentration exceeds 100 copies/ μL , add 1 μL template DNA
 Table 7 PreAmp Sample Mix solution

- Vortex the Preamplification Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Thermal Cycling of the 96-well PCR Plate

- 1 Place the PCR plate in a 96-well block thermal cycler and run the following preamplification protocol:

Preamplification Stages	Number of Cycles
95 °C 10 min	1
95 °C 15 sec 60 °C 4 min	2
95 °C 15 sec 72 °C 4 min	13
4 °C	hold

Table 8 PreAmp protocol

ExoSAP-IT Treatment of the Preamplification Products

- 1 Add 4 µL ExoSAP-IT enzyme mix to each sample well of the 96-well plate.
- 2 Vortex the ExoSAP-IT containing solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.
- 3 Return the PCR plate to the 96-well block thermal cycler and run the following ExoSAP-IT protocol:

ExoSAP-IT Stages	Number of Cycles
37 °C 15 min	1
80 °C 15 min	1
4 °C	hold

Table 9 ExoSAP-IT protocol

Prepare a 5-fold Dilution of the Preamplification Products

- 1 In a 96-well plate pipette 13 µL PCR certified water in 48 wells.
- 2 Add 5 µL of the ExoSAP-IT treated preamplification products.
- 3 Vortex the preamplification product dilutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The diluted preamplification products can serve as template material for multiple downstream target-enrichment amplifications on Access Array IFCs with subsets of 48 primer pairs that were included in the preamplification primer pool.



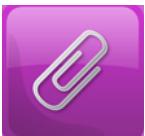
WARNING! Because the preamplification products are tagged with universal tags, it is recommended to carry out subsequent steps in the Post-PCR Lab to avoid contamination of the Pre-PCR Lab with universally amplifiable material.

Amplicon Tagging on the 48.48 Access Array IFC

Because the primer pairs used in the preamplification reaction are the same universally tagged primers that will be used in subsequent amplification on the Access Array IFC, the outer barcode primers cannot be added to the Access Array IFC at this point. Inclusion of the outer barcode primers will result in amplification of all products generated in the preamplification reaction, a reaction that will be dominated by spurious primer dimer products. The amplicon tagging strategy has therefore been separated out into two steps: target specific amplification on the Access Array IFC and incorporation of barcodes and sequencer specific adaptor sequences in a subsequent PCR.

Priming the 48.48 Access Array IFC

- 1 Inject control line fluid into both 48.48 Access Array IFC accumulators.



NOTE: Control line fluid on the IFC or in the inlets makes the IFC unusable. Use only 48.48 syringes with 300 µL of Control Line Fluid (PN 89000020)

- 2 Add 500 µL of 1X Access Array Harvest Reagent (Fluidigm, PN 100-1031) to the H1-H4 wells on the IFC.
- 3 Place the IFC into the Pre-PCR IFC Controller AX located in the Pre-PCR Lab and run Prime (151x).



NOTE: Load the IFC in the Post-PCR IFC Controller AX in the Post-PCR Lab within 60 minutes of priming.

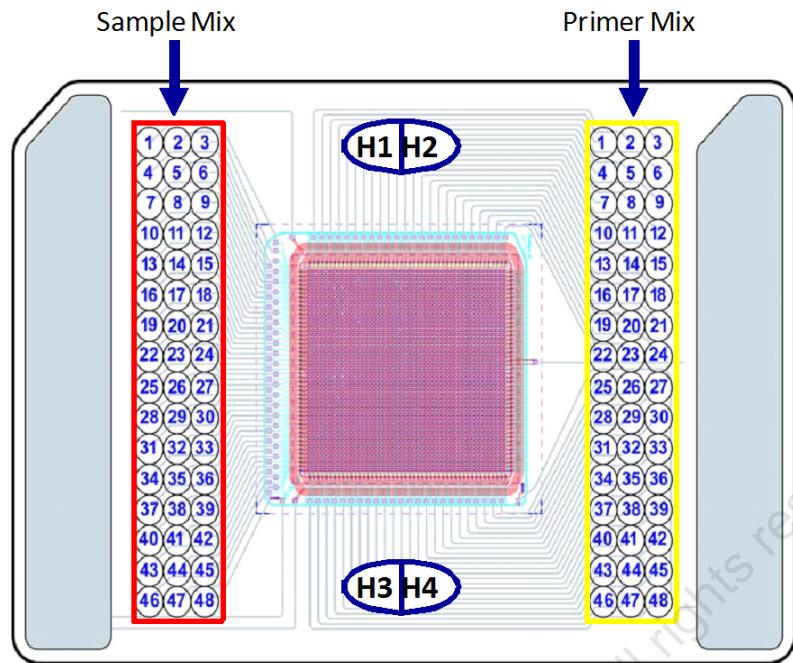


Figure 6 48.48 Access Array IFC Sample and Assay inlet pipetting map

Enrichment PCR with Target-Specific Primers on the 48.48 Access Array IFC

Prepare the 20X Primer Solutions

- In a DNA-free hood in the Pre-PCR Lab, prepare the 20X Primer solutions for the 48 primer pairs as shown in the table below. These will be loaded into the primer inlets of a 48.48 Access Array IFC.

Component	Volume (μL)	Final Concentration
50 μM CS1-Tagged TS Forward Primer & 50 μM CS2-Tagged TS Reverse Primer	6.0	6 μM
20X Access Array Loading Reagent (PN 100-0883) cap	2.5	1X
DNA Suspension Buffer (TEKnova, PN T0221)	41.5	
Total	50.0	

Table 10 20X Primer Solutions

- Vortex the 20X Primer solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final Tagged TS Forward and Reverse Primer concentrations are 6 µM in the 20X Primer solutions. The final TS Forward and Reverse Primer concentrations in the Access Array reaction chamber are 300 nM.

Load the Primer Inlets of the 48.48 Access Array IFC

- Pipette 4 µL of 20X Primer solution into each of the primer inlets.
- All subsequent steps should be carried out in the Post-PCR Lab.



NOTE: We recommend using an 8-channel pipette to load the Sample Mix and 20X Primer solutions. The recommended pipetting order is shown below.

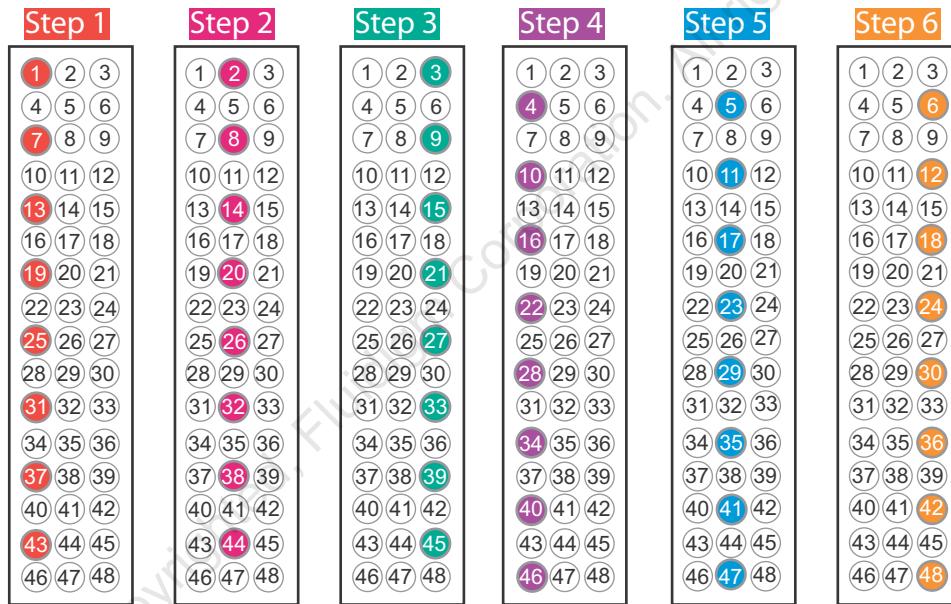


Figure 7 48.48 Access Array IFC Sample and Assay pipetting scheme

Prepare the Sample Mix Solutions

All DNA samples need to be added into the Sample Pre-Mix individually, prior to loading the Sample Mix solutions into the sample inlets of a 48.48 Access Array IFC.

Prepare the Sample Pre-Mix Solution

- 1 Working in a DNA-free hood, combine the components listed in the table below. This protocol prepares enough Sample Pre-Mix solution for 64 reactions. This is enough reagent to load one 48.48 Access Array IFC with additional reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 64 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer <u>without</u> MgCl ₂ (Roche)	0.5	32.0	1X
25 mM MgCl ₂ (Roche)	0.9	57.6	4.5 mM
DMSO (Roche)	0.25	16.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.1	6.4	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.05	3.2	0.05 U/ μL
20X Access Array Loading Reagent (Fluidigm)	0.25	16.0	1X
PCR Certified Water (TEKnova)	1.95	124.8	
Total	4.0	256.0	

Table 11 Sample Pre-Mix Solution

- 2 Vortex the Sample Pre-Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare the Sample Mix Solutions

- 1 Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume per Reaction (μL)
Sample Pre-Mix	4.0
Diluted Preamplification Products	1.0
Total	5.0

Table 12 Sample Mix

- 2 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Load the Sample Inlets of the 48.48 Access Array IFC

- 1 Pipette 4 µL of Sample Mix solution into each of the sample inlets. Follow the same pipetting scheme as outlined in Figure 7 on page 157.
- 2 Before loading the 48.48 Access Array IFC on the IFC Controller AX, clean the interface plate by grasping the tab of the red interface plate and removing it by pulling toward you. Clean the gasket-side of the interface plate with DNAZap solution, rinse with water and isopropanol and dry with a lint-free wipe. Replace the interface plate by sliding it into the IFC Controller and pressing firmly to ensure proper seating.
- 3 Place the 48.48 Access Array IFC into the Post-PCR IFC Controller AX in the Post-PCR Lab and run the **Load Mix (151x)** script.
- 4 When the script is finished, press **Eject** to remove the IFC from the IFC Controller.

Thermal Cycling the 48.48 Access Array IFC

- 1 Place the 48.48 Access Array IFC into the Fluidigm Thermal Cycler and start thermal cycling by selecting protocol **AA48v1** (for the SATC) or **48x48 Standard v1** (for the FC1).



NOTE: Contact Fluidigm Technical Support (1-866-358-4354 or techsupport@fluidigm.com) if you need assistance in programming your Stand-Alone Thermal Cycler or FC1 Thermal Cycler.

Harvest PCR Products from the 48.48 Access Array IFC

- 1 After the thermal cycling has finished, press **ENTER** and turn off the vacuum on the Fluidigm Thermal Cycler.
- 2 Pipette 2 µL of 1X Access Array Harvest Reagent into each of the sample inlets on the 48.48 Access Array IFC.
- 3 Remove the remaining 1X Access Array Harvest Reagent from the H1-H4 wells.
- 4 Pipette 600 µL of fresh 1X Access Array Harvest Reagent into each of the H1-H4 wells.
- 5 Repeat the cleaning of the Interface Plate of the IFC Controller AX as described previously.
- 6 Place the 48.48 Access Array IFC into the Post-PCR IFC Controller AX located in the Post-PCR Lab and run the **Harvest (151x)** script.
- 7 When the script has finished, remove the 48.48 Access Array IFC from the Post-PCR IFC Controller.
- 8 Label a 96-well plate with the 48.48 Access Array IFC barcode. Carefully transfer the harvested PCR products from each of the sample inlets into columns 1-6 of the 96-well PCR plate.



NOTE: Remove PCR products from the IFC using an 8-channel pipette in the same order as you loaded the IFC.

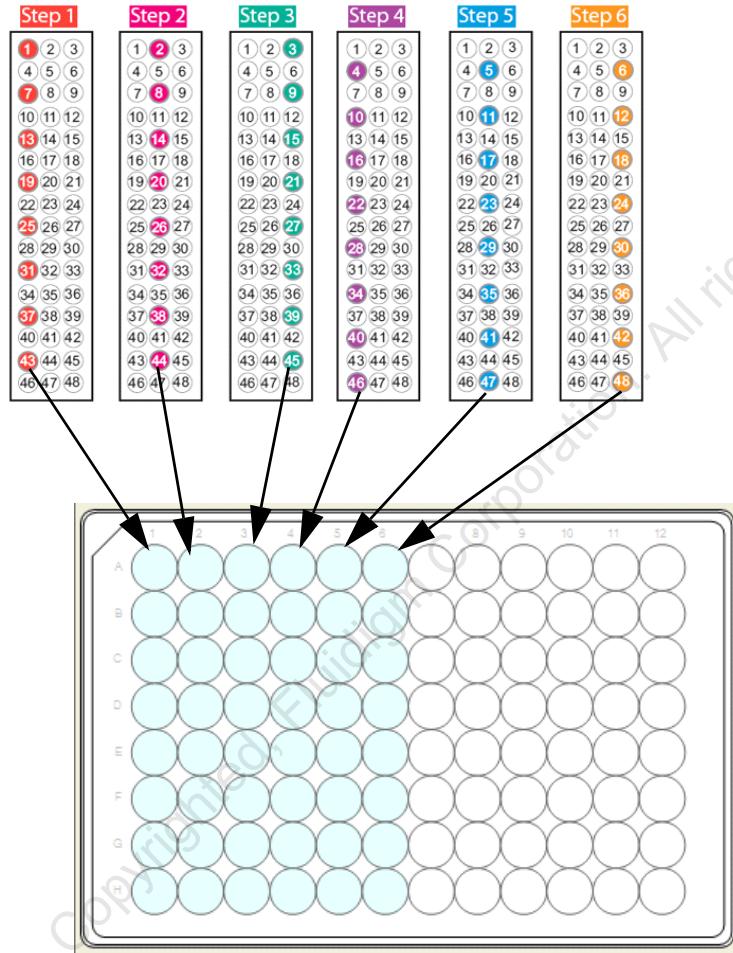


Figure 7 PCR product transfer map from the 48x48 Access Array IFC to a 96-well PCR plate

Attaching Sequence Tags and Sample Barcodes

Prepare the Sample Pre-Mix Solution

- Working in a DNA-free hood, combine the components listed in the table below. This protocol prepares enough Sample Pre-Mix for the 48 PCR product pools harvested from one 48.48 Access Array IFC with extra reactions to compensate for dead volume and pipetting error.

Component	Volume per Reaction (μL)	Volume for 60 Reactions (μL)	Final Concentration
10X FastStart High Fidelity Reaction Buffer <u>without</u> MgCl ₂ (Roche)	2.0	120.0	1X
25 mM MgCl ₂ (Roche)	3.6	216.0	4.5 mM
DMSO (Roche)	1.0	60.0	5%
10 mM PCR Grade Nucleotide Mix (Roche)	0.4	24.0	200 μM ea
5 U/ μL FastStart High Fidelity Enzyme Blend (Roche)	0.2	12.0	0.05 U/ μL
PCR Certified Water (TEKnova)	7.80	468.0	
Total	15.0	900.0	

Table 13 Sample Pre-Mix Solution

- Vortex the Pre-Sample Master Mix for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare a 100-Fold Dilution of the Harvested PCR Products

- In a 96-well plate, pipette 99 μL PCR certified water in 48 wells.
- Add 1 μL of PCR product harvested from the 48.48 Access Array IFC in the “Harvest PCR Products from the 48.48 Access Array IFC” section.
- Vortex the PCR product dilutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.

Prepare the Sample Mix Solutions

- 1 Combine the components listed below in a 96-well plate to prepare 48 individual Sample Mix solutions.

Component	Volume per Reaction (µL)
Sample Pre-Mix	15.0
96 Access Array Barcode Library for the 454 FLX Titanium Sequencer (2 µM each) or Access Array Barcode Library for Illumina Sequencers-384 (2 µM each)	4.0
Diluted Harvested PCR Product Pool	1.00
Total	20.0

Table 14 Sample Mix

- 2 Vortex the Sample Mix solutions for a minimum of 20 seconds, and centrifuge for 30 seconds to spin down all components.



NOTE: The final concentrations of the Access Array Barcode Forward and Reverse Primers are 400 nM per well. Each well should receive a unique barcode primer pair.

Thermal Cycling of the 96-Well PCR Plate

- 1 Place the PCR plate in the Thermal Cycler and run the following PCR protocol:

PCR Stages	Number of Cycles
95 °C 10 min	1
95 °C 15 sec 60 °C 30 sec 72 °C 1 min	15
72 °C 3 min	1
4 °C	hold

Table 15 Thermal cycling protocol

PCR Product Quantitation and Qualification

The PCR products generated on the Access Array IFC are first analyzed using an Agilent 2100 BioAnalyzer to check the quality and quantity of the PCR products. Next, the PCR products are pooled together to create one PCR product library. The PCR product library is purified using AMPure XP beads. After purification, the PCR product library is quantified before proceeding to emulsion PCR or cluster generation.

- 1 The PCR product library can be qualified and quantified using an Agilent 2100 BioAnalyzer with DNA 1000 Chips.
- 2 Run 1 μ L of the 48 pooled PCR products on a BioAnalyzer DNA 1000 Chip following the manufacturer's instructions.
 - a Ensure that amplicon sizes and distribution are within the expected range (+/- 5% for amplicons in the range of 200-325 bp). Because of the incorporation of the sequence tags, the expected PCR products will be 114 bp larger than the target region for 454 amplicon tagging and 107 bp for Illumina amplicon tagging.
 - b Primer dimer contamination in the PCR product pool (in the range of 50-130 bp) should be less than 25% based on the BioAnalyzer quantitation.
- 3 Define a region of interest in the electropherogram to determine the PCR product library concentration (See Figure 8 on page 164).
- 4 Select the **Region Table** sub-tab on the bottom panel of the **Electropherogram** tab.
 - a Select the **Global** tab in the right-hand panel. Select the **Advanced** settings in the dropdown menu.
 - b Scroll to the **Smear Analysis** section.
 - c Check the **Perform Smear Analysis** checkbox.
 - d Select the **Table** in the **Regions** line and enter the lower and upper values of the PCR product pool region.
 - e Check in the electropherogram that the region spans the entire PCR product pool. Adjust upper and lower values in the table if necessary.
 - f The **Region Table** listed below the electropherogram will show the concentration of the Region containing the PCR product library. Refer to the Agilent 2100 BioAnalyzer User Guide for additional information on Regions.

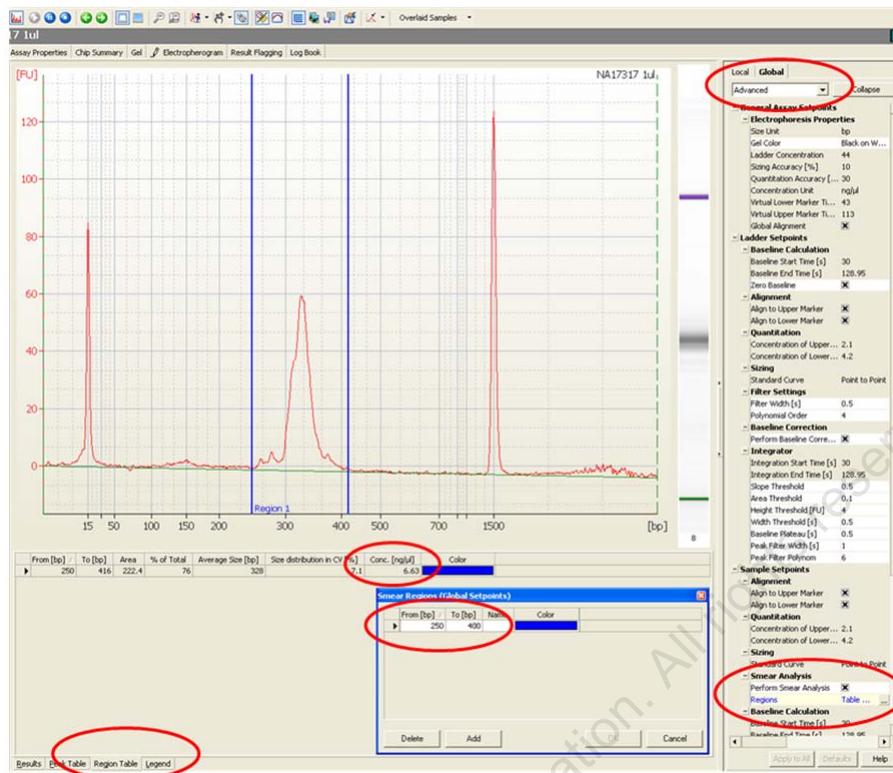


Figure 8 Example of PCR product pool quantitation using an Agilent 2100 BioAnalyzer with a DNA 1000 Chip.

PCR Product Pooling and Purification

- 1 Due to the highly variable quality and quantity of FFPE extracted genomic DNA, the resulting PCR product yields may not be uniform across different samples. In order to avoid underrepresentation and overrepresentation of the different samples on the sequencer, the sample pooling can be normalized based on the PCR product yields as determined with the BioAnalyzer. If the PCR product yield of the different pools is within 2-fold of average, it is recommended to pool volumetrically without correction to avoid introducing sample distribution noise due to pipetting error.
- 2 The pooled PCR product library is purified using AMPure XP beads according to the instructions in the *Fluidigm® Access Array™ System for 454 Sequencing Platform User Guide* (PN 68000158).

Section 5: BioAnalyzer Gel Image Example

- 1 Figure 9 shows BioAnalyzer gel images of PCR product pools harvested from Access Array IFCs. A selection of 25 FFPE samples and 1 cell line DNA sample were run either following the standard amplicon tagging procedure (Figure 10A) or following the preamplification approach (Figure 10B). Samples of poor quality such as samples 12 and 18 can be partially rescued. However, drop out of PCR products is to be expected. The remainder of the samples that showed little or no amplification following the standard amplicon tagging strategy show great improvement following the preamplification strategy.

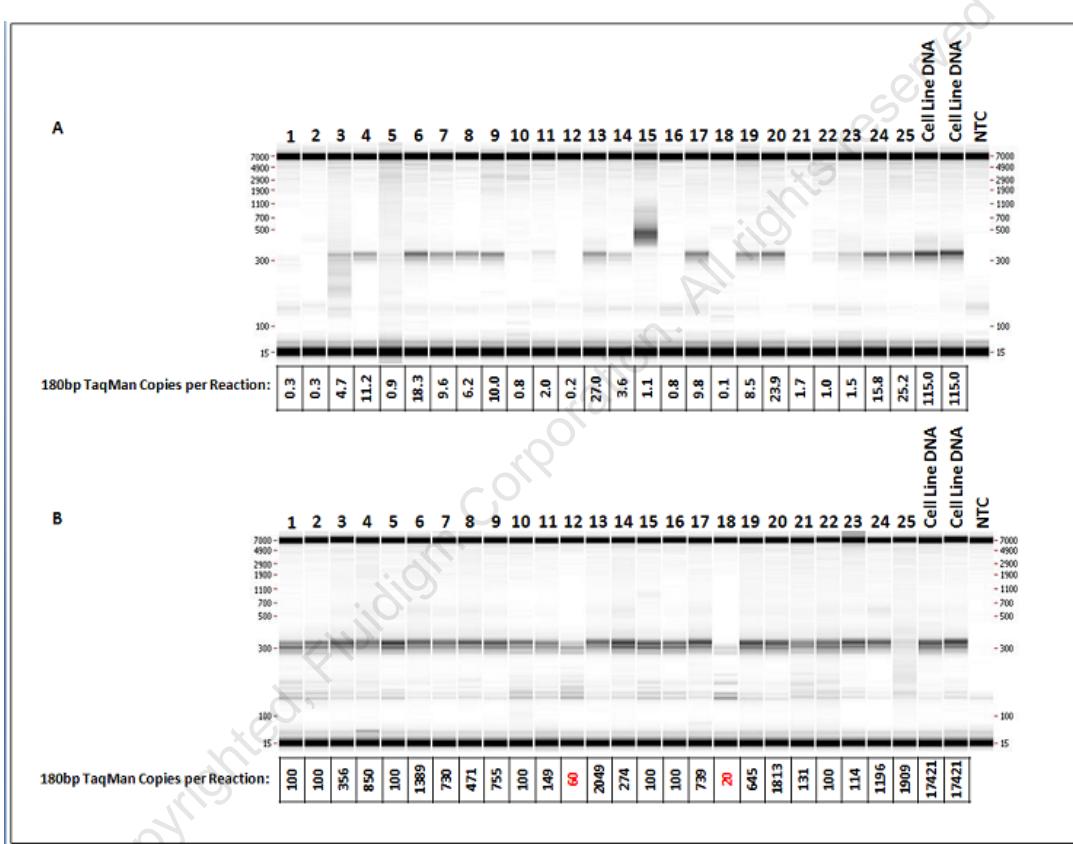


Figure 9 BioAnalyzer gel images of PCR product pools harvested from Access Array IFCs. Samples run following the standard amplicon tagging procedure are shown in A, samples run following the preamplification approach are shown in B. The table below A shows the estimated 180 bp copy number per Access Array sample chamber. The table below B shows the estimated 180 bp copy number per preamplification reaction. The number indicated in red correspond to samples for which less than 100 copies could be added to the reaction.

When template DNA copy numbers drop below a certain threshold, distribution across the sample chambers on the Access Array IFC becomes stochastic, i.e. certain sample chambers will not receive template DNA and therefore certain PCR products will be absent from the harvested PCR product pool. An informative way of assessing this effect is to calculate the fraction of PCR products for a given sample that have a sequence read coverage within two fold of the average sequence read coverage for that particular sample. If amplification has been uniform and no assays have dropped out, the fraction that is within two fold of average coverage should be close to 100%. If assays have dropped out, this fraction will decline. Figure 11 shows the effect of preamplification on the fraction that is within two fold of average coverage. For the standard amplicon tagging approach, a clear correlation is visible between the quality of the DNA template as determined with the 180bp amplicon TaqMan assay and the amplification uniformity. Such a correlation can also be observed for the samples that have undergone preamplification. However, for samples with greater than 10 copies/ μ L the preamplification strategy has rescued many PCR products that had dropped out in the standard amplicon tagging strategy.

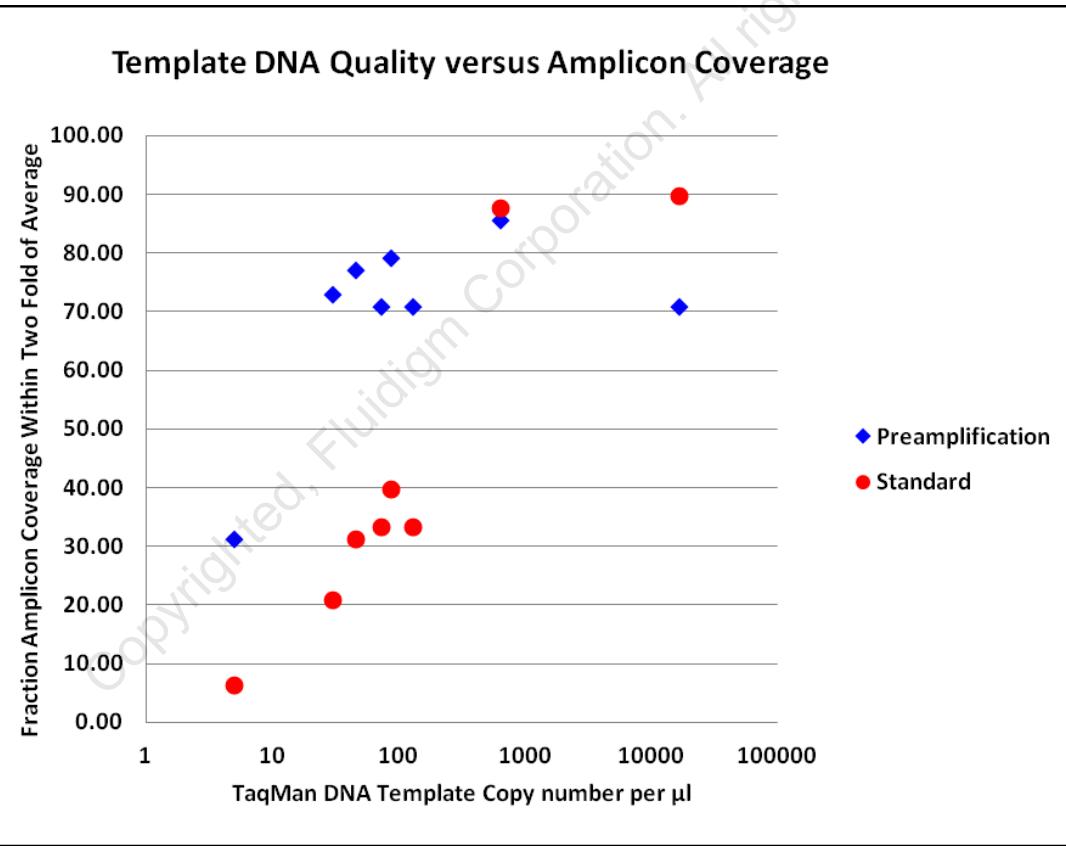


Figure 10 Correlation between template DNA quality as determined with the 180bp amplicon TaqMan assay and amplification efficiency. PCR products were generated with the standard amplicon tagging approach (red markers) or following the preamplification approach (blue markers).

Access Array Barcodes for the Illumina Sequencing Systems

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Access Array Barcode Library for Illumina Sequencers 168

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Access Array Barcode Library for Illumina Sequencers

The barcode library is a complete set of 384 unique barcodes designed to be used for single-direction or bidirectional amplicon preparation on the Access Array System. PCR products that have been generated on the Access Array™ System can be barcoded using the Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, PN 100-4876) for standard single-direction amplicon preparation on singleplex or multiplex reactions. PCR products that have been generated using the Access Array System can also be barcoded using the Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771) for bidirectional amplicon preparation.

Plate Number	Reagent Description	Volume
A1	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2_1-96 at 2 µM per oligo	50 µL
A2	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2_97-192 at 2µM per oligo	50 µL
A3	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/PE2_BC_CS2_193-288 at 2 µM per oligo	50 µL
A4	Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) PE1_CS1/ PE2_BC_CS2_289-384 at 2 µM per oligo	50 µL

Table 1 Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction) (Fluidigm, PN 100-4876)

Plate Number	Reagent Description	Volume
A1	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS1/PE2_BC_CS2_1-96 at 2 μM per oligo	50 μL
A2	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS1/PE2_BC_CS2_97-192 at 2 μM per oligo	50 μL
A3	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS1/PE2_BC_CS2_193-288 at 2 μM per oligo	50 μL
A4	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS1/ PE2_BC_CS2_289-384 at 2 μM per oligo	50 μL
B1	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS2/ PE2_BC_CS1_1-96 at 2 μM per oligo	50 μL
B2	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS2/ PE2_BC_CS1_97-192 at 2 μM per oligo	50 μL
B3	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS2/PE2_BC_CS1_193-288 at 2 μM per oligo	50 μL
B4	Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) PE1_CS2/ PE2_BC_CS1_289-384 at 2 μM per oligo	50 μL

Table 2 Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional) (Fluidigm, PN 100-3771)

Barcode Sequences for Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction), PN 100-4876

Barcode Name	Barcode Sequence	Barcode Well	Plate
FLD0001	GTATCGTCGT	A1	A1
FLD0002	GTGTATGCGT	B1	A1
FLD0003	TGCTCGTAGT	C1	A1
FLD0004	GTCGTCGTCT	D1	A1
FLD0005	GTGCGTGTGT	E1	A1
FLD0006	GCGTCGTGTA	F1	A1
FLD0007	GTCGTGTACT	G1	A1
FLD0008	GATGTAGCGT	H1	A1
FLD0009	GAGTGATCGT	A2	A1
FLD0010	CGCTATCAGT	B2	A1
FLD0011	CGCTGTAGTC	C2	A1
FLD0012	GCTAGTGAGT	D2	A1
FLD0013	GAGCTAGTGA	E2	A1
FLD0014	CGTGCTGTCA	F2	A1
FLD0015	GATCGTCTCT	G2	A1
FLD0016	GTGCGTGTGT	H2	A1
FLD0017	TGAGCGTGCT	A3	A1
FLD0018	CATGTCGTCA	B3	A1
FLD0019	TCAGTGTCTC	C3	A1
FLD0020	GTGCTCATGT	D3	A1
FLD0021	CGTATCTCGA	E3	A1
FLD0022	GTCATGCGTC	F3	A1
FLD0023	CTATGCGATC	G3	A1
FLD0024	TGCTATGCTG	H3	A1
FLD0025	TGTGTGCATG	A4	A1
FLD0026	GAGTGTCACT	B4	A1
FLD0027	CTAGTCTCGT	C4	A1
FLD0028	GAGTGCATCT	D4	A1
FLD0029	TGCGTAGTCG	E4	A1
FLD0030	CTGTGTCGTC	F4	A1
FLD0031	CTGTAGTGCG	G4	A1
FLD0032	GTGCGCTAGT	H4	A1
FLD0033	TGTGCTCGCA	A5	A1
FLD0034	GATGCGAGCT	B5	A1
FLD0035	CTGTACGTGA	C5	A1
FLD0036	GCGATGATGA	D5	A1
FLD0037	TGTCGAGTC	E5	A1
FLD0038	GTCTACTGTC	F5	A1
FLD0039	CAGTCAGAGT	G5	A1
FLD0040	CGCAGTCTAT	H5	A1
FLD0041	GTATGAGCAC	A6	A1
FLD0042	CGAGTGCTGT	B6	A1
FLD0043	TATAGCACGC	C6	A1
FLD0044	TCATGCGCGA	D6	A1
FLD0045	TATGCGCTGC	E6	A1
FLD0046	TCTCTGTGCA	F6	A1
FLD0047	CTATCGCGTG	G6	A1
FLD0048	TACGCTGCTG	H6	A1
FLD0049	CTGCATGATC	A7	A1
FLD0050	CGCGTATCAT	B7	A1
FLD0051	GTATCTCTCG	C7	A1
FLD0052	GCTCATATGC	D7	A1
FLD0053	CACTATGTCG	E7	A1
FLD0054	TAGCGCGTAG	F7	A1
FLD0055	CGTCACAGTA	G7	A1
FLD0056	TCGCGTGAGA	H7	A1
FLD0057	TACATCGCTG	A8	A1
FLD0058	GTGAGAGACA	B8	A1
FLD0059	GACTGTACGT	C8	A1
FLD0060	GCACGTAGCT	D8	A1
FLD0061	TCACGCTATG	E8	A1
FLD0062	CGTACTACGT	F8	A1
FLD0063	CAGCTGAGTA	G8	A1
FLD0064	GAGATCAGTC	H8	A1
FLD0065	TACTGAGCTG	A9	A1
FLD0066	TAGTAGCGCG	B9	A1
FLD0067	GACGTCTGCT	C9	A1

FLD0068	GTACTCGCGA	D9	A1
FLD0069	TCTGAGCGCA	E9	A1
FLD0070	TAGACGTGCT	F9	A1
FLD0071	GTGACTCGTC	G9	A1
FLD0072	TCGAGTAGCG	H9	A1
FLD0073	CGTATGATGT	A10	A1
FLD0074	TAGTCTGTCA	B10	A1
FLD0075	TGTCTCTATC	C10	A1
FLD0076	CTAGAGTATC	D10	A1
FLD0077	TATCATGTGC	E10	A1
FLD0078	CATGAGTGTA	F10	A1
FLD0079	TGTCGTCATA	G10	A1
FLD0080	TATCTCATGC	H10	A1
FLD0081	TGTGTCACTA	A11	A1
FLD0082	TATCGATGCT	B11	A1
FLD0083	TAGAGTCTGT	C11	A1
FLD0084	CATGCATCAT	D11	A1
FLD0085	TGATCAGTC	E11	A1
FLD0086	CGTCTATGAT	F11	A1
FLD0087	GTGATACTGA	G11	A1
FLD0088	CTAGATCTGA	H11	A1
FLD0089	TATCAGTCTG	A12	A1
FLD0090	TCAGATGCTA	B12	A1
FLD0091	TATGTACGTG	C12	A1
FLD0092	CTATACAGTG	D12	A1
FLD0093	TGATACTCTG	E12	A1
FLD0094	TCAGCGATAT	F12	A1
FLD0095	CTACTGATGA	G12	A1
FLD0096	GTAGTACACA	H12	A1
FLD0097	TGCTACATCA	A1	A2
FLD0098	AGTGTGTCTA	B1	A2
FLD0099	TCATATCGCG	C1	A2
FLD0100	TACGTATAGC	D1	A2
FLD0101	CAGCTATAGC	E1	A2
FLD0102	TCGATGCGCT	F1	A2
FLD0103	GCACCGCGTAT	G1	A2
FLD0104	GCAGTATGCG	H1	A2
FLD0105	TGATAGAGAG	A2	A2
FLD0106	GCTACTAGCG	B2	A2
FLD0107	TGCGAGACGT	C2	A2
FLD0108	CGATGACAGA	D2	A2
FLD0109	GACTCATGCT	E2	A2
FLD0110	GTCTGATACG	F2	A2
FLD0111	ACTAGCTGTC	G2	A2
FLD0112	GCGTAGACGA	H2	A2
FLD0113	CTCAGCAGTG	A3	A2
FLD0114	CAGTCTACAT	B3	A2
FLD0115	TACTGCAGCG	C3	A2
FLD0116	TACACAGTAG	D3	A2
FLD0117	CACATACAGT	E3	A2
FLD0118	CACAGTGTG	F3	A2
FLD0119	CGAGCTAGCA	G3	A2
FLD0120	GAGACTATGC	H3	A2
FLD0121	CAGAGCTAGT	A4	A2
FLD0122	CGCAGAGCAT	B4	A2
FLD0123	TGTACAGCGA	C4	A2
FLD0124	ACGTCAGTAT	D4	A2
FLD0125	TCACAGCATA	E4	A2
FLD0126	ACTGCGTGT	F4	A2
FLD0127	CGATCGACTG	G4	A2
FLD0128	GCGAGATGTA	H4	A2
FLD0129	CTGATGCGAGA	A5	A2
FLD0130	GTGACGCTACG	B5	A2
FLD0131	CGACGCTGAT	C5	A2
FLD0132	CTACGATCAG	D5	A2
FLD0133	GCACTAGACA	E5	A2
FLD0134	CTAGCAGATG	F5	A2
FLD0135	CATGATACGC	G5	A2

Barcode Sequences for Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction), PN 100-4876

FLD0136	GCAGCTGTCA	H5	A2
FLD0137	ACGTATCATC	A6	A2
FLD0138	AGTATCGTAC	B6	A2
FLD0139	GATAACTGAA	C6	A2
FLD0140	GAATAGTCAG	D6	A2
FLD0141	GATGACTACG	E6	A2
FLD0142	CAGAGAGTCA	F6	A2
FLD0143	TCGATCGACA	G6	A2
FLD0144	ACTGATGTAG	H6	A2
FLD0145	ACTCGATAGT	A7	A2
FLD0146	GACGATCGCA	B7	A2
FLD0147	TCATCATGCG	C7	A2
FLD0148	ACATGTCTGA	D7	A2
FLD0149	AGTCATCGCA	E7	A2
FLD0150	TAGCATACAG	F7	A2
FLD0151	AGAGTCGCGT	G7	A2
FLD0152	TCTACGACAT	H7	A2
FLD0153	CACGAGATGA	A8	A2
FLD0154	ACGGCACATAT	B8	A2
FLD0155	ACGTGCTCTG	C8	A2
FLD0156	ACGATCACAT	D8	A2
FLD0157	AGTGTACTCA	E8	A2
FLD0158	TGATGTATGT	F8	A2
FLD0159	GATATATGTC	G8	A2
FLD0160	TAGTACTAGA	H8	A2
FLD0161	TATAGAGATC	A9	A2
FLD0162	TCGATATCTA	B9	A2
FLD0163	TACATGATAG	C9	A2
FLD0164	TGAGATCATA	D9	A2
FLD0165	CTACATACTA	E9	A2
FLD0166	ATCACTGTAT	F9	A2
FLD0167	ATCATATCTC	G9	A2
FLD0168	AGTAGATCAT	H9	A2
FLD0169	ACATAGTATC	A10	A2
FLD0170	ATGTATAGTC	B10	A2
FLD0171	ACAGTCATAT	C10	A2
FLD0172	ACATATACTG	D10	A2
FLD0173	AGCATCTATA	E10	A2
FLD0174	AGACTATATC	F10	A2
FLD0175	CAGCATCTAG	G10	A2
FLD0176	CGAGACGACA	H10	A2
FLD0177	ATCACTCATA	A11	A2
FLD0178	AGCTCTGTGA	B11	A2
FLD0179	ATGTCATGCT	C11	A2
FLD0180	GCTGACAGAG	D11	A2
FLD0181	ATACAGTCTC	E11	A2
FLD0182	CATAGACGTG	F11	A2
FLD0183	AGAGATATCA	G11	A2
FLD0184	ATGCTGCGCT	H11	A2
FLD0185	AGTCAGACGC	A12	A2
FLD0186	ACGATACACT	B12	A2
FLD0187	AGCGAGTATG	C12	A2
FLD0188	ATCGCTACAT	D12	A2
FLD0189	ATGCTAGAGA	E12	A2
FLD0190	AGCAGTACTC	F12	A2
FLD0191	ATCTAGATCA	G12	A2
FLD0192	ATCCGCATAGA	H12	A2
FLD0193	TTGTTGCTGT	A1	A3
FLD0194	GTGTGGTTGT	B1	A3
FLD0195	TAGGTGGAAT	C1	A3
FLD0196	TGTAGGTGGA	D1	A3
FLD0197	TTAGTGGTGA	E1	A3
FLD0198	GTGAAGGTAA	F1	A3
FLD0199	TGTTGTGGTA	G1	A3
FLD0200	GTTGATGAGT	H1	A3
FLD0201	GGTCAGTGTA	A2	A3
FLD0202	GTAATGGAGT	B2	A3
FLD0203	CTCGTTATTG	C2	A3

FLD0204	GGAAGTAAGG	D2	A3
FLD0205	CGGTGTGTGT	E2	A3
FLD0206	CGTCTTCTTA	F2	A3
FLD0207	TGTGAATCTC	G2	A3
FLD0208	CTAATCGTGT	H2	A3
FLD0209	CTCTTAGTTTC	A3	A3
FLD0210	GGATAGGATC	B3	A3
FLD0211	GGTGTCTTGT	C3	A3
FLD0212	GATGGTTGTA	D3	A3
FLD0213	CCTCGTTGTT	E3	A3
FLD0214	GGTGGAGTT	F3	A3
FLD0215	TGGTGTCCGT	G3	A3
FLD0216	CGTTAGCGTA	H3	A3
FLD0217	TACTAGGATC	A4	A3
FLD0218	GTCTCAATGT	B4	A3
FLD0219	GATGAGGTAT	C4	A3
FLD0220	GGTGTAGTG	D4	A3
FLD0221	CATTCTCTGA	E4	A3
FLD0222	CATCTGGAGT	F4	A3
FLD0223	GAATGGAAGA	G4	A3
FLD0224	GGCTGTGATC	H4	A3
FLD0225	TGGTGTCTGGA	A5	A3
FLD0226	TATGGTAAGG	B5	A3
FLD0227	GTTCGATTGT	C5	A3
FLD0228	GGTAGAAATGA	D5	A3
FLD0229	TTCTCATCGT	E5	A3
FLD0230	CTCAATCGTA	F5	A3
FLD0231	CGCTAACATGTA	G5	A3
FLD0232	GCGTCTGAAT	H5	A3
FLD0233	TTCTGTTGCC	A6	A3
FLD0234	TTGTCCTTGC	B6	A3
FLD0235	CCTGTGTTAGA	C6	A3
FLD0236	GATAAGAAGG	D6	A3
FLD0237	CAGGTACACAT	E6	A3
FLD0238	GCCATGTCAT	F6	A3
FLD0239	TCTGCCTATA	G6	A3
FLD0240	CTTAGTTCGC	H6	A3
FLD0241	CGTAATGAGC	A7	A3
FLD0242	TTGCTTAGTC	B7	A3
FLD0243	TCTTGTTCAC	C7	A3
FLD0244	GTGGCTTCGT	D7	A3
FLD0245	TGTCGATAG	E7	A3
FLD0246	TCATTCAGTG	F7	A3
FLD0247	GTGGAGAGCT	G7	A3
FLD0248	GTAGAACGTGG	H7	A3
FLD0249	TGGAGCATGT	A8	A3
FLD0250	GAAAGGAGATA	B8	A3
FLD0251	CGAATGTATG	C8	A3
FLD0252	TCGTGAATGA	D8	A3
FLD0253	GAATAGCTGA	E8	A3
FLD0254	TTGTCACATC	F8	A3
FLD0255	CTGGAGGCTA	G8	A3
FLD0256	TGTCAGCTTA	H8	A3
FLD0257	GTTCTTCGTA	A9	A3
FLD0258	TTACACGTT	B9	A3
FLD0259	GTAGCCAGTA	C9	A3
FLD0260	TGAGAACGTA	D9	A3
FLD0261	CCATATGATC	E9	A3
FLD0262	CGATCCTATA	F9	A3
FLD0263	TGACTAGCTT	G9	A3
FLD0264	TAACCTGTCT	H9	A3
FLD0265	TCGAATGTGC	A10	A3
FLD0266	TCGCTGAACA	B10	A3
FLD0267	GCGTTATTGC	C10	A3
FLD0268	GAACTATCAC	D10	A3
FLD0269	TCGAGGTTACT	E10	A3
FLD0270	TGCGGATGGT	F10	A3
FLD0271	TTCGAGCTAT	G10	A3

FLD0272	GGCTGGTGT	H10	A3
FLD0273	CTAAGTCATG	A11	A3
FLD0274	TTGCAGATCA	B11	A3
FLD0275	CTGCGAACAT	C11	A3
FLD0276	CTGTTCTAGC	D11	A3
FLD0277	CACTTGTGTG	E11	A3
FLD0278	TGGATGACAT	F11	A3
FLD0279	GATCCGTGAGC	G11	A3
FLD0280	GTCGGTCTGA	H11	A3
FLD0281	TGTTACGATC	A12	A3
FLD0282	GTCTGGCTC	B12	A3
FLD0283	GGTCGTGCAT	C12	A3
FLD0284	CAGGCTCAGT	D12	A3
FLD0285	TAGCTTCACT	E12	A3
FLD0286	CAGATGTCCT	F12	A3
FLD0287	TTACGCAGTG	G12	A3
FLD0288	TTCGTTCTG	H12	A3
FLD0289	CACTGTTGA	A1	A4
FLD0290	TCTAGCGTGG	B1	A4
FLD0291	GCATAATCGC	C1	A4
FLD0292	GTCGTAACAC	D1	A4
FLD0293	GAGATTGCTA	E1	A4
FLD0294	GGACAGATGG	F1	A4
FLD0295	CTTACGTTGC	G1	A4
FLD0296	GTGTTCGGTC	H1	A4
FLD0297	CTCAAGAACG	A2	A4
FLD0298	TCTCGGATAG	B2	A4
FLD0299	CTCTGGACGA	C2	A4
FLD0300	CGAGCATTGT	D2	A4
FLD0301	CCAAGAACGAA	E2	A4
FLD0302	TCCTTGTCT	F2	A4
FLD0303	GTAACGATGT	G2	A4
FLD0304	TGGACTCAGA	H2	A4
FLD0305	GGCATCATGC	A3	A4
FLD0306	GTATAACGCT	B3	A4
FLD0307	GCAGATAAGT	C3	A4
FLD0308	GTCCGGCTCA	D3	A4
FLD0309	TTCGATAGCA	E3	A4
FLD0310	GTCTAGCAGG	F3	A4
FLD0311	GGAACACAGG	G3	A4
FLD0312	TGGTCGCTG	H3	A4
FLD0313	CACATTAGCG	A4	A4
FLD0314	GAAGCGCACT	B4	A4
FLD0315	GCATGCCAGT	C4	A4
FLD0316	GGAGACTGTA	D4	A4
FLD0317	TCGAACGTCA	E4	A4
FLD0318	GAGAGGACAT	F4	A4
FLD0319	GAGCACGGAA	G4	A4
FLD0320	GCTCTAACAT	H4	A4
FLD0321	TGCTGGCTTG	A5	A4
FLD0322	TGCATGGAGC	B5	A4
FLD0323	GTACTAACAG	C5	A4
FLD0324	GAAGTCAAGC	D5	A4
FLD0325	GCGCATTATG	E5	A4
FLD0326	GTCCAGACAT	F5	A4
FLD0327	GAGACCTCTA	G5	A4
FLD0328	TTGCACTCAG	H5	A4
FLD0329	TGCGCGATA	A6	A4
FLD0330	AGTTGCTAGT	B6	A4
FLD0331	AGGATTGAGG	C6	A4
FLD0332	CCAGAACAGA	D6	A4
FLD0333	CGTCAAGCAT	E6	A4
FLD0334	TTGTCGAGAC	F6	A4
FLD0335	GACAGGTGAC	G6	A4
FLD0336	CTGACAAGTG	H6	A4
FLD0337	CACGAAGAGC	A7	A4
FLD0338	CATACCTGAT	B7	A4
FLD0339	GACGTGCTTC	C7	A4

Barcode Sequences for Access Array Barcode Library for Illumina Sequencers - 384 (Single Direction), PN 100-4876

FLD0340	ATTGTGGAGT	D7	A4
FLD0341	TCTGGTCTCA	E7	A4
FLD0342	AGGTAAGAGG	F7	A4
FLD0343	TCCTGACAGA	G7	A4
FLD0344	GCACTGTTGC	H7	A4
FLD0345	ACCATGAGTC	A8	A4
FLD0346	AATGCAGTGT	B8	A4
FLD0347	ATATGGTGGA	C8	A4
FLD0348	ACTCAGTTAC	D8	A4
FLD0349	AAGTGCAGTG	E8	A4
FLD0350	CCACAGAGTG	F8	A4
FLD0351	AGTGGTGATC	G8	A4
FLD0352	ACTTCTTAGC	H8	A4
FLD0353	GCCACATATA	A9	A4
FLD0354	ACGCAGGAGT	B9	A4
FLD0355	AATATGCTGC	C9	A4
FLD0356	AAGCGTAGAA	D9	A4
FLD0357	GACAGCAAGC	E9	A4
FLD0358	CTGACCGAGA	F9	A4
FLD0359	CGCGACTTGT	G9	A4
FLD0360	CATCAACATG	H9	A4
FLD0361	TGGCTACGCT	A10	A4
FLD0362	ACGCGGACTA	B10	A4
FLD0363	AGAGGTCGGA	C10	A4
FLD0364	AATCGAGCGT	D10	A4
FLD0365	AAGTACACTC	E10	A4
FLD0366	AGCTGAATGA	F10	A4
FLD0367	ATGCCTATCA	G10	A4
FLD0368	ACTGTAGGAC	H10	A4
FLD0369	ATAGCCGTGT	A11	A4
FLD0370	TCACGACGAA	B11	A4
FLD0371	ATCTGTCCAT	C11	A4
FLD0372	ACTTAGAGAG	D11	A4
FLD0373	AGTGGCAGGT	E11	A4
FLD0374	ATGAGGTCGT	F11	A4
FLD0375	AGGAGAAGGA	G11	A4
FLD0376	ACAAC TGCAA	H11	A4
FLD0377	ATTAGCGAGT	A12	A4
FLD0378	ACAACGAACA	B12	A4
FLD0379	AGAGCGCCAA	C12	A4
FLD0380	AGGTAGCTCA	D12	A4
FLD0381	AACGCCAAGA	E12	A4
FLD0382	AAGGTATGAG	F12	A4
FLD0383	ATGGAGCACT	G12	A4
FLD0384	ACGGTGCTAG	H12	A4

Barcode Sequences for Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional), PN 100-3771

Barcode Name	Barcode Sequence	Barcode Well	Plate
FLD0001	GTATCGTCGT	A1	A1 & B1
FLD0002	GTGTATGCGT	B1	A1 & B1
FLD0003	TGCTCGTAGT	C1	A1 & B1
FLD0004	GTCGTCGTCT	D1	A1 & B1
FLD0005	GTGCGTGTGT	E1	A1 & B1
FLD0006	GCGTCGTGTA	F1	A1 & B1
FLD0007	GTCGTGTACT	G1	A1 & B1
FLD0008	GATGTAGCGT	H1	A1 & B1
FLD0009	GAGTGATCGT	A2	A1 & B1
FLD0010	CGCTATCAGT	B2	A1 & B1
FLD0011	CGCTGTAGTC	C2	A1 & B1
FLD0012	GCTAGTGAGT	D2	A1 & B1
FLD0013	GAGCTAGTGA	E2	A1 & B1
FLD0014	CGTGCTGTCA	F2	A1 & B1
FLD0015	GATCGTCTCT	G2	A1 & B1
FLD0016	GTGCGTGTGT	H2	A1 & B1
FLD0017	TGAGCGTGCT	A3	A1 & B1
FLD0018	CATGTCGTCA	B3	A1 & B1
FLD0019	TCAGTGTCTC	C3	A1 & B1
FLD0020	GTGCTCATGT	D3	A1 & B1
FLD0021	CGTATCTCGA	E3	A1 & B1
FLD0022	GTCATGCGTC	F3	A1 & B1
FLD0023	CTATGCGATC	G3	A1 & B1
FLD0024	TGCTATGCTG	H3	A1 & B1
FLD0025	TGTGTGCATG	A4	A1 & B1
FLD0026	GAGTGTCACT	B4	A1 & B1
FLD0027	CTAGTCTCGT	C4	A1 & B1
FLD0028	GAGTGCATCT	D4	A1 & B1
FLD0029	TGCGTAGTCG	E4	A1 & B1
FLD0030	CTGTGTCGTC	F4	A1 & B1
FLD0031	CTGTAGTGCG	G4	A1 & B1
FLD0032	GTGCGCTAGT	H4	A1 & B1
FLD0033	TGTGCTCGCA	A5	A1 & B1
FLD0034	GATGCGAGCT	B5	A1 & B1
FLD0035	CTGTACGTGA	C5	A1 & B1
FLD0036	GCGATGATGA	D5	A1 & B1
FLD0037	TGTCGAGTC	E5	A1 & B1
FLD0038	GTCTACTGTC	F5	A1 & B1
FLD0039	CAGTCAGAGT	G5	A1 & B1
FLD0040	CGCAGTCTAT	H5	A1 & B1
FLD0041	GTATGAGCAC	A6	A1 & B1
FLD0042	CGAGTGTGT	B6	A1 & B1
FLD0043	TATAGCACGC	C6	A1 & B1
FLD0044	TCATGCGCGA	D6	A1 & B1
FLD0045	TATGCGCTGC	E6	A1 & B1
FLD0046	TCTCTGTGCA	F6	A1 & B1
FLD0047	CTATGCGGTG	G6	A1 & B1
FLD0048	TACGCGCTG	H6	A1 & B1
FLD0049	CTGCATGATC	A7	A1 & B1
FLD0050	CGCGTATCAT	B7	A1 & B1
FLD0051	GTATCTCTCG	C7	A1 & B1
FLD0052	GCTCATATGC	D7	A1 & B1
FLD0053	CACTATGTCG	E7	A1 & B1
FLD0054	TAGCGCGTAG	F7	A1 & B1
FLD0055	CGTCACAGTA	G7	A1 & B1
FLD0056	TCGCGTGAGA	H7	A1 & B1
FLD0057	TACATCGCTG	A8	A1 & B1
FLD0058	GTGAGAGACA	B8	A1 & B1
FLD0059	GACTGTACGT	C8	A1 & B1
FLD0060	GCACGTAGCT	D8	A1 & B1
FLD0061	TCACGCTATG	E8	A1 & B1
FLD0062	CGTACTACGT	F8	A1 & B1
FLD0063	CAGCTGAGTA	G8	A1 & B1
FLD0064	GAGATCAGTC	H8	A1 & B1
FLD0065	TACTGAGCTG	A9	A1 & B1
FLD0066	TAGTAGCGCG	B9	A1 & B1
FLD0067	GACGTCTGCT	C9	A1 & B1

FLD0068	GTACTCGCGA	D9	A1 & B1
FLD0069	TCTGAGCGCA	E9	A1 & B1
FLD0070	TAGACGTGCT	F9	A1 & B1
FLD0071	GTGACTCGTC	G9	A1 & B1
FLD0072	TCGAGTAGCG	H9	A1 & B1
FLD0073	CGTATGATGT	A10	A1 & B1
FLD0074	TAGTCTGTCA	B10	A1 & B1
FLD0075	TGTCCTATC	C10	A1 & B1
FLD0076	CTAGAGTATC	D10	A1 & B1
FLD0077	TATCATGTGC	E10	A1 & B1
FLD0078	CATGAGTGTA	F10	A1 & B1
FLD0079	TGTCGTCATA	G10	A1 & B1
FLD0080	TATCTCATGC	H10	A1 & B1
FLD0081	TGTGTCACTA	A11	A1 & B1
FLD0082	TATCGATGCT	B11	A1 & B1
FLD0083	TAGAGTCTGT	C11	A1 & B1
FLD0084	CATGCATCAT	D11	A1 & B1
FLD0085	TGATCAGTC	E11	A1 & B1
FLD0086	CGTCTATGAT	F11	A1 & B1
FLD0087	GTGATACTGA	G11	A1 & B1
FLD0088	CTAGATCTGA	H11	A1 & B1
FLD0089	TATCAGTCTG	A12	A1 & B1
FLD0090	TCAGATGCTA	B12	A1 & B1
FLD0091	TATGTACGTG	C12	A1 & B1
FLD0092	CTATACAGTG	D12	A1 & B1
FLD0093	TGATACTCTG	E12	A1 & B1
FLD0094	TCAGCGATAT	F12	A1 & B1
FLD0095	CTACTGATGA	G12	A1 & B1
FLD0096	GTAGTACACA	H12	A1 & B1
FLD0097	TGCTACATCA	A1	A2 & B2
FLD0098	AGTGTGTCTA	B1	A2 & B2
FLD0099	TCATATCGCG	C1	A2 & B2
FLD0100	TACGTATAGC	D1	A2 & B2
FLD0101	CAGCTATAGC	E1	A2 & B2
FLD0102	TCGATGCGCT	F1	A2 & B2
FLD0103	GCACCGCGTAT	G1	A2 & B2
FLD0104	GCAGTTATGCG	H1	A2 & B2
FLD0105	TGATAGAGAG	A2	A2 & B2
FLD0106	GCTACTAGCG	B2	A2 & B2
FLD0107	TGCGAGACGT	C2	A2 & B2
FLD0108	CGATGACAGA	D2	A2 & B2
FLD0109	GACTCATGCT	E2	A2 & B2
FLD0110	GTCTGATACG	F2	A2 & B2
FLD0111	ACTAGCTGTC	G2	A2 & B2
FLD0112	GCGTAGACGA	H2	A2 & B2
FLD0113	CTCAGCAGTG	A3	A2 & B2
FLD0114	CAGTCTACAT	B3	A2 & B2
FLD0115	TACTGCAGCG	C3	A2 & B2
FLD0116	TACACAGTAG	D3	A2 & B2
FLD0117	CACATACAGT	E3	A2 & B2
FLD0118	CACAGTGTATG	F3	A2 & B2
FLD0119	CGAGCTAGCA	G3	A2 & B2
FLD0120	GAGACTATGC	H3	A2 & B2
FLD0121	CAGAGCTAGT	A4	A2 & B2
FLD0122	CGCAGAGCAT	B4	A2 & B2
FLD0123	TGTACAGCGA	C4	A2 & B2
FLD0124	ACGTCAGTAT	D4	A2 & B2
FLD0125	TCACAGCATA	E4	A2 & B2
FLD0126	ACTGCGTGT	F4	A2 & B2
FLD0127	CGATCGACTG	G4	A2 & B2
FLD0128	GCGAGATGTA	H4	A2 & B2
FLD0129	CTGATGCAGA	A5	A2 & B2
FLD0130	GTGACGCTACG	B5	A2 & B2
FLD0131	CGACGCTGAT	C5	A2 & B2
FLD0132	CTACGATCAG	D5	A2 & B2
FLD0133	GCACTAGACA	E5	A2 & B2
FLD0134	CTAGCAGATG	F5	A2 & B2
FLD0135	CATGATACGC	G5	A2 & B2

Barcode Sequences for Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional), PN 100-3771

FLD0136	GCAGCTGTCA	H5	A2 & B2
FLD0137	ACGTATCATC	A6	A2 & B2
FLD0138	AGTATCGTAC	B6	A2 & B2
FLD0139	GATACACTGA	C6	A2 & B2
FLD0140	GAATGTCAG	D6	A2 & B2
FLD0141	GATGACTACG	E6	A2 & B2
FLD0142	CAGAGAGTCA	F6	A2 & B2
FLD0143	TCGATCGACA	G6	A2 & B2
FLD0144	ACTGATGTAG	H6	A2 & B2
FLD0145	ACTCGATAGT	A7	A2 & B2
FLD0146	GACGATCGCA	B7	A2 & B2
FLD0147	TCATCATGCG	C7	A2 & B2
FLD0148	ACATGTCTGA	D7	A2 & B2
FLD0149	AGTCATCGCA	E7	A2 & B2
FLD0150	TAGCATACAG	F7	A2 & B2
FLD0151	AGAGTCGCGT	G7	A2 & B2
FLD0152	TCTACGACAT	H7	A2 & B2
FLD0153	CACCGAGATGA	A8	A2 & B2
FLD0154	ACGGCACATAT	B8	A2 & B2
FLD0155	ACGTGCTCTG	C8	A2 & B2
FLD0156	ACGATCACAT	D8	A2 & B2
FLD0157	AGTGTACTCA	E8	A2 & B2
FLD0158	TGATGTATGT	F8	A2 & B2
FLD0159	GATATATGTC	G8	A2 & B2
FLD0160	TAGTACTAGA	H8	A2 & B2
FLD0161	TATAGAGATC	A9	A2 & B2
FLD0162	TCGATATCTA	B9	A2 & B2
FLD0163	TACATGATAG	C9	A2 & B2
FLD0164	TGAGATCATA	D9	A2 & B2
FLD0165	CTACATACTA	E9	A2 & B2
FLD0166	ATCACTGTAT	F9	A2 & B2
FLD0167	ATCATATCTC	G9	A2 & B2
FLD0168	AGTAGATCAT	H9	A2 & B2
FLD0169	ACATAGTATC	A10	A2 & B2
FLD0170	ATGTATAGTC	B10	A2 & B2
FLD0171	ACAGTCATAT	C10	A2 & B2
FLD0172	ACATATACTG	D10	A2 & B2
FLD0173	AGCATCTATA	E10	A2 & B2
FLD0174	AGACTATATC	F10	A2 & B2
FLD0175	CAGCATCTAG	G10	A2 & B2
FLD0176	CGAGACGACA	H10	A2 & B2
FLD0177	ATCACTCATA	A11	A2 & B2
FLD0178	AGCTCTGTGA	B11	A2 & B2
FLD0179	ATGTCATGCT	C11	A2 & B2
FLD0180	GCTGACAGAG	D11	A2 & B2
FLD0181	ATACAGTCTC	E11	A2 & B2
FLD0182	CATAGACGTG	F11	A2 & B2
FLD0183	AGAGATATCA	G11	A2 & B2
FLD0184	ATGCTGCGCT	H11	A2 & B2
FLD0185	AGTCAGACGC	A12	A2 & B2
FLD0186	ACGATACACT	B12	A2 & B2
FLD0187	AGCGAGTATG	C12	A2 & B2
FLD0188	ATCGCTACAT	D12	A2 & B2
FLD0189	ATGCTAGAGA	E12	A2 & B2
FLD0190	AGCAGTACTC	F12	A2 & B2
FLD0191	ATCTAGATCA	G12	A2 & B2
FLD0192	ATCCGCATAGA	H12	A2 & B2
FLD0193	TTGTTGCTGT	A1	A3 & B3
FLD0194	GTGTTGTTGT	B1	A3 & B3
FLD0195	TAGGTGGAAT	C1	A3 & B3
FLD0196	TGTAGGTGGA	D1	A3 & B3
FLD0197	TTAGTGGTGA	E1	A3 & B3
FLD0198	GTGAAGGTAA	F1	A3 & B3
FLD0199	TGTTGTGGTA	G1	A3 & B3
FLD0200	GTTGATGAGT	H1	A3 & B3
FLD0201	GGTCAGTGTA	A2	A3 & B3
FLD0202	GTAATGGAGT	B2	A3 & B3
FLD0203	CTCGTTATTG	C2	A3 & B3

FLD0204	GGAAGTAAGG	D2	A3 & B3
FLD0205	CGGTGTGTGT	E2	A3 & B3
FLD0206	CGTCTTCTTA	F2	A3 & B3
FLD0207	TGTGAATCTC	G2	A3 & B3
FLD0208	CTAATCGTGT	H2	A3 & B3
FLD0209	CTCTTAGTTTC	A3	A3 & B3
FLD0210	GGATAGGATC	B3	A3 & B3
FLD0211	GGTGTCTTGT	C3	A3 & B3
FLD0212	GATGGTTGTA	D3	A3 & B3
FLD0213	CCTCGTTGTT	E3	A3 & B3
FLD0214	GGTGGAGTT	F3	A3 & B3
FLD0215	TGGTGTCCGT	G3	A3 & B3
FLD0216	CGTTAGCGTA	H3	A3 & B3
FLD0217	TACTAGGATC	A4	A3 & B3
FLD0218	GTCTCAATGT	B4	A3 & B3
FLD0219	GATGAGGTAT	C4	A3 & B3
FLD0220	GGTGTAGTG	D4	A3 & B3
FLD0221	CATTCTCTGA	E4	A3 & B3
FLD0222	CATCTGGAGT	F4	A3 & B3
FLD0223	GAATGGAAGA	G4	A3 & B3
FLD0224	GGCTGTGATC	H4	A3 & B3
FLD0225	TGGTGCTGGA	A5	A3 & B3
FLD0226	TATGTAAGG	B5	A3 & B3
FLD0227	GTTCGATTGT	C5	A3 & B3
FLD0228	GGTAGAACATG	D5	A3 & B3
FLD0229	TTCTCATCGT	E5	A3 & B3
FLD0230	CTCAATCGTA	F5	A3 & B3
FLD0231	CGCTAAATGTA	G5	A3 & B3
FLD0232	GCGTCTGAAT	H5	A3 & B3
FLD0233	TTCTGTTGCC	A6	A3 & B3
FLD0234	TTGTCCTTGC	B6	A3 & B3
FLD0235	CCTGTGTTAGA	C6	A3 & B3
FLD0236	GATAAGAACAGG	D6	A3 & B3
FLD0237	CAGGTACACAT	E6	A3 & B3
FLD0238	GCCATGTCAT	F6	A3 & B3
FLD0239	TCTGCCTATA	G6	A3 & B3
FLD0240	CTTAGTTCGC	H6	A3 & B3
FLD0241	CGTAATGAGC	A7	A3 & B3
FLD0242	TTGCTTAGTC	B7	A3 & B3
FLD0243	TCTTGTTCAC	C7	A3 & B3
FLD0244	GTGGCTTCGT	D7	A3 & B3
FLD0245	TGTCGATAG	E7	A3 & B3
FLD0246	TCATTCAGTG	F7	A3 & B3
FLD0247	GTGGAGAGGCT	G7	A3 & B3
FLD0248	GTAGAACGTGG	H7	A3 & B3
FLD0249	TGGAGCATGT	A8	A3 & B3
FLD0250	GAAAGGAGATA	B8	A3 & B3
FLD0251	CGAATGTATG	C8	A3 & B3
FLD0252	TCGTGAATGA	D8	A3 & B3
FLD0253	GAATAGCTGA	E8	A3 & B3
FLD0254	TTGTCACATC	F8	A3 & B3
FLD0255	CTGGAGGCTA	G8	A3 & B3
FLD0256	TGTCAAGCTTA	H8	A3 & B3
FLD0257	GTTCTTCGTA	A9	A3 & B3
FLD0258	TTACACGTT	B9	A3 & B3
FLD0259	GTAGCCAGTA	C9	A3 & B3
FLD0260	TGAGAACGTA	D9	A3 & B3
FLD0261	CCATATGATC	E9	A3 & B3
FLD0262	CGATCCTATA	F9	A3 & B3
FLD0263	TGACTAGCTT	G9	A3 & B3
FLD0264	TAACCTGCT	H9	A3 & B3
FLD0265	TCGAATGTGC	A10	A3 & B3
FLD0266	TCGCTGAACA	B10	A3 & B3
FLD0267	GCGTTATTGC	C10	A3 & B3
FLD0268	GAACTATCAC	D10	A3 & B3
FLD0269	TCGAGGTACT	E10	A3 & B3
FLD0270	TGCGGATGGT	F10	A3 & B3
FLD0271	TTCGAGCTAT	G10	A3 & B3

FLD0272	GGTCTGGTGT	H10	A3 & B3
FLD0273	CTAAGTCATG	A11	A3 & B3
FLD0274	TTGCAGATCA	B11	A3 & B3
FLD0275	CTGCGAATGT	C11	A3 & B3
FLD0276	CTGTTCTAGC	D11	A3 & B3
FLD0277	CACTTGTGTG	E11	A3 & B3
FLD0278	TGGATGACAT	F11	A3 & B3
FLD0279	GATCCGTGAGC	G11	A3 & B3
FLD0280	GTCGGTCTGA	H11	A3 & B3
FLD0281	TGTTACGATC	A12	A3 & B3
FLD0282	GTCTGGCTC	B12	A3 & B3
FLD0283	GGTCGTGCAT	C12	A3 & B3
FLD0284	CAGGCTCAGT	D12	A3 & B3
FLD0285	TAGCTTCACT	E12	A3 & B3
FLD0286	CAGATGTCCT	F12	A3 & B3
FLD0287	TTACGCAGTG	G12	A3 & B3
FLD0288	TTCGTTCTG	H12	A3 & B3
FLD0289	CACTGTTGA	A1	A4 & B4
FLD0290	TCTAGCGTGG	B1	A4 & B4
FLD0291	GCATAATCGC	C1	A4 & B4
FLD0292	GTCGTAACAC	D1	A4 & B4
FLD0293	GAGATTGCTA	E1	A4 & B4
FLD0294	GGACAGATGG	F1	A4 & B4
FLD0295	CTTACGTTGC	G1	A4 & B4
FLD0296	GTGTTCGGTC	H1	A4 & B4
FLD0297	CTCAAGAACG	A2	A4 & B4
FLD0298	TCTCGGATAG	B2	A4 & B4
FLD0299	CTCTGGACGA	C2	A4 & B4
FLD0300	CGAGCATTGT	D2	A4 & B4
FLD0301	CCAAGAACGAA	E2	A4 & B4
FLD0302	TCCTTGTCT	F2	A4 & B4
FLD0303	GTAACGATGT	G2	A4 & B4
FLD0304	TGGACTCAGA	H2	A4 & B4
FLD0305	GGCATCATGC	A3	A4 & B4
FLD0306	GTATAACGCT	B3	A4 & B4
FLD0307	GCAGATAAGT	C3	A4 & B4
FLD0308	GTCCGGCTCA	D3	A4 & B4
FLD0309	TTCGATAGCA	E3	A4 & B4
FLD0310	GTCTAGCAGG	F3	A4 & B4
FLD0311	GGAACACAGG	G3	A4 & B4
FLD0312	TGGTCGCTG	H3	A4 & B4
FLD0313	CACATTAGCG	A4	A4 & B4
FLD0314	GAAGCGCACT	B4	A4 & B4
FLD0315	GCATGCCAGT	C4	A4 & B4
FLD0316	GGAGACTGTA	D4	A4 & B4
FLD0317	TCGAAC TGCA	E4	A4 & B4
FLD0318	GAGAGGACAT	F4	A4 & B4
FLD0319	GAGCACGGAA	G4	A4 & B4
FLD0320	GCTCTAACAT	H4	A4 & B4
FLD0321	TGCTGGCTTG	A5	A4 & B4
FLD0322	TGCATGGAGC	B5	A4 & B4
FLD0323	GTACTAACAG	C5	A4 & B4
FLD0324	GAAGTCAAGC	D5	A4 & B4
FLD0325	GCGCATTATG	E5	A4 & B4
FLD0326	GTCCAGACAT	F5	A4 & B4
FLD0327	GAGACCTCTA	G5	A4 & B4
FLD0328	TTGCACTCAG	H5	A4 & B4
FLD0329	TGCGCGATA	A6	A4 & B4
FLD0330	AGTTGCTAGT	B6	A4 & B4
FLD0331	AGGATTGAGG	C6	A4 & B4
FLD0332	CCAGAACAGA	D6	A4 & B4
FLD0333	CGTCAAGCAT	E6	A4 & B4
FLD0334	TTGTCGAGAC	F6	A4 & B4
FLD0335	GACAGGTGAC	G6	A4 & B4
FLD0336	CTGACAAGTG	H6	A4 & B4
FLD0337	CACGAAGAGC	A7	A4 & B4
FLD0338	CATACCTGAT	B7	A4 & B4
FLD0339	GACGTGCTTC	C7	A4 & B4

Barcode Sequences for Access Array Barcode Library for Illumina Sequencers - 384 (Bidirectional), PN 100-3771

FLD0340	ATTGTGGAGT	D7	A4 & B4
FLD0341	TCTGGTCTCA	E7	A4 & B4
FLD0342	AGGTAAGAGG	F7	A4 & B4
FLD0343	TCCTGACAGA	G7	A4 & B4
FLD0344	GCACTGTTGC	H7	A4 & B4
FLD0345	ACCATGAGTC	A8	A4 & B4
FLD0346	AATGCAGTGT	B8	A4 & B4
FLD0347	ATATGGTGGA	C8	A4 & B4
FLD0348	ACTCAGTTAC	D8	A4 & B4
FLD0349	AAGTGCAGATG	E8	A4 & B4
FLD0350	CCACAGAGTG	F8	A4 & B4
FLD0351	AGTGGTGATC	G8	A4 & B4
FLD0352	ACTTCTTAGC	H8	A4 & B4
FLD0353	GCCACATATA	A9	A4 & B4
FLD0354	ACGCAGGAGT	B9	A4 & B4
FLD0355	AATATGCTGC	C9	A4 & B4
FLD0356	AAGCGTAGAA	D9	A4 & B4
FLD0357	GACAGCAAGC	E9	A4 & B4
FLD0358	CTGACCGAGA	F9	A4 & B4
FLD0359	CGCGACTTGT	G9	A4 & B4
FLD0360	CATCAACATG	H9	A4 & B4
FLD0361	TGGCTACGCT	A10	A4 & B4
FLD0362	ACGCGGACTA	B10	A4 & B4
FLD0363	AGAGGTGGGA	C10	A4 & B4
FLD0364	AATCGAGCGT	D10	A4 & B4
FLD0365	AAGTACACTC	E10	A4 & B4
FLD0366	AGCTGAATGA	F10	A4 & B4
FLD0367	ATGCCTATCA	G10	A4 & B4
FLD0368	ACTGTAGGAC	H10	A4 & B4
FLD0369	ATAGCCGTGT	A11	A4 & B4
FLD0370	TCACGACGAA	B11	A4 & B4
FLD0371	ATCTGTCCAT	C11	A4 & B4
FLD0372	ACTTAGAGAG	D11	A4 & B4
FLD0373	AGTGGCAGGT	E11	A4 & B4
FLD0374	ATGAGGTCGT	F11	A4 & B4
FLD0375	AGGAGAAGGA	G11	A4 & B4
FLD0376	ACAAC TGCAA	H11	A4 & B4
FLD0377	ATTAGCGAGT	A12	A4 & B4
FLD0378	ACAACGAACA	B12	A4 & B4
FLD0379	AGAGCGCCAA	C12	A4 & B4
FLD0380	AGGTAGCTCA	D12	A4 & B4
FLD0381	AACGCCAAGA	E12	A4 & B4
FLD0382	AAGGTATGAG	F12	A4 & B4
FLD0383	ATGGAGCACT	G12	A4 & B4
FLD0384	ACGGTGCTAG	H12	A4 & B4

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