Fluent BioSciences

PIPseq™ UDI-96 Kit

User Guide

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Introduction

The Fluent BioSciences PIPseq™ UDI-96 Kit enables multiplex sample preparation of PIPseq Single Cell 3′ gene expression libraries for next-generation sequencing on the Illumina platform. Each kit provides 96 pre-mixed unique pairs of i5 and i7 index primers packaged in a single-use 96-well breakable plate with a pierceable foil seal.

Recommended Use

The recommended application for this kit is PIPseq library preparation of Single Cell 3′ gene expression libraries to be sequenced on an Illumina platform. Users can break away a single column of 8 pre-mixed, unique indexes without thawing the entire plate for each experiment. Further, the pierceable foil seal ensures single use of each pre-mixed index pair to avoid index contamination across wells. The volumes provided are sufficient for the preparation of up to 96 reactions. **The 96-well plate should be stored at -20°C.**

The PIPseq UDI-96 Kit should only be used with Fluent BioSciences PIPseq 3′ Single Cell RNA Kits chemistry versions 4.0PLUS and V. These indexes have not been tested for compatibility with other single-cell library preparation kits or technologies.

Note: Users should not mix index primers provided by the PIPseq v4.0PLUS 3′ Single Cell RNA Kits and the index primers in the UDI-96 Kit, as the lengths of the sample indexes differ. Instead, it is recommended to utilize index primers from one kit or the other, as determined by the user's desired number of samples for multiplexing.

Users should refer to the kit-specific User Guide before using the PIPseq UDI-96 Kit primers for PIPseq library preparation. Users should verify they have the latest User Guide revision downloaded at www.fluentbio.com.

Compatible PIPseq 3′ Single Cell RNA Kits

Product Name	Catalog Number	User Guide
PIPseq T2 3′ Single Cell RNA Kit v4.0PLUS	FBS-SCR-T2-8-V4.05	FB0001026 Rev 12.11+
PIPseq T20 3′ Single Cell RNA Kit v4.0PLUS	FBS-SCR-T20-4-V4.05	FB0002130 Rev 8.9+
PIPseq T100 3′ Single Cell RNA Kit v4.0PLUS	FBS-SCR-T100-2-V4.05	FB0003657 Rev 2.9+
PIPseq V T2 3′ Single Cell RNA Kit Bundle	FB0005384	FB0005260 Rev 1.5+
PIPseq V T10 3′ Single Cell RNA Kit Bundle	FB0005385	FB0004762 Rev 1.5+
PIPseq V T20 3′ Single Cell RNA Kit Bundle	FB0005386	FB0005261 Rev 1.5+
PIPseq V T100 3′ Single Cell RNA Kit Bundle	FB0005387	FB0005262 Rev 1.5+

Index Plate Overview

The PIPseq UDI-96 plate provides 96 pre-mixed unique pairs of i5 and i7 index primers that are compatible with the PIPseq 3′ Single Cell RNA Kits versions 4.0PLUS and V. These pairs are sequentially labeled by column (Figure 1). **NOTE: Index primers provided in the PIPseq V 3' Single Cell RNA Kit are identical to column 3 of the UDI-96 plate**. Users should be sure to use indexes from the other columns if using both index sets together for their pooled libraries.

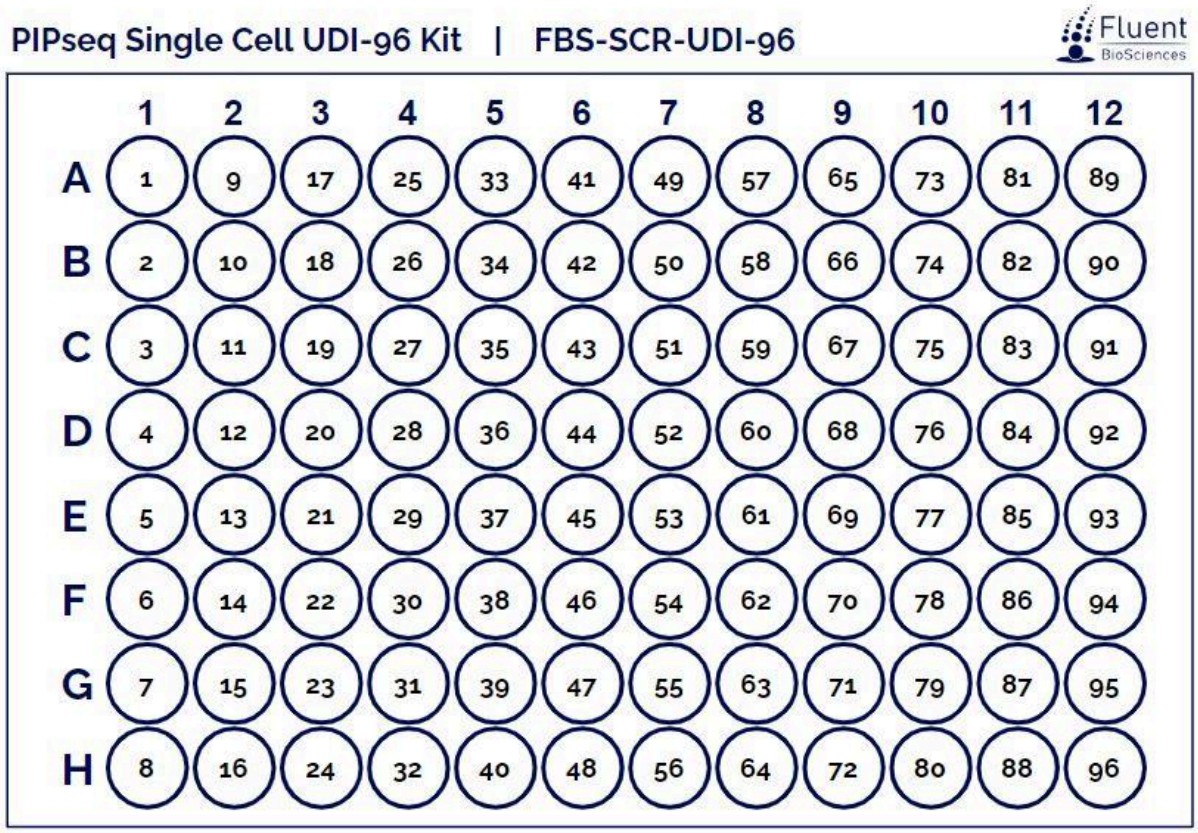

Figure 1. Plate map for the UDI-96 kit where each well contains a unique i5/i7 index pair and the wells are labeled sequentially by column. This configuration is intended to allow users to break apart the plate by column so that index pairs can be used most efficiently one row at a time.

PIPseq Single Cell Gene Expression Library Overview

The PIPseq 3′ Single Cell gene expression library comprises the standard Illumina paired-end sequences P5 and P7. Read 1 contains the barcode information and is distinct between PIPseq chemistry versions while Read 2 contains the gene expression information. **PIPseq utilizes a unique mixture of Nextera and TruSeq sequences for Read 1 and Read 2**, and these libraries are compatible with standard Illumina sequencing. It is recommended to maximize the use of the flow cell with longer reads when possible.

PIPseq version 4.0PLUS: the libraries prepared in this protocol using the UDI-96 Kit for Illumina are **dual-indexed with 10-base i5 and i7 indexes**. **Read 1 length must be ≥ 54 bases** and the recommended **Read 2 length ≥ 63 bases (Figure 2)**.

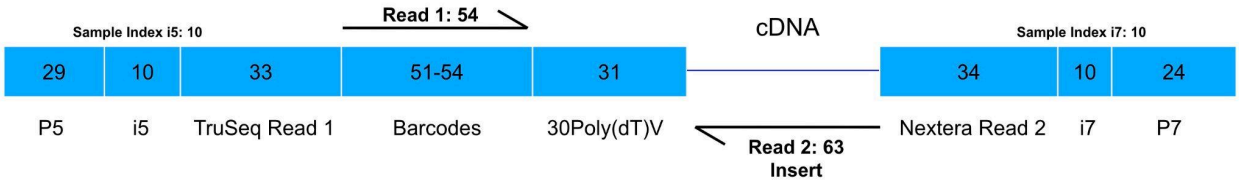

Figure 2. Structure of the PIPseq single-cell gene expression library for 4.0 and 4.0PLUS libraries prepared using the UDI-96 kit with 10 bp indices.

PIPseq version V: Read 1 length must be ≥ 45 bases and the recommended read 2 length ≥ 72 bases (Figure 3). It is recommended to maximize the use of the flow cell with longer reads when possible. **Please note that the index primers provided in the PIPseq V 3' Single Cell RNA Kit are identical to column 3 of the UDI-96 plate.** Users should be sure to use indexes from the other columns if using both index sets together for their pooled libraries.

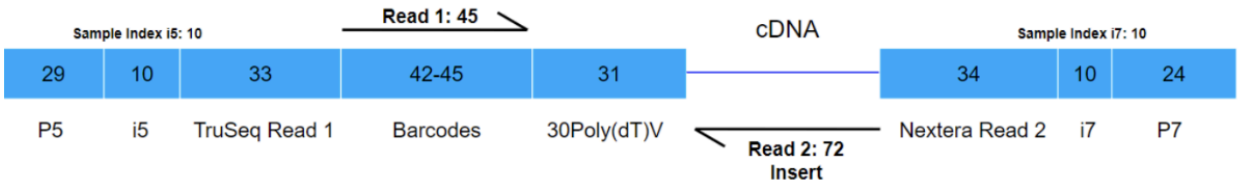

Figure 3. Structure of the PIPseq single-cell gene expression library for version V chemistry.

PIPseq UDI-96 Kit Protocol

Users should refer to the PIPseq kit-specific User Guide before using the PIPseq UDI-96 Kit primers for PIPseq library preparation. The protocol presented below is an example sample indexing PCR that should replace the corresponding section within the PIPseq kit-specific User Guide.

Reagent Preparation

Upon receipt of the PIPseq UDI-96 Kit, store it in a -20°C freezer.

Before each experiment, determine the number of indexes necessary for sample multiplexing (see Index Pooling Guidelines). To break apart a column (strip), remove the plate from the -20°C freezer, securely grip the desired strip and press along the perforation while pulling the strip away from the rest of the plate. Return the remainder of the plate to the -20°C freezer. Thaw the index strip for 10 min at room temperature before use.

Sample Index PCR (< 96 samples)

⚠ **Before starting the Sample Index PCR, choose the appropriate sample index sets to ensure that index combinations are color balanced and that no sample index combinations overlap in a multiplexed sequencing run (Index Pooling Guidelines for recommendations). There are 96 pre-mixed i5 and i7 indexes provided with this kit to allow for unique dual indexing of 96 samples.**

1. Determine the number of libraries that will be amplified and pooled for sequencing while ensuring that a valid index combination is chosen according to color balance guidelines in the Index Pooling Guidelines section below.
2. Thaw the selected number of index strips at room temperature for 10 minutes.
3. Mix briefly by vortexing and then centrifuge the index strip on a benchtop minifuge to collect all of the primers at the bottom of the well.
4. Thaw 4X PCR Master Mix (alternatively, (HC) Library Prep Mix B) on ice. Once thawed, flick the tubes several times, pipette mix 10 times, and then briefly centrifuge to collect (DO NOT VORTEX).
5. Orient the strip according to the provided plate map (Fig 1) to ensure the correct indexes will be removed. With a pipette tip, pierce the desired wells and transfer the volume of primer mix required for the PCR reaction (see step 6).

   ⚠ **CAUTION: Ensure pipette tips are not reused across wells to avoid cross-contamination of indexed primers. Do not reuse primer if the seal has been previously pierced to avoid cross-contamination of indexed primers.**

6. To the 32.5 µL of each PIPseq gene expression library (volume depends on PIPseq protocol), add the following reagents in the order in which they appear in the table(s) below. **CAUTION: Please review which kit you have purchased to confirm which table is appropriate**.

PIPseq V Protocol

Reagent	Volume Per Sample (µL)
Cleaned library DNA	32.5
UDI-XX (one well of UDI-96 plate)	5
4X PCR Master Mix	12.5
Total	**50**

PIPseq V4.0PLUS Protocol

Reagent	Volume Per Sample (µL)
Cleaned library DNA	32.5
UDI-XX (one well of UDI-96 plate)	5
HC Library Prep Mix B **(V4.0PLUS)**	12.5
Total	**50**

7. Mix the reactions by pipetting up and down 10 times at the 25 µL stroke, and briefly centrifuge to collect all liquid at the bottom of the tubes.
8. Proceed with the sample index PCR according to the specific PIPseq Kit specific User Guide.

Index Pooling Guidelines

The PIPseq 3′ Single Cell gene expression libraries may be pooled for sequencing, taking into account the differences in cell number and read depth requirements. Refer to Illumina documentation as the primary source for appropriate color balance combinations for the selected sequencing platform. **Fluent-validated index combinations are listed in the "Pooling Recommendations" section below.** These libraries are dual-indexed with 10-base i5 and i7 indexes. Sequencing depth will vary based on your application needs but it is recommended to start with a depth of 20,000 reads per input cell.

Users should note that PIPseq index sequences provided with v4.0PLUS 3' Single Cell RNA kits are dual-indexed with 8-base i5 and i7 indexes. This is distinct from the 10-base i5 and i7 indexes provided with PIPseq V 3' Single Cell RNA kits and the PIPseq UDI-96 Kit. Refer to the table below for the read length requirements when using the 10-base indexes provided in the UDI-96 kit. Do not use UDI-17 - UDI-24 from the UDI-96 kit when combining with libraries that were prepared using the indexes provided in the PIPseq V 3' Single Cell RNA kits.

PIPseq chemistry	Read 1 Length	Read 2 Length	i5/i7 index adapter length
v4.0PLUS	Min of 54	Min of 63	10
V	Min of 45	Min of 72	10

Users may download a sample sheet with the index sequences for use with Illumina Experiment Manager from the PIPseq UDI-96 Kit product page at www.fluentbio.com.

Well position	Index ID	i5 bases in adapter sequence	i7 bases in adapter sequence
A1	UDI-1	TCGTGGAGCG	CGCTCAGTTC
B1	UDI-2	CTACAAGATA	GAATTGAGTG
C1	UDI-3	TATAGTAGCT	ATATGAGACG
D1	UDI-4	TGCCTGGTGG	CTTATGGAAT
E1	UDI-5	ACATTATCCT	TAATCTCGTC
F1	UDI-6	GTCCACTTGT	GCGCGATGTT
G1	UDI-7	TGGAACAGTA	AGAGCACTAG
H1	UDI-8	CCTTGTTAAT	TGCCTTGATC
A2	UDI-9	GTTGATAGTG	CTACTCAGTC
B2	UDI-10	ACCAGCGACA	TTCTACAGAA
C2	UDI-11	CATACACTGT	GAACATACGG
D2	UDI-12	GTGTGGCGCT	CCTATGACTC
E2	UDI-13	ATCACGAAGG	TAATGGCAAG
F2	UDI-14	CGGCTCTACT	GTGCCGCTTC

Well position	Index ID	i5 bases in adapter sequence	i7 bases in adapter sequence
G2	UDI-15	GAATGCACGA	CGGCAATGGA
H2	UDI-16	AAGACTATAG	GCCGTAACCG
A3	UDI-17	TCGGCAGCAA	AACCATTCTC
B3	UDI-18	CTAATGATGG	TCCAATTCTA
C3	UDI-19	GGTTGCCTCT	CTAATGATGG
D3	UDI-20	CGCACATGGC	TCGGCCTATC
E3	UDI-21	GGCCTGTCCT	AGTCAACCAT
F3	UDI-22	CTGTGTTAGG	GAGCGCAATA
G3	UDI-23	TAAGGAACGT	AACAAGGCGT
H3	UDI-24	CTAACTGTAA	GTATGTAGAA
A4	UDI-25	GGCGAGATGG	TTCTATGGTT
B4	UDI-26	AATAGAGCAA	CCTCGCAACC
C4	UDI-27	TCAATCCATT	TGGATGCTTA
D4	UDI-28	TCGTATGCGG	ATGTCGTGGT
E4	UDI-29	TCCGACCTCG	AGAGTGCGGC
F4	UDI-30	CTTATGGAAT	TGCCTGGTGG
G4	UDI-31	GCTTACGGAC	TGCGTGTCAC
H4	UDI-32	GAACATACGG	CATACACTGT
A5	UDI-33	GTCGATTACA	CGTATAATCA
B5	UDI-34	ACTAGCCGTG	TACGCGGCTG
C5	UDI-35	AAGTTGGTGA	GCGAGTTACC
D5	UDI-36	TGGCAATATT	TACGGCCGGT
E5	UDI-37	GATCACCGCG	GTCGATTACA
F5	UDI-38	TACCATCCGT	CTGTCTGCAC
G5	UDI-39	GCTGTAGGAA	CAGCCGATTG
H5	UDI-40	CGCACTAATG	TGACTACATA
A6	UDI-41	GACAACTGAA	ATTGCCGAGT
B6	UDI-42	AGTGGTCAGG	GCCATTAGAC
C6	UDI-43	TTCTATGGTT	GGCGAGATGG
D6	UDI-44	AATCCGGCCA	TGGCTCGCAG
E6	UDI-45	CCATAAGGTT	TAGAATAACG
F6	UDI-46	ATCTCTACCA	CAGTAGTTGT
G6	UDI-47	CGGTGGCGAA	TATCCAGGAC
H6	UDI-48	TAACAATAGG	AGTGCCACTG

Well position	Index ID	i5 bases in adapter sequence	i7 bases in adapter sequence
A7	UDI-49	CTGGTACACG	GGCCATCATA
B7	UDI-50	TCAACGTGTA	ACATGGTGTC
C7	UDI-51	ACTGTTGTGA	GACAGACAGG
D7	UDI-52	GTGCGTCCTT	TCTTACATCA
E7	UDI-53	AGCACATCCT	TTACAATTCC
F7	UDI-54	TTCCGTCGCA	AAGCTTATGC
G7	UDI-55	CTTAACCACT	TATTCCTCAG
H7	UDI-56	GCCTCGGATA	CTCGTGCGTT
A8	UDI-57	CGTCGACTGG	TTAGGATAGA
B8	UDI-58	TACTAGTCAA	CCGAAGCGAG
C8	UDI-59	ATAGACCGTT	GGACCAACAG
D8	UDI-60	ACAGTTCCAG	TTCCAGGTAA
E8	UDI-61	AGGCATGTAG	TGATTAGCCA
F8	UDI-62	GCAAGTCTCA	TAACAGTGTT
G8	UDI-63	TTGGCTCCGC	ACCGCGCAAT
H8	UDI-64	AACTGATACT	GTTCGCGCCA
A9	UDI-65	GTAAGGCATA	AGACACATTA
B9	UDI-66	AATTGCTGCG	GCGTTGGTAT
C9	UDI-67	TTACAATTCC	GATAACAAGT
D9	UDI-68	AACCTAGCAC	TTGTTCCGTG
E9	UDI-69	TCTGTGTGGA	AAGTACTCCA
F9	UDI-70	GGAATTCCAA	ACGTCAATAC
G9	UDI-71	AAGCGCGCTT	GGTGTACAAG
H9	UDI-72	TGAGCGTTGT	CCACCTGTGT
A10	UDI-73	ATCATAGGCT	GTTCCGCAGG
B10	UDI-74	TGTTAGAAGG	ACCTTATGAA
C10	UDI-75	GATGGATGTA	CGCTGCAGAG
D10	UDI-76	ACGGCCGTCA	GTAGAGTCAG
E10	UDI-77	CGTTGCTTAC	GGATACCAGA
F10	UDI-78	TGACTACATA	CGCACTAATG
G10	UDI-79	CGGCCTCGTT	TCCTGACCGT
H10	UDI-80	CAAGCATCCG	CTGGCTTGCC
A11	UDI-81	TCGTCTGACT	ACCAGCGACA
B11	UDI-82	CTCATAGCGA	TTGTAACGGT

Well position	Index ID	i5 bases in adapter sequence	i7 bases in adapter sequence
C11	UDI-83	AGACACATTA	GTAAGGCATA
D11	UDI-84	GCGCGATGTT	GTCCACTTGT
E11	UDI-85	CATGAGTACT	TTAGGTACCA
F11	UDI-86	ACGTCAATAC	GGAATTCCAA
G11	UDI-87	GATACCTCCT	CATGTAGAGG
H11	UDI-88	ATCCGTAAGT	TACACGCTCC
A12	UDI-89	CGTGTATCTT	GCTTACGGAC
B12	UDI-90	GAACCATGAA	CGCTTGAAGT
C12	UDI-91	GGCCATCATA	CGCCTTCTGA
D12	UDI-92	ACATACTTCC	ATACCAACGC
E12	UDI-93	TATGTGCAAT	CTGGATATGT
F12	UDI-94	GATTAAGGTG	CAATCTATGA
G12	UDI-95	ATGTAGACAA	GGTGGAATAC
H12	UDI-96	CACATCGGTG	TGGACGGAGG

Pooling Recommendations

Fluent has validated *in silico* the following index combinations in the PIPseq UDI-96 Kit for color balance according to Illumina guidelines for the NextSeq2000 and the NovaSeqX. Not all combinations have been experimentally validated.

NextSeq 2000	
Plexity	Recommended well pairs
2	A1,B1 : C1,D1 : E1,F1 : G1,H1 (Adjacent well pairs from top)
	G2,H2
	C3,D3
	G3,H3
	A4,B4
	E4,F4
	G4,H4
	A5,B5
	C5,D5
	A6,B6
	C6,D6
	E6,F6
	A7,B7
	C7,D7

NextSeq 2000	
Plexity	Recommended well pairs
	G7,H7
	A8,B8
	E8,F8
	G8,H8
	A9,B9
	E9,F9
	G9,H9
	A10,B10
	C10,D10
	E10,F10
	A11,B11
	G11,H11
	A12,B12
	G12,H12
3	A1,B1,C1 : D1,E1,F1 : F1,G1,H1 (Adjacent wells from top)
	COLUMN 2 Adjacent wells from top
	COLUMN 3 Adjacent wells from top
	COLUMN 4 Adjacent wells from top
	A5,B5,C5
	F5,G5,H5
	COLUMN 6 Adjacent wells from top
	COLUMN 7 Adjacent wells from top
	COLUMN 8 Adjacent wells from top
	COLUMN 9 Adjacent wells from top
	COLUMN 10 Adjacent wells from top
	COLUMN 11 Adjacent wells from top
	COLUMN 12 Adjacent wells from top
4	The first 4 or last 4 wells in any column
5	The first 5 or last 5 wells in any column
6	The first 6 or last 6 wells in any column
7	The first 7 or last 7 wells in any column
8	The entire column, any column

NovaSeq X	
Plexity	Recommended well pairs
2	G12,H12
3	D2,E2,F2
3	D10,E10,F10
3	F12,G12,H12
4	E1,F1,G1,H1

NovaSeq X	
Plexity	Recommended well pairs
	A2,B2,C2,D2
	E2,F2,G2,H2
	A3,B3,C3,D3
	E3,F3,G3,H3
	A4,B4,C4,D4
	A5,B5,C5,D5
	E8,F8,G8,H8
	A9,B9,C9,D9
	E9,F9,G9,H9
	A10,B10,C10,D10
	E12,F12,G12,H12
5	The first 5 or last 5 wells in COLUMNS 1, 2 or 3
	A4 - E4
	The first 5 or last 5 wells in COLUMN 5
	A6 - E6
	A7 - E7
	The first 5 or last 5 wells in COLUMN 8, 9 or 10
	D12 - H12
6	The first 6 or last 6 wells in COLUMNS 1, 2 or 3
	A4 - F4
	The first 6 or last 6 wells in COLUMN 5
	A6 - F6
	The first 6 or last 6 wells in COLUMNS 7, 8, 9, 10, 11 or 12
8	The entire column, any column

Oligonucleotide Sequences

Name	Sequence (5′ - 3′)
UDI-## P7 Index	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
UDI-## P5 Index	AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXXACACTCTTTCCCTACACGACGC

Document Revision Summary

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General Changes:

- Updated Pooling recommendations and added PIPseq V compatibility

Legal Notices

Support

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