Technical Note

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Title:	Guideline for designing multiplexing fusion primers for Roche 454 GS FLX Titanium/Junior
Summary:	This document describes how to design fusion barcoded PCR primers for multiplexing amplicon sequencing by 454 Titanium and Junior.
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Disclaimer

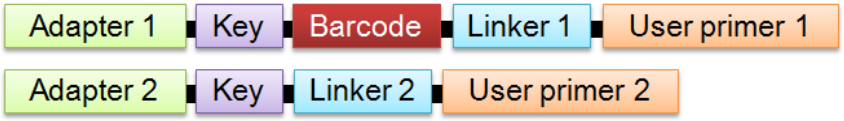
Guideline for designing multiplexing fusion primers for Roche 454 GS FLX Titanium/Junior

This document describes how to design amplification primers for ***uni-directional*** sequencing using Roche 454 GS FLX Titanium/Junior.

Fusion primers for 454 should contain the following sequences:

Oligonucleotide	Sequence	Comment
454 Adapter 1:	CCATCTCATCCCTGCGTGTCTCCGAC	Sequencing is carried out from this adapter
454 Adapter 2:	CCTATCCCCTGTGTGCCTTGGCAGTC	
Key sequence:	TCAG	

The general structure of fusion primer is as follows:



Note that sequencing is carried out only from the fusion primer 1. Fusion primer 2 is used for all of fusion primer 1 with different barcodes.

Considerations:

- Roche provides 10 nt long barcode for general amplicon sequencing. At Chunlab, Inc., we use the barcodes with variable lengths (7 to 11 nt). **In a single sequencing reaction, we recommend to mix barcodes with a variety of lengths to get the best amplicon sequencing results.**
- Always use them as an even mixture of different lengths. If you need 10 barcodes, use two 7, 8, 9, 10 and 11 nt long barcodes (2x5=10).
- When barcodes are selected, make sure that you do not generate any homopolymers (e.g. AA, CC, GG, TT). Since key sequence (TCAG) ends with 'G', barcode starting 'G' should not be selected.
- Linkers 1 and 2 should be designed NOT to match the template genomic sequences. This can be easily designed from multiple sequence alignment of your target gene which you want to amplify. Avoid homopolymer whiling designing linker.

Typical examples of mistakes (here, user primer is CTTACCGCGGCTGCTsGG).

Primer	What went wrong?
CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCTCTGTATG-AA-CTTACCGCGGCTGCTGG	Linker AA is a homopolymer sequence. Change the linker.
CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCTCTGTATC-CA-CTTACCGCGGCTGCTGG	Barcode AGCTCTGTATC and linker CA form a homopolymer sequence (i.e. CC). Use other barcode.
CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCTCTGTATG-GC-CTTACCGCGGCTGCTGG	Linker GC and user primer CTTACCGCGGCTGCTGG forms a homopolymer sequence (i.e. CC). Change the linker.

For sequencing your amplicons using Roche 454 and related bioinformatic analysis, please contact chunlab.com@gmail.com. Chunlab, Inc. provides comprehensive microbial community analysis solution. Please check our latest product and free trial event at http://www.chunlab.com/.

Q: How many multiplexing barcodes are suitable for my experiment?

A: It depends on the number of reads that you want to obtain for each sample. Generally, 454 Titanium can produce 500,000 reads (1/2 plate) or 240,000 reads (1/4 plate), and 454 Junior (whole plate) can generate 100,000 reads. Since combining >30 different (purified) PCR products in equal quantity (as moles) in a single 454 sequencing reaction is not an easy task, A 454 Titanium 1/4 plate or Junior run with 30 or less multiplexing barcodes can be a good choice. If 30 different barcodes are used, an average of 3,300 reads will be obtained per a 454 Junior run. After trimming low quality and chimeric reads, you may end up with average 2,500 reads per each sample. Further information and sequencing services, contact chunlab.com@gmail.com.

The followings are the lists of fusion primers used at Chunlab, Inc. for rRNA gene amplification and sequencing. Note that the barcodes used are 7-11 bp long whereas Roche's barcodes are 10 bp long. All primers listed below have barcode different each other, so you can mix bacterial, archaeal and eukaryal primers in the same sequencing batch.

Primers for Bacterial 16S rRNA genes

Fusion primer 2

B16S-F CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-GAGTTTGATCMTGGCTCAG

This primer is used with all other barcoded reverse primers (so, make it large amount of it).

Bif16S-F CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-GGGTTCGATTCTGGCTCAG

The primer B16S-F has mismatches to some *Bifidobacterium* 16S rRNA genes. If you want to make sure of the amplification of bifidobacterial 16S rRNA genes, you may use the mixture of B16S-F and Bif16S-F (9:1 ratio is recommended).

Fusion primer 1 (barcoded primers)

Name	Sequence
B16-7-1	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGTCAT-AC-WTTACCGCGGCTGCTGG
B16-7-4	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGAGCTG-AC-WTTACCGCGGCTGCTGG
B16-7-7	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCAGATG-AC-WTTACCGCGGCTGCTGG
B16-7-8	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGATGAG-AC-WTTACCGCGGCTGCTGG
B16-7-12	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCTGCAG-AC-WTTACCGCGGCTGCTGG
B16-7-13	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCGATG-AC-WTTACCGCGGCTGCTGG
B16-8-3	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATGCTGAG-AC-WTTACCGCGGCTGCTGG
B16-8-4	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TACAGCAG-AC-WTTACCGCGGCTGCTGG
B16-8-18	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATCGTGTG-AC-WTTACCGCGGCTGCTGG
B16-8-21	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTACACAG-AC-WTTACCGCGGCTGCTGG
B16-8-24	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TAGCTACG-AC-WTTACCGCGGCTGCTGG
B16-8-27	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGAGTAG-AC-WTTACCGCGGCTGCTGG
B16-9-4	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGTGTACTG-AC-WTTACCGCGGCTGCTGG
B16-9-5	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTGTCTACG-AC-WTTACCGCGGCTGCTGG
B16-9-8	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGTCACTAG-AC-WTTACCGCGGCTGCTGG
B16-9-12	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCTCACTG-AC-WTTACCGCGGCTGCTGG
B16-9-15	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATACGTACG-AC-WTTACCGCGGCTGCTGG
B16-9-21	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CACTATGTG-AC-WTTACCGCGGCTGCTGG
B16-10-1	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CACACGATAG-AC-WTTACCGCGGCTGCTGG
B16-10-2	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATGTGTCTAG-AC-WTTACCGCGGCTGCTGG
B16-10-3	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATGTACGATG-AC-WTTACCGCGGCTGCTGG
B16-10-4	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATCGTCTGTG-AC-WTTACCGCGGCTGCTGG
B16-10-5	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATCGTAGCAG-AC-WTTACCGCGGCTGCTGG
B16-10-6	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATCACGTGCG-AC-WTTACCGCGGCTGCTGG
B16-11-1	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCATATACGCG-AC-WTTACCGCGGCTGCTGG
B16-11-2	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TAGATAGTGCG-AC-WTTACCGCGGCTGCTGG
B16-11-3	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ACGTCTCTACG-AC-WTTACCGCGGCTGCTGG
B16-11-6	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATACACGAGCG-AC-WTTACCGCGGCTGCTGG
B16-11-23	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTCGATAGACG-AC-WTTACCGCGGCTGCTGG
B16-11-27	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGACAGTACAG-AC-WTTACCGCGGCTGCTGG

Primers are named as Gene prefix-barcode length-unique id (B16-7-4 means [bacterial 16S rRNA gene-7 nt long barcode-unique id=4]).

Primers for Archaeal 16S rRNA genes

Fusion primer 2

A16S-F CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AG-TCCGGTTGATCCYGCCGG

This primer is used with all other barcoded reverse primers (so, make it large amount of it).

Fusion primer 1 (barcoded primers)

Name	Sequence
A16-7-11	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCACAT-GA-GGTDTTACCGCGGCKGCTG
A16-7-18	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CAGAGCA-GA-GGTDTTACCGCGGCKGCTG
A16-7-19	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGTCAGA-GA-GGTDTTACCGCGGCKGCTG
A16-7-22	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CATGTCA-GA-GGTDTTACCGCGGCKGCTG
A16-7-25	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGATGCA-GA-GGTDTTACCGCGGCKGCTG
A16-7-29	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CAGCTGA-GA-GGTDTTACCGCGGCKGCTG
A16-8-1	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCATGCA-GA-GGTDTTACCGCGGCKGCTG
A16-8-15	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATCTGCGA-GA-GGTDTTACCGCGGCKGCTG
A16-8-20	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ACTGTCGA-GA-GGTDTTACCGCGGCKGCTG
A16-8-29	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCAGTGA-GA-GGTDTTACCGCGGCKGCTG
A16-8-31	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ACGAGCTA-GA-GGTDTTACCGCGGCKGCTG
A16-8-33	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CATAGCGA-GA-GGTDTTACCGCGGCKGCTG
A16-9-6	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CAGTCTCGA-GA-GGTDTTACCGCGGCKGCTG
A16-9-10	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ACGACAGTA-GA-GGTDTTACCGCGGCKGCTG
A16-9-13	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGTGCGATA-GA-GGTDTTACCGCGGCKGCTG
A16-9-18	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTGTCGCTA-GA-GGTDTTACCGCGGCKGCTG
A16-9-20	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TAGCGCTCA-GA-GGTDTTACCGCGGCKGCTG
A16-9-24	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCTAGCGA-GA-GGTDTTACCGCGGCKGCTG
A16-10-55	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGTCGTCGCA-GA-GGTDTTACCGCGGCKGCTG
A16-10-56	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGTCACACGA-GA-GGTDTTACCGCGGCKGCTG
A16-10-57	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGTAGTGTGA-GA-GGTDTTACCGCGGCKGCTG
A16-10-58	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGAGTCAGTA-GA-GGTDTTACCGCGGCKGCTG
A16-10-59	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCTGACGTCA-GA-GGTDTTACCGCGGCKGCTG
A16-10-60	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGCTGCGTA-GA-GGTDTTACCGCGGCKGCTG
A16-11-11	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCTGCGTGACA-GA-GGTDTTACCGCGGCKGCTG
A16-11-12	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ACACGATCTGA-GA-GGTDTTACCGCGGCKGCTG
A16-11-20	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGCTAGTGTA-GA-GGTDTTACCGCGGCKGCTG
A16-11-21	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCAGTACTGA-GA-GGTDTTACCGCGGCKGCTG
A16-11-22	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGCGTAGTCTA-GA-GGTDTTACCGCGGCKGCTG
A16-11-24	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGCTCGTAGTA-GA-GGTDTTACCGCGGCKGCTG

Primer for Eukaryotes (including fungi)

This primer set targets eukaryotic large subunit D1D2 region. It will amplify fungi and other eukaryotes.

Fusion primer 1

EL-F CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-TG-ACCCGCTGAAYTTAAGCATAT

This primer is used with all other barcoded reverse primers (so, make it large amount of it).

Fusion primer 2 (barcoded primers)

Name	Sequence
EL-7-32	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CATCAGT-GA-CTTGGTCCGTGTTTCAAGAC
EL-7-33	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATGCGCA-GA-CTTGGTCCGTGTTTCAAGAC
EL-7-34	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCTCTCT-GA-CTTGGTCCGTGTTTCAAGAC
EL-7-35	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTCATGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-7-45	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGACGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-7-46	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CAGAGAT-GA-CTTGGTCCGTGTTTCAAGAC
EL-8-37	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGTGTGTA-GA-CTTGGTCCGTGTTTCAAGAC
EL-8-38	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGCGATA-GA-CTTGGTCCGTGTTTCAAGAC
EL-8-39	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCGTGTA-GA-CTTGGTCCGTGTTTCAAGAC
EL-8-40	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATCGCACA-GA-CTTGGTCCGTGTTTCAAGAC
EL-8-59	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGCAGTCA-GA-CTTGGTCCGTGTTTCAAGAC
EL-8-64	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTAGTCGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-9-31	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCGACACA-GA-CTTGGTCCGTGTTTCAAGAC
EL-9-40	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CACTCACTA-GA-CTTGGTCCGTGTTTCAAGAC
EL-9-41	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TACTCGACA-GA-CTTGGTCCGTGTTTCAAGAC
EL-9-45	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ATGACACGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-9-58	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCGTAGTA-GA-CTTGGTCCGTGTTTCAAGAC
EL-9-59	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-ACACGTGTA-GA-CTTGGTCCGTGTTTCAAGAC
EL-10-61	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCGATAGTGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-10-62	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TCACGCGAGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-10-63	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TATGCTAGTA-GA-CTTGGTCCGTGTTTCAAGAC
EL-10-64	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TATATATACA-GA-CTTGGTCCGTGTTTCAAGAC
EL-10-65	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TAGTCGCATA-GA-CTTGGTCCGTGTTTCAAGAC
EL-10-66	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TACGTCATCA-GA-CTTGGTCCGTGTTTCAAGAC
EL-11-26	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCGTATAGCA-GA-CTTGGTCCGTGTTTCAAGAC
EL-11-38	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-AGAGAGCGACA-GA-CTTGGTCCGTGTTTCAAGAC
EL-11-39	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TACGTAGTGCA-GA-CTTGGTCCGTGTTTCAAGAC
EL-11-41	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-TGCGTCTCAGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-11-45	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CGCATCTATGA-GA-CTTGGTCCGTGTTTCAAGAC
EL-11-53	CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-CTCGTGCACTA-GA-CTTGGTCCGTGTTTCAAGAC