

Amplicon Deep Sequencing on the Illumina MiSeq/NextSeq Platform

Detect introduced or expected mutations
Discover rare genomic variations with high confidence

Introduction

Amplicon deep sequencing using next generation sequencing (NGS) technologies has become a powerful tool to study a wide variety of research questions. Typical applications include (i) CRISPR

genome editing protocols of eukaryotes, (ii) genome-wide transposon insertion analysis in microorganisms, (iii) human leukocyte antigen (HLA) typing and (iv) screening of specific somatic muta-

tions in tumor tissues. Common to all these approaches is that a PCR is used to amplify fragments, which are then sequenced on a single-molecule level.

The Two-Step PCR Approach – How it Works

The two-step PCR approach combined with Illumina's dual indexing strategy allows to process up to 384 samples in parallel (see **Figure 1**).

The first-step PCR uses primers containing a locus-specific sequence as well as a universal 5' tail as specified in the Nextera library protocol from Illumina (see **Table 1**). Instead of using only one single forward and reverse primer, some protocols make use of up to 3 forward primers that differ in length by adding wobble bases (Ns) between the locus-specific and common 5' tail. This might be especially useful in high-throughput projects where the sequencing throughput is especially critical and many samples are pooled. However, for most projects the sequencing throughput is high enough by simply using one forward and one reverse primer. If you need further background about this particular topic, please contact us.

The resulting PCR amplicons are then used as templates within the second-step PCR for further amplification, but also to

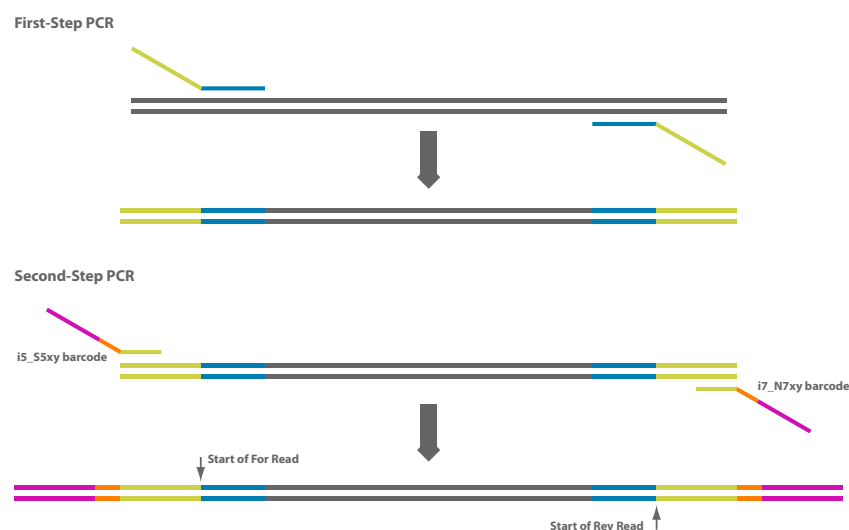


Figure 1. Overview of the double indexing strategy used in the Illumina two-step protocol. During the Illumina sequencing step the amplified genomic sequence including the specific primers (grey and blue bars) as well as the forward and reverse barcodes (orange bar) are read out.

include the indexes (barcodes) as well as the Illumina adaptors. The Illumina indexing strategy for the second-step PCR consists of 16 forward primers and 24 reverse primers. The combinatorial

use of these primers (16 x 24) defines the maximal number of 384 samples which can be pooled and sequenced on one Illumina MiSeq/NextSeq run.

Table 1. 5' tails used for the first-step PCR. Oligonucleotide sequences © 2007-2013 Illumina, Inc. All rights reserved.

Primer Name	Sequence (5'-3')
1st_PCR_for	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence]
1st_PCR_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]

Table 2. Indexed forward primers for the second-step PCR. Oligonucleotide sequences © 2007-2013 Illumina, Inc. All rights reserved.

Primer Name	Sequence (5'-3')	Index Name	Index Sequence
NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACAC CTCTCTATT CGTCGGCAGCGTC	S502 (set A/B)	CTCTCTAT
NGS_i5_S503	AATGATACGGCGACCACCGAGATCTACAC TATCCTCTT CGTCGGCAGCGTC	S503 (set A/B)	TATCCTCT
NGS_i5_S505	AATGATACGGCGACCACCGAGATCTACAC GTAAGGAGT CGTCGGCAGCGTC	S505 (set A/B)	GTAAGGAG
NGS_i5_S506	AATGATACGGCGACCACCGAGATCTACAC ACTGCATAT CGTCGGCAGCGTC	S506 (set A/B)	ACTGCATA
NGS_i5_S507	AATGATACGGCGACCACCGAGATCTACAC AAGGAGTAT CGTCGGCAGCGTC	S507 (set A/B)	AAGGAGTA
NGS_i5_S508	AATGATACGGCGACCACCGAGATCTACAC CTAAGCCTT CGTCGGCAGCGTC	S508 (set A/B)	CTAAGCCT
NGS_i5_S510	AATGATACGGCGACCACCGAGATCTACAC CGTCTAATT CGTCGGCAGCGTC	S510 (set A/B)	CGTCTAAT
NGS_i5_S511	AATGATACGGCGACCACCGAGATCTACAC TCTCTCCGT CGTCGGCAGCGTC	S511 (set A/B)	TCTCTCCG
NGS_i5_S513	AATGATACGGCGACCACCGAGATCTACAC TCGACTAGT CGTCGGCAGCGTC	S513 (set C/D)	TCGACTAG
NGS_i5_S515	AATGATACGGCGACCACCGAGATCTACAC TTCTAGCTT CGTCGGCAGCGTC	S515 (set C/D)	TTCTAGCT
NGS_i5_S516	AATGATACGGCGACCACCGAGATCTACAC CCTAGAGTT CGTCGGCAGCGTC	S516 (set C/D)	CCTAGAGT
NGS_i5_S517	AATGATACGGCGACCACCGAGATCTACAC CGGTAAGAT CGTCGGCAGCGTC	S517 (set C/D)	GCGTAAGA
NGS_i5_S518	AATGATACGGCGACCACCGAGATCTACAC CTATTAAGT CGTCGGCAGCGTC	S518 (set C/D)	CTATTAAG
NGS_i5_S520	AATGATACGGCGACCACCGAGATCTACAC AAGGCTATT CGTCGGCAGCGTC	S520 (set C/D)	AAGGCTAT
NGS_i5_S521	AATGATACGGCGACCACCGAGATCTACAC GAGCCTTAT CGTCGGCAGCGTC	S521 (set C/D)	GAGCCTTA
NGS_i5_S522	AATGATACGGCGACCACCGAGATCTACAC TTATGCGAT CGTCGGCAGCGTC	S522 (set C/D)	TTATGCGA

Table 3. Indexed reverse primers for the second-step PCR. Oligonucleotide sequences © 2007-2013 Illumina, Inc. All rights reserved.

Primer Name	Sequence (5'-3')	Index Name	Index Sequence
NGS_i7_N701	CAAGCAGAAGACGGCATAACGAGAT TCGCCTTAGT TCTCGTGGGCTCGG	N701 (set A/C)	TCGCCTTA
NGS_i7_N702	CAAGCAGAAGACGGCATAACGAGAT CTAGTACGGT TCTCGTGGGCTCGG	N702 (set A/C)	CTAGTACG
NGS_i7_N703	CAAGCAGAAGACGGCATAACGAGAT TTCTGCCTT GTCTCGTGGGCTCGG	N703 (set A/C)	TTCTGCCT
NGS_i7_N704	CAAGCAGAAGACGGCATAACGAGAT GCTCAGGAGT TCTCGTGGGCTCGG	N704 (set A/C)	GCTCAGGA
NGS_i7_N705	CAAGCAGAAGACGGCATAACGAGAT AGGAGTCCGT TCTCGTGGGCTCGG	N705 (set A/C)	AGGAGTCC
NGS_i7_N706	CAAGCAGAAGACGGCATAACGAGAT CATGCCTAGT TCTCGTGGGCTCGG	N706 (set A/C)	CATGCCTA
NGS_i7_N707	CAAGCAGAAGACGGCATAACGAGAT GTAGAGAGT TCTCGTGGGCTCGG	N707 (set A/C)	GTAGAGAG
NGS_i7_N710	CAAGCAGAAGACGGCATAACGAGAT CAGCCTCGT TCTCGTGGGCTCGG	N710 (set A/C)	CAGCCTCG
NGS_i7_N711	CAAGCAGAAGACGGCATAACGAGAT TGCCTCTT GTCTCGTGGGCTCGG	N711 (set A/C)	TGCCTCTT
NGS_i7_N712	CAAGCAGAAGACGGCATAACGAGAT TCCTCTACGT TCTCGTGGGCTCGG	N712 (set A/C)	TCCTCTAC
NGS_i7_N714	CAAGCAGAAGACGGCATAACGAGAT TCATGAGCGT TCTCGTGGGCTCGG	N714 (set A/C)	TCATGAGC
NGS_i7_N715	CAAGCAGAAGACGGCATAACGAGAT CCTGAGATG TCTCGTGGGCTCGG	N715 (set A/C)	CCTGAGAT
NGS_i7_N716	CAAGCAGAAGACGGCATAACGAGAT TAGCGAGT TCTCGTGGGCTCGG	N716 (set B/D)	TAGCGAGT
NGS_i7_N718	CAAGCAGAAGACGGCATAACGAGAT GTAGTCCGT TCTCGTGGGCTCGG	N718 (set B/D)	GTAGTCC
NGS_i7_N719	CAAGCAGAAGACGGCATAACGAGAT TACTACGCGT TCTCGTGGGCTCGG	N719 (set B/D)	TACTACGC
NGS_i7_N720	CAAGCAGAAGACGGCATAACGAGAT AGGCTCCGT TCTCGTGGGCTCGG	N720 (set B/D)	AGGCTCCG
NGS_i7_N721	CAAGCAGAAGACGGCATAACGAGAT GCAGCGTAGT TCTCGTGGGCTCGG	N721 (set B/D)	GCAGCGTA
NGS_i7_N722	CAAGCAGAAGACGGCATAACGAGAT CTGCGCATG TCTCGTGGGCTCGG	N722 (set B/D)	CTGCGCAT
NGS_i7_N723	CAAGCAGAAGACGGCATAACGAGAT GAGCGTAGT TCTCGTGGGCTCGG	N723 (set B/D)	GAGCGCTA
NGS_i7_N724	CAAGCAGAAGACGGCATAACGAGAT CGCTCAGT TCTCGTGGGCTCGG	N724 (set B/D)	CGCTCAGT
NGS_i7_N726	CAAGCAGAAGACGGCATAACGAGAT GTCTTAGGT TCTCGTGGGCTCGG	N726 (set B/D)	GTCTTAGG
NGS_i7_N727	CAAGCAGAAGACGGCATAACGAGAT ACTGATCGT TCTCGTGGGCTCGG	N727 (set B/D)	ACTGATCG
NGS_i7_N728	CAAGCAGAAGACGGCATAACGAGAT TAGCTGCA TCTCGTGGGCTCGG	N728 (set B/D)	TAGCTGCA
NGS_i7_N729	CAAGCAGAAGACGGCATAACGAGAT GACGTCGAGT TCTCGTGGGCTCGG	N729 (set B/D)	GACGTCGA

How to Order the NGS Primers?

First, download the specific "OrderForm_IlluminaAmpliconDeepSeq" from Microsynth's website (see Amplicon Deep Sequencing within the NGS menu). Next, specify the locus-specific sequences for your first-step PCR primers, and then

select your desired indexed forward and reverse primers. Send the upload sheet to info@microsynth.ch and request your specific offer. Alternatively, directly order the oligos in our webshop using the prefix "NGS_" in the 0.2 µmol scale, HPLC purified.

Example of Primer Pipetting Scheme for 96 Samples (8 x 12 indexes)

Table 4. Pipetting scheme for barcoding 96 samples using 8 i5 indexes (vertical) and 12 i7 indexes (horizontal). Using the 16 x 24 described indexes of Illumina it is also possible to unequivocally identify up to 384 samples.

	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502	S502
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503	S503
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505	S505
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506	S506
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507	S507
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508	S508
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510	S510
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511	S511
	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715

PCR Design Considerations

In general, the first-step PCR is a standard PCR using a proofreading polymerase and 5' tailed PCR primers. The only point to consider is the length (up to 500 bp) of the amplified product including the

locus-specific parts of the forward and reverse primers. If the sequence of the entire amplicon is of interest, the Illumina forward and reverse reads may be merged to reconstruct the full length

molecule. Single-end run configurations are also possible depending on your specific question. Please contact us to discuss your options.

Microsynth Competences and Services

One of Microsynth's core skills is in the field of amplicon deep sequencing. Microsynth is able to offer its customers a non-stop service covering the entire process from experimental design planning, DNA isolation, PCR amplification and sequencing up to bioinformatics analysis of the generated data for typical deep sequencing projects (see **Figure 2**).

DNA Isolation: Either the customer provides isolated DNA or outsources this step to Microsynth (>13 years of experience in DNA/RNA isolation from various and demanding matrices).

PCR Amplification: The PCR amplifica-

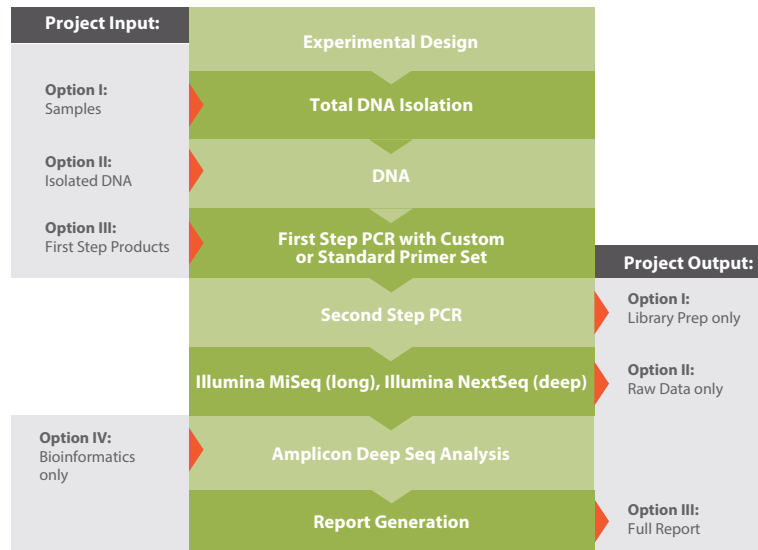
tion will use a state-of-the-art high-fidelity polymerase resulting in high-quality multiplex amplicon libraries. The customer either provides the DNA, the first-step PCR products, or the second-step products. The advantage of providing the first-step PCR products is that you will only need two primers per locus and do not need to order any indexed primers. PCR products are purified, quantified with fluorescence spectroscopy and pooled in equimolar amounts.

NGS Sequencing: Sequencing is done using Illumina MiSeq/NextSeq sequencing technology. Both technologies allow

high-throughput profiling at low costs, and the MiSeq has the additional advantage of long reads.

Bioinformatics Analyses: Depending on customer requirements, Microsynth can offer customized analysis of the data including demultiplexing, QC of the sequence data, paired-end read merging, mapping and identification of InDels and SNVs and analysis of their likelihood. For gene editing specific analysis (e.g. CRISPR/Cas), please also consult the gene editing application note.

Figure 2. Typical steps in a amplicon deep-sequencing project. Depending on researcher needs, Microsynth can deliver a one-stop service encompassing DNA isolation, PCR amplification and sequencing, and customized data analysis.



Example Outputs for an Amplicon Deep-Sequencing Project

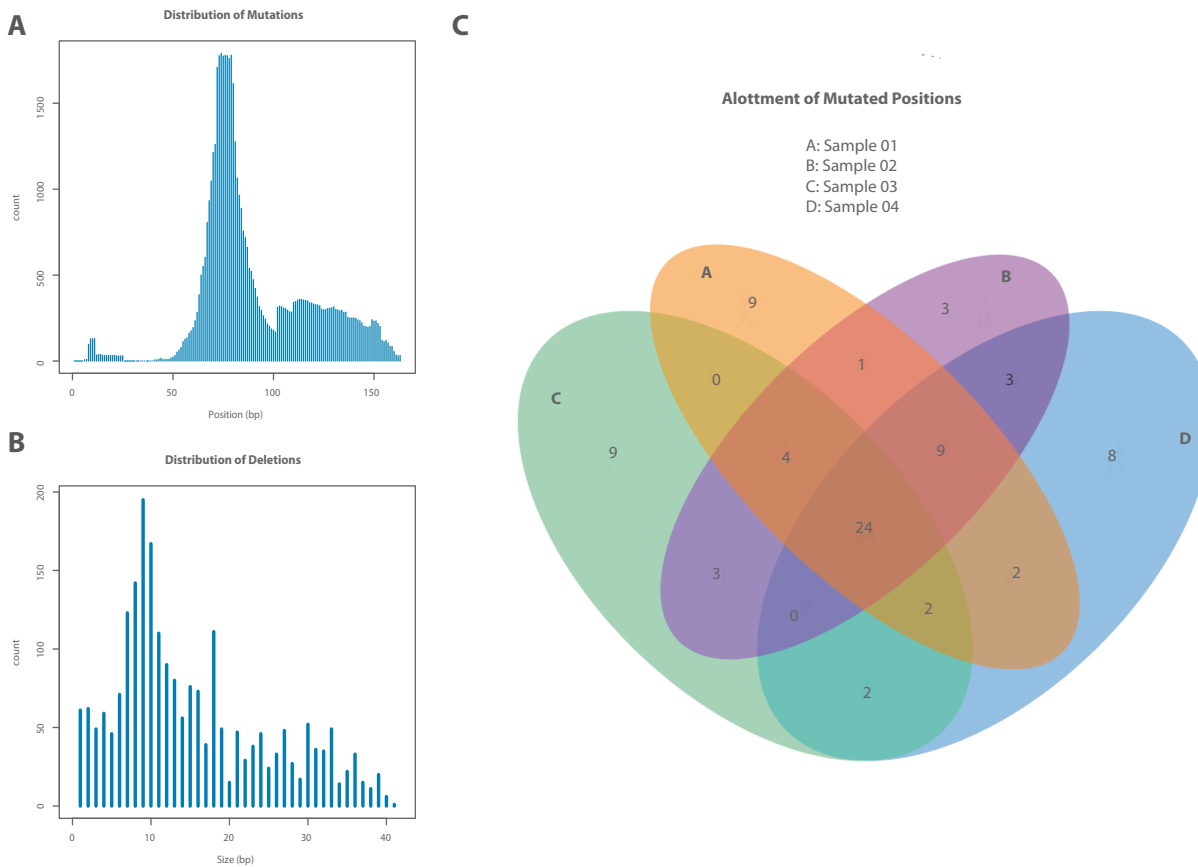


Figure 3. Example output of a deep-sequencing project. Sequencing reads of individual samples are aligned to the reference, and the distribution of SNPs (3A) and Indels (3B) are reported. In addition, Venn diagrams are displayed to visualize the number of mutations shared by the different samples included in the analysis (3C).