Our goal is to fragment the genome, amplify those fragments and then sequence them. For Illumina sequencing we want fragments in the 200-500bp range. We need to make sure the fragments have the correct Illumina sequencing primers on each end (note there are different primers for single-end and paired-end sequencing).

1. Digest doublestranded DNA with restriction enzymes. We use SPHI-HF and MluCl

NNN-NNN represents genomic DNA of unknown length

```
5' GCATGCNNNNNN-NNNNNNAATT 3'
3' CGTACGNNNNNN-NNNNNNTTAA 5'
```

```
5'      CNNNNN-NNNNNN      3'
3'  GTACGNNNNN-NNNNNNTTAA 5'
```

Example fragment of genomic DNA after digestion

2. Ligate adaptors to fragments of genomic DNA. Adaptors are designed so that (i) they can ligate to the cutsite, (ii) primers we design can be used to amplify fragments in PCR and add an index on the right side, and (iii) the illumina primers can match the adaptors during sequencing. We make adaptors by ordering the oligos and annealing them together.
With a barcode on the left side and an index on the right side, you can pool samples from many individuals.

Example "left" adaptor 1 | Fragment | Example "right" adaptor 2

mistake here missing C

Primer site for PCR and Illumina seqencing >>> | Cutsite SPHI-HF | Cutsite MluCl | Divergent Y

```
5'    CTCTTTCCCTACACGACGCTCTTCCGATCTAACGCCAACCATG  CNNN-NNNN  AATTCGAGATCGGAAGAGC GAGAACAA 3'
3' TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGATTGCGGTTG  GTACGNNN-NNNNTTAA  GGCTCTAGCCTTCTCGTGTGCACACTTGAGGTCAGTG 5'
```

Barcode and Protector base | Protector base | <<< Primer site for PCR and Illumina seqencing

3. After size selection (300-400bp), amplify fragments with Illumina PCR Primers, note that the left primer (Illpcr1) is not specific to the barcode, but the right primer is specific to the index that you want the sample to have. The Divergent Y functions to make sure the first PCR step only synthesizes the sense (top) strand using the antisense (bottom) strand as a template (fragment shown after 2nd round of PCR).

PCR primer 1 adds on bases needed for Illumina machine (Illpcr1)

```
5'A*A*TGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'>>>
                          5'    CTCTTTCCCTACACGACGCTCTTCCGATCTAACGCCAACCATGCNNN-NNNAATTCCGAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3'
                          3' TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGATTGCGGTTGGTACGNNN-NNNTTAAGGCTCTAGCCTTCTCGTGTGCACACTTGAGGTCAGTG 5'
                                                                                <<< 3' CGTGTGCACACTTGAGGTCAGTGTAGTGCTAGAGCATACGGCAGAAGACGAAC 5'
```

Index part of PCR primer

PCR primer 2 adds on index and bases needed for Illumina machine (Illpcr1)

4. Example fragment after PCR is ready for paired-end Illumina sequencing with multiplexing.

Illumina Read 1 sequencing primer | Illumina Index Read Sequencing Primer will read up to 7(?) base index after read 1

```
                          5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3' >>>          5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC 3' >>>
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTAACGCCAACCATGCNNN-NNNAATTCCGAGATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG 3'
3' TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGATTGCGGTTGGTACGNNN-NNNTTAAGGCTCTAGCCTTCTCGTGTGCACACTTGAGGTCAGTGTAGTGCTAGAGCATACGGCAGAAGACGAAC 5'
                                                                                <<<<3' TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG 5'
```

Illumina Read 2 Sequencing primer