A) SphI-HF and MspI/HpaII

```
5' GCATG^CNNNNNN-NNNNNNC^CGG 3'
3' C^GTACGNNNNNN-NNNNNNGGC^C 5'

Fragment after digestion:
5'       CNNNNN-NNNNNNC     3'
3'  GTACGNNNNNN-NNNNNNGGC   5'
```

Ligation (P5 on left and P7 on right):

```
5' ACACTCTTTCCCTACACGACGCTCTTCCGATCTBARCODINSCATG  CNNN-NNNNC  CGINCCIINNNNAGATCGGAAGAGCGAGAACAA 3'
3' TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGAOCRABDSNI  GTACGNNN-NNNNGGC  NIGGMHNNNNTCTAGCCTTCTCGTGTGCACACTTGAGGTCAGTG 5'
```

The insertion site (0-3bp) functions to offset and increase sequence variability to preserve the lasers of the sequencer from blinding by the identical bases in the restriction sites.

(M) A or C
(H) A, C, or T
(N) A, C, T, or G
(I) 2'-deoxyinosine (bonds C, A, T, then G)

For P5: Make sure barcode or insertion site (whichever is last) does not end with a "G" or restriction site will be re-created.
For P7: Make sure barcode or insertion site (whichever is last) does not begin with "G" or restriction site will be re-created.

BARCOD is the 6bp barcode

NNNNNIIICC (from Schweyen paper) On the top strand five Ns are located next to three 2'-deoxyinosine (I), which can pair with any of the four DNA bases, preferring, in decreasing order, C, A, and T (Watkins and SantaLucia, 2005), followed by two C-residues: 5' NNNNNIIICC 3'. The design of this fragment is expected to minimize mispairings of the top and the bottom strands during hybridization. Hybridization of both strands was performed according to Peterson *et al.* (2012), with the exception of the temperature profile (97 °C for 5 min followed by a decrease in temperature of 2 °C per min in a 1.5-ml Eppendorf tube in a thermal block.

PCR:

```
5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT 3'>>
                          5' ACACTCTTTCCCTACACGACGCTCTTCCGATCTBARCOINSCATG  CNNN-NNNNC  CGINCCIINNNNAGATCGGAAGAGCGAGAACAA 3'          "DIVERGENT Y"
                          3' TGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGAOCRABINS  GTACGNNN-NNNNGGC  NIGGMHNNNNTCTAGCCTTCTCGTGTGCACACTTGAGGTCAGTG 5'
                                                                                                <<3' CGTGTGCAGACTTGAGGTCAGTGXXEDNIAGAGCATACGGCAGAAGACGAAC 5'


          Illum. Read-1 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3' >>>                          5' GATCGGAAGAGCACACGTCTGAACTCCAGTCAC >> Illumina index read after read 1
5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTBARCOINSCATGCNNN-NNNCCGINCCIINNNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCACINDEXXTCTCGTATGCCGTCTTCTGCTTG 3'
3' TTACTATGCCGCTGGTGGCTCTAGATGTGAGAAAGGGATGTGCTGCGAGAAGGCTAGAOCRABINSGTACGNNN-NNNGGCNIGGMHNNNNTCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTGXXEDNIAGAGCATACGGCAGAAGACGAAC 5'
                                                                  <<<<3' TCTAGCCTTCTCGTGTGCAGACTTGAGGTCAGTG 5' Illumina Read 2
```

OLIGOS

P7 PCR primers

	Peterson	
Generic	CAAGCAGAAGACGGCATACGAGATINDEXXGTGACTGGAGTTCAGACGTGTG*C	12 of these

P7 adapters

After annealing separately, these adapters will be applied to each individual at random.

```
CGCCIIINNNNAGATCGGAAGAGCGAGAACAA                    GTGACTGGAGTTCACACGTGTGCTCTTCCGATCTNNNNHMMGG
CGCCIIINNNNCAGATCGGAAGAGCGAGAACAA                   GTGACTGGAGTTCACACGTGTGCTCTTCCGATCTGNNNNHMMGG
CGCCIIINNNNATAGATCGGAAGAGCGAGAACAA                  GTGACTGGAGTTCACACGTGTGCTCTTCCGATCTANNNNHMMGG
```

(M) A or C
(H) A, C, or T
(N) A, C, T, or G
(I) 2'-deoxyinosine

P5 primer

P5_primer_PCR1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	1 of these

P5 adapters

Generic	ACACTCTTTCCCTACACGACGCTCTTCCGATCTBARCOINSCAT*G	16 of these
Generic reverse complement	INSOCRABAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT	