Protocol: Tn5-on-beads DNA tagmentation

Version: v1.3, June 2020

By: Marek Kucka, Chan Lab, Friedrich Miescher Laboratory, Max Planck Campus Tübingen

Contact: mkucka@tuebingen.mpg.de; frank.chan@tue.mpg.de; http://fml.tuebingen.mpg.de/chan-group.html

Coupling of assembled Tn5 with magnetic beads.

Beads: 5ml of NEB, S1421, Hydrophilic Streptavidin Magnetic Beads

- Move 5 ml (or less/more) of NEB-beads from the bottle into a 50 ml falcon tube (shake the beads well before to re-suspend). Use a 15 ml falcon if using 2ml or less of beads, then put on magnetic stand and remove the bead-storage buffer.
- Add 20 ml of *streptavidin binding buffer*, mix to re-suspend the beads, put on magnet and remove the buffer.
- Add 30 ml of *streptavidin binding buffer* and the calculated amount of assembled Tn5.

Depending on the strength of the current batch of Tn5, e.g. I used 0.45 µl Tn5 per 5 µl NEB-beads => 450 µl Tn5 in total for 5ml of NEB-beads in 30 ml of streptavidin binding buffer).

- Close the tube and immediately mix by inverting and let rotate the tube at 10 rpm at RT for 30 minutes.
- Put on magnet for 2-4 min and decant/remove the buffer.
- Add 30 ml of *Dialysis buffer* and rotate at 10 rpm at RT for 5 min.
- Put on magnet and remove buffer.
- Gently re-suspend the Tn5-on-beads in a desired amount of *Dialysis buffer*. I usually store the Tn5-on-beads in **4-times the original volume of NEB-bead** => 20 ml of dialysis buffer for every 5 ml of beads.
  - ➔ 10 µl of Tn5-on-beads in dialysis buffer equals to 2.5 ul of original NEB-beads
  - ➔ 20 µl equals to 5 ul of original NEB-beads.

Notes: For an easier resuspension of the beads, store the beads in a small bottle (e.g. glass 50 or 100 ml bottle), not a falcon tube. Store at 4C.

***5 ml of original NEB beads** => enough for 1000 samples when using 20ul of Tn5-on-beads per sample, or 2000 samples (if using 10 ul Tn5-on-bedas per sample).*

***5ul of Tn5-0n-beads** cuts (tagments) maximum of approximately 8 ng of your input DNA (the rest will be discarded during washes).*

Biotinylated-oligos used to assemble Tn5:

```
Biot-25xT-Tn5ME-A          /5BiosG/TTTTTTTTTTTTTTTTTTTTTTTTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG '3
Tn5ME-rev                                                   /5Phos/CTGTCTCTTATACACATCT '3

Biot-24xT-Tn5ME-B          /5BiosG/TTTTTTTTTTTTTTTTTTTTTTTGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG '3
 Tn5ME-rev                                                  /5Phos/CTGTCTCTTATACACATCT '3
```

DNA Tagmentation with Tn5-on-beads

Transfer 20 µl or 10 µl of the Stock Tn5-on-beads per sample *(20 µl = 5 µl of NEB beads; 10 µl equals to 2.5 µl of original NEB beads) into a 1.5 ml Eppendorf tube on magnet*

- *for a 96 samples experiment, you need 1 ml or 2 ml of Tn5-on-beads, then exchange the storage buffer for* ***1 ml of WASH buffer****. Keep on ice.*

Note: Pre-warm the plate shaker and PCR-thermocycler to 55 ºC.

Pipette 20 µl of tagmentation buffer per sample in a 96-well plate, then add DNA (1-4µl) and only then add 10 ul of Tn5-on-beads in Wash buffer:

1st: pipette 20 µl of **tagmentation buffer** in each well, per sample*:* ***14 µl H2O***
6 µl 5xTAPS-DMF-MgCl2 buffer

2nd: pipette 1-4 µl of gDNA to each well (but at least 5 ng per sample)
3rd: pipette 10 µl of Tn5-on-beads (in WASH buffer)

Total = 30-34 µl

Seal and m**ix the plate in a plate shaker (>1500rpm), 30 sec, then incubate in PCR-thermocycler for 10 minutes at 55ºC, *then hold at 10ºC.***

Tip: put the plate on magnetic stand for 2 seconds and then straight into the plate-shaker if the beads have settled already and are difficult to re-suspend just by shaking in plate-shaker.

Note: shake the plate with at least 1500 rpm for 30 second (or by pipetting, or inverting the strip tubes if fewer samples) until the beads are evenly re-suspended.

OPTIONAL: mix in a plate shaker again after 5 minutes incubation to re-suspend the beads, and then continue the incubation at 55ºC.

Stripping of the Tn5 from the DNA

Add 20 µl of "**0.6% SDS- Wash**" buffer to each sample.
Seal the plate and mix in the plate shaker or by inverting the tubes to re-suspend the beads.
Incubate at **55ºC for 10 minutes**.

Put plate on magnet, remove supernatant.
Add 150 µl WASH buffer to the beads, incubate at RT for 1 minute, and then remove the wash buffer
Add 100 µl WASH buffer.
Keep in wash buffer until ready to pipet Q5 PCR-mix.

PCR amplification and adapter extension.

- *Note: Pre-warm the plate shaker and PCR-thermocycler to 72 ºC.*

- Prepare PCR-mix with the same N50X for all samples of a plate (note: 96-well plate = almost 2.4 ml of PCR mix!):

24 µl of PCR Mix per one sample:	
17 µl	H2O
5 µl	5x Q5 buffer
0.5 µl	10 mM dNTP
1.5 µl	10 uM N50X nextera primer
0.25 µl	Q5 polymerase

- Remove WASH buffer from the tagmented-DNA-on-beads and place the plate/tubes on ice.
- **Pipette 24 µl of PCR Mix into each well.**

- With a multichannel pipette, **pipette 1.5 µl of 10 uM N70(1-96) nextera primer** *(each sample gets different N70X barcoded primer from your barcoded 96-well-N70X-primer plate)*

=> PCR volume = 25.5 ul

Mix the plate in plate-mixer preheated to 72ºC or by inverting the tubes, and place in a preheated PCR-cycler.

Tip: put the plate on magnetic stand for 2 seconds and then straight into the plate-shaker if the beads have settled already and are difficult to re-suspend just by shaking in plate-shaker.

Important: The 1st step, 10 min at 72ºC is absolutely crucial so that the polymerase fills-in the holes made by Tn5. Never heat-denaturate tagmented samples before this step!

1. 72ºC….10 minutes (this fills in 9bp holes made by transposase) (note: **NEVER use HOT-start** polymerase)
2. 98ºC….30 sec
----
4. 98ºC… 15 sec
5. 65ºC… 20 sec
6. 72ºC… 60 sec
----
7. Go to 4 8x (= 9 cycles in total)

Note: At the end of 10 min 72 ºC incubation, pause the cycler and mix the plate in plate-mixer one more time, then un-pause the cycler and continue the protocol.

Spin the plate after PCR to collect all the liquid.

After PCR run 5 µl of several samples on 1.5% agarose gel to see if tagmentation/PCR worked and the size-distribution of the libraries, then add 5 µl of 10mM Tris Buffer back into these samples to make the volume 25 µl again.

Clean-up and size select each sample (see next page) with magnetic beads to get 350-600 bp library -> then pool 5 or 10 ng of each library into the Final Library Pool. Then do one last clean-up with 1:1 ratio of magnetic beads to remove any residual primers and if available run on BioAnalyzer.

Adjust some of the final library pool to 0.8 ng/µl (2.5 nM) to submit at least 15 µl for sequencing (HiSeq300).

SIZE selection of libraries after PCR

1. Add 30 µl of H2O or 10 mM Tris to each of the 25 µl PCR sample to make the volume 55 µl.

Prepare two 96 well plates: **Plate1**. Add 25 µl of size-selection magnetic beads in each well, short spin the plate
Plate2. Add 12 µl of size-selection magnetic beads in each well, short spin the plate

2. Transfer 50 µl of each PCR sample into **Plate1** and mix by pipetting up/down 5-10 times.
- incubate 5 min at RT *(here ration of Beads : Sample = 0.5 => beads bind only DNA above approx. 600+ bp)*

- put on magnet for 3-5 minutes.

3. Transfer the SUPERNATANT (75 µl) into the **Pate2** and mix by pipetting up/down 5-10 times.
- incubate 10-15 min at RT (here the ration of Beads : Sample increased to 0.74) => binds everything from approx. 350-600 bp)

-Put on magnet, discard supernatant and wash the beads 2-times with 150 µl of 80% EtOH (not disturbing the beads).
-Remove all the EtOH by pipetting out liquid 2-5 time with the same pipet tip.
-Let stand to evaporate any left-over EtOH (approx.. 2-5 minutes, do not over-dry the beads)

-Take the plate away from the magnet and add 40 µl of 10mM Tris buffer, pH=8.0 to the beads.
-Mix on plate shaker or until the beads re-suspend evenly and incubate 5 min at RT to elute the library DNA.
-Shortly spin the plate down in a centrifuge and put the plate on magnet; let stand 3-5 minutes, and then transfer the supernatant (DNA library) to your final plate.
-Measure concentration of library in 1 or 2 µl of each sample with PicoGreen (Quant-iT PicoGreen dsDNA Assay Kit).

-Pool together 5 ng of each library sample into one 1.5ml eppi to make a “Final Library Pool”.

-Check the Final Pool's volume, and do a final clean-up/concentrating of the Final Pool with 1:1 (Pool:Beads) ratio.

If the Final Pool is, let's say, 500 µl and it is expected to have 480 ng of library DNA (e.g. from 96 samples, 5 ng each), then, to this 500 µl Final Pool, I would add: 200 µl of Magnetic-beads + 300ul of Magnetic-Bead-Buffer.

- Vortex and let stand at RT for 10-15 minutes, short spin down and put on the magnet for 3-5 min.

- Remove supernatant; wash the beads on magnet twice with 80% EtOH,

- Evaporate the left-over EtOH for 4 minutes,

- Elute the final library to approximately 1.5-2.5 ng/µl expected concentration (in this case, if expecting 480 ng of library, I would use 200 µl of 10mM Tris buffer, to make a concentrated STOCK-Library pool).

- Adjust the whole pool, or a smaller part (but at least 15 µl), of the STOCK-Library pool to **0.8 ng/µl** , which equals to approximately **2.5nM Library-pool**, required for the sequencing on ***HiSeq3000/NovaSeq***.

Buffers / Recipes

DNA extraction/Size-selection-magnetic beads

STOCK BEADS: 15ml, SpeedBeads™(*GE65152105050250 SIGMA) from Sigma /or/ GElifesciences Sera-Mag SpeedBead (65152105050250)*

BEAD BUFFER: *for 50 ml of Bead buffer, dissolve in 35 ml of mol.biol. grade H_2O:*

9 g	*PEG8000*
7.3 g	*NaCl*
500 µl	*1M Tris-HCl, pH=8.0*
100 µl	*0.5M EDTA, pH=8.0*
➔	*dissolve and add mol.biol. grade H_2O to 50 ml*

- *on magnetic stand, add 1 ml of the Stock Beads in an 1.5 ml Eppendorf tube*
- *after 1 min remove the solution and wash beads with 1 ml of 10mM Tris-HCl, pH=8*
- *on magnet, remove Tris buffer and use some of the Bead Buffer to transfer and re-suspend the beads into the rest 50 ml of Bead Buffer.*
- *Beads are ready to use, store at 4C, use at RT.*

1 : 1 ratio (50 µl sample + 50 µl beads) => binds all above 200 bp
1 : 0.54 ration (50 µl sample + 27 µl beads) => binds all above 500 bp
1 : 0.76 ratio (50 µl sample + 38 µl beads) => binds all above 250 pb

5xTn5 buffer = 5x TAPS-Mg-DMF (contains: 50mM TAPS, 25mM MgCl2, 50% DMF),

DMF = N,N-Dimethylformamide

TAPS = *T5130 SIGMA*

1M $MgCl_2$ = e.q. SERVA ,1 M Magnesium chloride, 38772.01

1M TAPS STOCK (50 ml):

1M TAPS in H_2O, adjust to pH:8.5 with 10M NaOH (for 25 ml of 1M TAPS you will need ca 5 ml of 3M NaOH)
⇨ 0.2/0.45 um Filter sterilize the 1M TAPS

To prepare 10 ml of 5x Tn5 buffer:

***Mix on ice in same order*:**

4.25 ml H_2O + 500 µl 1M TAPS (pH=8.5) + 250 µl 1M $MgCl_2$ + 5 ml DMF

Note: Buffer becomes warm after adding DMF (keep in ice)

Aliquot in 1.5 ml tubes, store at 4C (for short time, 1-2 months), for longer storage at -20C (buffer doesn't freeze)

Strept. Binding buffer
0.6 M NaCl
10 mM Tris, pH=8.0
0.5 mM EDTA
0.1 % Triton X-100

Dialysis / Tn-bead storage buffer
50 mM HEPES-KOH pH 7.2
0.2 M NaCl
0.2 mM EDTA
2 mM DTT
0.2 % Triton X-100
20 % glycerol

WASH buffer:
10 mM Tris,pH=8,
30 mM NaCl,
0.1 % Triton X-100

"0.6% SDS-Wash"
WASH buffer + 0.6% SDS (from stock 20% SDS in H2O)

Barcoding oligos

	If possible: N50X oligos order with PAGE purification and bigger scale especially if planning to pool more than 96 samples per lane into sequencing…	on sample sheet HiSeq3000	on sample sheet NovaSeq
Nextera_N501	AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC	GCGATCTA	TAGATCGC
Nextera_N502	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC	ATAGAGAG	CTCTCTAT
Nextera_N503	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC	AGAGGATA	TATCCTCT
Nextera_N504	AATGATACGGCGACCACCGAGATCTACACAGAGTAGATCGTCGGCAGCGTC	TCTACTCT	AGAGTAGA
Nextera_N505	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC	CTCCTTAC	GTAAGGAG
Nextera_N506	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC	TATGCAGT	ACTGCATA
Nextera_N507	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGTC	TACTCCTT	AAGGAGTA
Nextera_N508	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC	AGGCTTAG	CTAAGCCT

		barcode in primer		barcode on sample sheet
N701	1	TCGCCTTA	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG	TAAGGCGA
N702	2	CTAGTACG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG	CGTACTAG
N703	3	TTCTGCCT	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG	AGGCAGAA
N704	4	GCTCAGGA	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG	TCCTGAGC
N705	5	AGGAGTCC	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG	GGACTCCT
N706	6	CATGCCTA	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG	TAGGCATG
N707	7	GTAGAGAG	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG	CTCTCTAC
N708	8	CCTCTCTG	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGG	CAGAGAGG
N709	9	AGCGTAGC	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGG	GCTACGCT
N710	10	CAGCCTCG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG	CGAGGCTG
N711	11	TGCCTCTT	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG	AAGAGGCA
N712	12	TCCTCTAC	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG	GTAGAGGA
N714	13	TCATAGGC	CAAGCAGAAGACGGCATACGAGATTCATAGGCGTCTCGTGGGCTCGG	GCCTATGA
N715	14	CCTGAGAT	CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCGTGGGCTCGG	ATCTCAGG
N716	15	TAGCGAGT	CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCGG	ACTCGCTA
N718	16	GTAGCTCC	CAAGCAGAAGACGGCATACGAGATGTAGCTCCGTCTCGTGGGCTCGG	GGAGCTAC
N719	17	CATCAGGC	CAAGCAGAAGACGGCATACGAGATCATCAGGCGTCTCGTGGGCTCGG	GCCTGATG
N720	18	AGGCTCCG	CAAGCAGAAGACGGCATACGAGATAGGCTCCGGTCTCGTGGGCTCGG	CGGAGCCT
N721	19	GCAGCGTA	CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCGTGGGCTCGG	TACGCTGC
N722	20	CTGCGCAT	CAAGCAGAAGACGGCATACGAGATCTGCGCATGTCTCGTGGGCTCGG	ATGCGCAG
N723	21	GAGCGCTA	CAAGCAGAAGACGGCATACGAGATGAGCGCTAGTCTCGTGGGCTCGG	TAGCGCTC
N724	22	CGCTCAGT	CAAGCAGAAGACGGCATACGAGATCGCTCAGTGTCTCGTGGGCTCGG	ACTGAGCG
N726	23	TACAGGAT	CAAGCAGAAGACGGCATACGAGATTACAGGATGTCTCGTGGGCTCGG	ATCCTGTA
N727	24	ACTGATCG	CAAGCAGAAGACGGCATACGAGATACTGATCGGTCTCGTGGGCTCGG	CGATCAGT
N728	25	TAGCTGCA	CAAGCAGAAGACGGCATACGAGATTAGCTGCAGTCTCGTGGGCTCGG	TGCAGCTA
N729	26	GACGTCGA	CAAGCAGAAGACGGCATACGAGATGACGTCGAGTCTCGTGGGCTCGG	TCGACGTC
N730	27	TGTGGTTG	CAAGCAGAAGACGGCATACGAGATTGTGGTTGGTCTCGTGGGCTCGG	CAACCACA
N731	28	TAGTCTTG	CAAGCAGAAGACGGCATACGAGATTAGTCTTGGTCTCGTGGGCTCGG	CAAGACTA
N732	29	TTCCATTG	CAAGCAGAAGACGGCATACGAGATTTCCATTGGTCTCGTGGGCTCGG	CAATGGAA
N733	30	TCGAAGTG	CAAGCAGAAGACGGCATACGAGATTCGAAGTGGTCTCGTGGGCTCGG	CACTTCGA
N734	31	TAACGCTG	CAAGCAGAAGACGGCATACGAGATTAACGCTGGTCTCGTGGGCTCGG	CAGCGTTA
N735	32	TTGGTATG	CAAGCAGAAGACGGCATACGAGATTTGGTATGGTCTCGTGGGCTCGG	CATACCAA
N736	33	TGAACTGG	CAAGCAGAAGACGGCATACGAGATTGAACTGGGTCTCGTGGGCTCGG	CCAGTTCA
N737	34	TAGTTCGG	CAAGCAGAAGACGGCATACGAGATTAGTTCGGGTCTCGTGGGCTCGG	CCGAACTA
N738	35	TCTCACGG	CAAGCAGAAGACGGCATACGAGATTCTCACGGGTCTCGTGGGCTCGG	CCGTGAGA
N739	36	TCATGAGG	CAAGCAGAAGACGGCATACGAGATTCATGAGGGTCTCGTGGGCTCGG	CCTCATGA
N740	37	TAAGTTCG	CAAGCAGAAGACGGCATACGAGATTAAGTTCGGTCTCGTGGGCTCGG	CGAACTTA
N741	38	TCCAGTCG	CAAGCAGAAGACGGCATACGAGATTCCAGTCGGTCTCGTGGGCTCGG	CGACTGGA
N742	39	TGTATGCG	CAAGCAGAAGACGGCATACGAGATTGTATGCGGTCTCGTGGGCTCGG	CGCATACA
N743	40	TCATTGAG	CAAGCAGAAGACGGCATACGAGATTCATTGAGGTCTCGTGGGCTCGG	CTCAATGA
N744	40	TGACTCAG	CAAGCAGAAGACGGCATACGAGATTGACTCAGGTCTCGTGGGCTCGG	CTGAGTCA
N745	42	TATGCCAG	CAAGCAGAAGACGGCATACGAGATTATGCCAGGTCTCGTGGGCTCGG	CTGGCATA
N746	43	TCAGATTC	CAAGCAGAAGACGGCATACGAGATTCAGATTCGTCTCGTGGGCTCGG	GAATCTGA
N747	44	TACTAGTC	CAAGCAGAAGACGGCATACGAGATTACTAGTCGTCTCGTGGGCTCGG	GACTAGTA
N748	45	TTCAGCTC	CAAGCAGAAGACGGCATACGAGATTTCAGCTCGTCTCGTGGGCTCGG	GAGCTGAA
N749	46	TGTCTATC	CAAGCAGAAGACGGCATACGAGATTGTCTATCGTCTCGTGGGCTCGG	GATAGACA
N750	47	TATGTGGC	CAAGCAGAAGACGGCATACGAGATTATGTGGCGTCTCGTGGGCTCGG	GCCACATA
N751	48	TTACTCGC	CAAGCAGAAGACGGCATACGAGATTTACTCGCGTCTCGTGGGCTCGG	GCGAGTAA
N752	49	TCGTTAGC	CAAGCAGAAGACGGCATACGAGATTCGTTAGCGTCTCGTGGGCTCGG	GCTAACGA

N753	50	TACGGAGC	CAAGCAGAAGACGGCATACGAGATTACGGAGCGTCTCGTGGGCTCGG	GCTCCGTA
N754	51	TGTTCTCC	CAAGCAGAAGACGGCATACGAGATTGTTCTCCGTCTCGTGGGCTCGG	GGAGAACA
N755	52	TTCGCACC	CAAGCAGAAGACGGCATACGAGATTTCGCACCGTCTCGTGGGCTCGG	GGTGCGAA
N756	53	TTGCGTAC	CAAGCAGAAGACGGCATACGAGATTTGCGTACGTCTCGTGGGCTCGG	GTACGCAA
N757	54	TCTACGAC	CAAGCAGAAGACGGCATACGAGATTCTACGACGTCTCGTGGGCTCGG	GTCGTAGA
N758	55	TGACAGAC	CAAGCAGAAGACGGCATACGAGATTGACAGACGTCTCGTGGGCTCGG	GTCTGTCA
N759	55	TAGAAAAC	CAAGCAGAAGACGGCATACGAGATTAGAAAACGTCTCGTGGGCTCGG	GTTTTCTA
N760	57	TCATCCTA	CAAGCAGAAGACGGCATACGAGATTCATCCTAGTCTCGTGGGCTCGG	TAGGATGA
N761	58	TGCTGATA	CAAGCAGAAGACGGCATACGAGATTGCTGATAGTCTCGTGGGCTCGG	TATCAGCA
N762	59	TAGACGGA	CAAGCAGAAGACGGCATACGAGATTAGACGGAGTCTCGTGGGCTCGG	TCCGTCTA
N763	60	TGTGAAGA	CAAGCAGAAGACGGCATACGAGATTGTGAAGAGTCTCGTGGGCTCGG	TCTTCACA
N764	61	TCTCTTCA	CAAGCAGAAGACGGCATACGAGATTCTCTTCAGTCTCGTGGGCTCGG	TGAAGAGA
N765	62	TTGTTCCA	CAAGCAGAAGACGGCATACGAGATTTGTTCCAGTCTCGTGGGCTCGG	TGGAACAA
N766	63	TGAAGCCA	CAAGCAGAAGACGGCATACGAGATTGAAGCCAGTCTCGTGGGCTCGG	TGGCTTCA
N767	64	TACCACCA	CAAGCAGAAGACGGCATACGAGATTACCACCAGTCTCGTGGGCTCGG	TGGTGGTA
N768	65	TGCGTGAA	CAAGCAGAAGACGGCATACGAGATTGCGTGAAGTCTCGTGGGCTCGG	TTCACGCA
N769	66	GGTGAGTT	CAAGCAGAAGACGGCATACGAGATGGTGAGTTGTCTCGTGGGCTCGG	AACTCACC
N770	67	GATCTCTT	CAAGCAGAAGACGGCATACGAGATGATCTCTTGTCTCGTGGGCTCGG	AAGAGATC
N771	68	GTGTCCTT	CAAGCAGAAGACGGCATACGAGATGTGTCCTTGTCTCGTGGGCTCGG	AAGGACAC
N772	69	GACGGATT	CAAGCAGAAGACGGCATACGAGATGACGGATTGTCTCGTGGGCTCGG	AATCCGTC
N773	70	GCAACATT	CAAGCAGAAGACGGCATACGAGATGCAACATTGTCTCGTGGGCTCGG	AATGTTGC
N774	71	GGTCGTGT	CAAGCAGAAGACGGCATACGAGATGGTCGTGTGTCTCGTGGGCTCGG	ACACGACC
N775	72	GAATCTGT	CAAGCAGAAGACGGCATACGAGATGAATCTGTGTCTCGTGGGCTCGG	ACAGATTC
N776	73	GTACATCT	CAAGCAGAAGACGGCATACGAGATGTACATCTGTCTCGTGGGCTCGG	AGATGTAC
N777	74	GAGGTGCT	CAAGCAGAAGACGGCATACGAGATGAGGTGCTGTCTCGTGGGCTCGG	AGCACCTC
N778	75	GCATGGCT	CAAGCAGAAGACGGCATACGAGATGCATGGCTGTCTCGTGGGCTCGG	AGCCATGC
N779	76	GTTAGCCT	CAAGCAGAAGACGGCATACGAGATGTTAGCCTGTCTCGTGGGCTCGG	AGGCTAAC
N780	77	GTCGCTAT	CAAGCAGAAGACGGCATACGAGATGTCGCTATGTCTCGTGGGCTCGG	ATAGCGAC
N781	78	GGAATGAT	CAAGCAGAAGACGGCATACGAGATGGAATGATGTCTCGTGGGCTCGG	ATCATTCC
N782	79	GAGCCAAT	CAAGCAGAAGACGGCATACGAGATGAGCCAATGTCTCGTGGGCTCGG	ATTGGCTC
N783	80	GCTCCTTG	CAAGCAGAAGACGGCATACGAGATGCTCCTTGGTCTCGTGGGCTCGG	CAAGGAGC
N784	81	GTAAGGTG	CAAGCAGAAGACGGCATACGAGATGTAAGGTGGTCTCGTGGGCTCGG	CACCTTAC
N785	82	GAGGATGG	CAAGCAGAAGACGGCATACGAGATGAGGATGGGTCTCGTGGGCTCGG	CCATCCTC
N786	83	GTTGTCGG	CAAGCAGAAGACGGCATACGAGATGTTGTCGGGTCTCGTGGGCTCGG	CCGACAAC
N787	84	GGATTAGG	CAAGCAGAAGACGGCATACGAGATGGATTAGGGTCTCGTGGGCTCGG	CCTAATCC
N788	85	GATAGAGG	CAAGCAGAAGACGGCATACGAGATGATAGAGGGTCTCGTGGGCTCGG	CCTCTATC
N789	86	GTGTGTCG	CAAGCAGAAGACGGCATACGAGATGTGTGTCGGTCTCGTGGGCTCGG	CGACACAC
N790	87	GCAATCCG	CAAGCAGAAGACGGCATACGAGATGCAATCCGGTCTCGTGGGCTCGG	CGGATTGC
N791	88	GACCTTAG	CAAGCAGAAGACGGCATACGAGATGACCTTAGGTCTCGTGGGCTCGG	CTAAGGTC
N792	89	GCCTGTTC	CAAGCAGAAGACGGCATACGAGATGCCTGTTCGTCTCGTGGGCTCGG	GAACAGGC
N793	90	GCACTGTC	CAAGCAGAAGACGGCATACGAGATGCACTGTCGTCTCGTGGGCTCGG	GACAGTGC
N794	91	GCTAACTC	CAAGCAGAAGACGGCATACGAGATGCTAACTCGTCTCGTGGGCTCGG	GAGTTAGC
N795	92	GATTCATC	CAAGCAGAAGACGGCATACGAGATGATTCATCGTCTCGTGGGCTCGG	GATGAATC
N796	93	GTCTTGGC	CAAGCAGAAGACGGCATACGAGATGTCTTGGCGTCTCGTGGGCTCGG	GCCAAGAC
N797	94	TGCGATCT	CAAGCAGAAGACGGCATACGAGATTGCGATCTGTCTCGTGGGCTCGG	AGATCGCA
N798	95	TTCCTGCT	CAAGCAGAAGACGGCATACGAGATTTCCTGCTGTCTCGTGGGCTCGG	AGCAGGAA
N799	96	TAGACACT	CAAGCAGAAGACGGCATACGAGATTAGACACTGTCTCGTGGGCTCGG	AGTGTCTA

NOTES:

N50X primers with PAGE purification can prevent data loss if multiplexing more than 96 samples, with several different N50X primers. In that case, you have to also use the N50X barcode to demultiplex data, not just the N70X barcode. PAGE purification keeps the errors in the barcode at minimum and thus helps with data recovery. It cost more and one needs to order bigger scale, but it is worth it. Of course, ideal would be to order not just N50X primers with PAGE purification, but also the N70X primers, but that would cost a lot.

Normally, if you have less than 96 samples in a sequencing pool, you can use the cheap, NOT-PAGE-purified-N50X , because you can ignore N50X barcode during demultiplexing (all samples have the same N50X) and thus you can be de-multiplex only using N70X barcode and recover more data (e.g. you will not lose any reads due to errors in N50X barcode, which you would, if you demultiplex using both N70X and N50X barcodes).