

Library Preparation for Medium- to High-throughput DNA Sequencing on the Illumina Sequencing Platform

A LABORATORY PROTOCOL

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1 INTRODUCTION AND SUMMARY

This protocol achieves parallel, cost-effective preparation of Illumina sequencing libraries using only standard laboratory equipment. Costs are less than \$10 per sample, and the intended scale is dozens to hundreds of samples. End-products are sequencing libraries, where each library is individually indexed with a molecular barcode. These libraries can be pooled for sequencing and subsequently demultiplexed in-silico. We provide 24 forward and 36 reverse primer sequences with a combinatorial barcoding capacity for up to 864 samples.

The library preparation protocol is based on Illumina's Nextera protocol using the Illumina's Nextera Transposase Enzyme. For understanding the principle, please consult the respective Nextera manuals and user guides (online). Similarly, size selection and clean-up is performed through a two-sided bead clean-up using commercially available beads (Agencourt Ampure beads). Details are described in the manufacturer's manuals and user guides.

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Contributors to the working draft

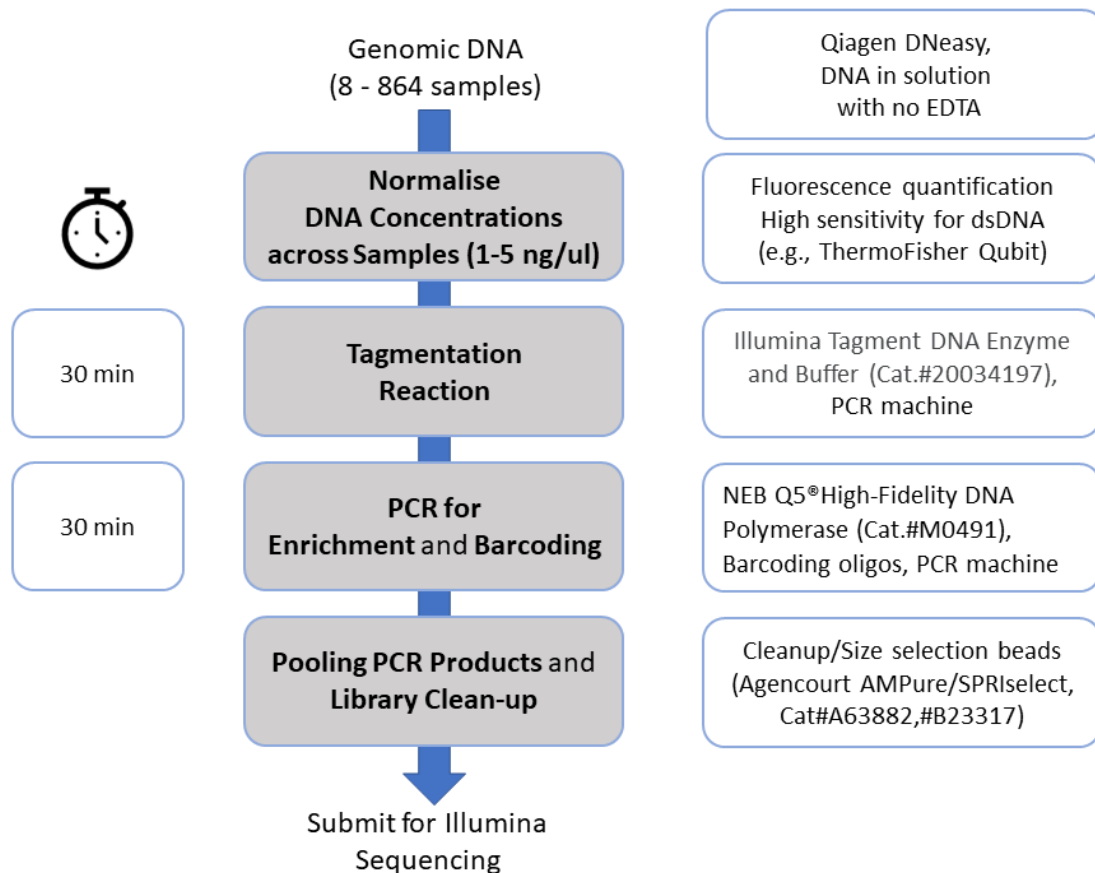
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1.1 OVERVIEW OF THE PROTOCOL

The library preparation protocol consists of the following steps:

- **DNA normalisation** (achieving equal DNA concentrations across samples)
- **Tagmentation** (Shearing and DNA-Adaptor attachment)
- **PCR** (Enrichment and sample-indexing with PCR-primers containing molecular barcodes)
- **Pooling** (combining equivolumes of individual libraries to form a library pool)
- **Library pool clean-up and fragment size selection**
- **QC** (Quality control for size distribution and amount)
- [Optional/if required: **Additional library enrichment**]



1.2 NOTES TO THE USER:

- This protocol is developed for performing all steps in 96-well format by manual pipetting. However, concentrations and volumes allow for straight-forward adaptations to other formats such as individual tubes or 384-well plates and automation by liquid handling robots.
- Starting point for this protocol is DNA. We strictly recommend using Qiagen DNeasy kits for upstream DNA extractions followed by fluorescence-based quantification of double stranded DNA.

- Note that EDTA inhibits the Tagmentation reaction. DNA should therefore be eluted/diluted with water or buffers with no or little amounts of EDTA.
- We found that initial normalisation of DNA is sufficient. I.e., starting with equal amounts of uniform DNA per sample will result in reasonably similar amounts of sequencing data per sample.
- Care must be taken to not cross-contaminate samples prior to the pooling step.

2 REQUIRED EQUIPMENT AND REAGENTS

Only standard laboratory equipment and commercially available reagents are required.

2.1 REQUIRED EQUIPMENT

For best results the following should be accessible:

- Multi-channel pipettes (8-channel and 12-channel, volume 1-10 µl)
- Plate reader and reagents for fluorescence-based DNA quantification in plates
- PCR tubes (~0.2 ml) in strips-of-8
- Magnetic stand for 1.5 ml tubes
- Agilent Bioanalyzer (or equivalent Fragment Analyzer)

However, the protocol can also be conducted with the following minimum equipment: 8-channel pipettes, PCR machine with heated lid, Qubit for fluorescence-based DNA quantification, gel electrophoresis equipment.

2.2 REQUIRED CONSUMABLES

This is a generic list. A list of specific consumables used at PBGL is in Appendix 1.

DNA normalisation (Achieving equal DNA concentrations across samples at the beginning)

- Reagents for fluorescence-based DNA quantification capable of quantifying DNA amounts down to 1 ng, such as Picogreen or Quant-IT dsDNA Assay kit, high sensitivity.

Tagmentation (Shearing and DNA-Adaptor attachment)

- Nextera Tagmentation Enzyme (Illumina #15027865)

PCR (Enrichment and sample-indexing with PCR-primers containing molecular barcodes)

- Enrichment/barcoding primers (for oligo list see Appendix 2)
- high-fidelity, proof-reading DNA polymerase; do not use a hot-start version. In PBGL we use NEB Q5 polymerase.

Pooling (combining equivolumes of individual libraries to form a library pool)

Library pool clean-up and fragment size selection

- Agencourt SPRI beads or equivalent,
- Magnetic Rack for bead cleanup (e.g., Life Technologies).

Alternatively, this size selection can be achieved by gel electrophoresis, or the size selection can be performed by the sequencing service provider.

QC (Quality control for size distribution and amount)

- Agilent consumables
- Agilent bioanalyzer

Alternatively, this QC can be achieved by gel electrophoresis, or QC can be performed by the sequencing service provider.

3 PROTOCOL

3.1 DNA NORMALISATION

Purpose is to adjust all individual DNA samples to a uniform DNA concentration. Note that high concentrations of EDTA inhibit subsequent reactions, hence Tris-buffer or water should be used for dilutions. When planning the individual concentrations, consider the total amount of DNA across all samples at the end. We have good experience with total amount of DNA between 200 and 400 ng total, i.e., when going into the Tagmentation reactions for 80+ samples, use 1.2 ng/μl per sample, for 24 samples use 3 ng/μl. Higher DNA concentrations will require more Tagmentation enzyme, increasing the cost per sample.

The normalisation is a crucial step and we encourage to dilute the DNA samples in a stepwise fashion until all samples are roughly equal. For a 1.2 ng target concentration we found a range of 1.0 to 1.7 ng/μl acceptable). If quantification is performed in plates, have the DNA standards on every plate for measurement. Tip: When performing this protocol on more than 80 samples spread them evenly over more than one plate and maintain this layout throughout the protocol. When handling full plates of 96, split into 2x48. Using only the DNA standards necessary for the respective range will increase accuracy.

3.2 TAGMENTATION

The Nextera Tagmentation Enzyme is a Transposase loaded with Illumina adapters. It tags DNA in a stoichiometric fashion. Consequently, the ratio of Nextera Tagmentation Enzyme to DNA molecules will determine the size distribution of the resulting fragments, with more enzyme per DNA resulting in smaller fragments. Unequal DNA concentrations between samples for a given amount of enzyme will influence the representation of the library in the final sequencing data. Hence, we recommend uniform upstream DNA extractions and good quantification and normalisation. For the Tagmentation Enzyme to DNA ratio we have good

experience with 0.10 to 0.16 μ l of enzyme per ng of genomic DNA (Tagmentation enzyme as purchased from Illumina and plant genomic DNA prepared with the Qiagen DNeasy plant Mini Kit). If you find your DNA overdigested in your setup, i.e., the size distribution smaller than desired, reduce the amount of enzyme, and vice versa.

3.2.1 Preparing the Tagmentation Reaction mix

- Array the sample DNAs into 96-well plates. For simplicity, spread the samples evenly across plates, i.e., have an equal number of samples on every plate. Document and maintain the plate layout(s) throughout the protocol.
- Prepare a Tagmentation Reaction Mastermix according to the table below. This is an example for 96 samples. Consult the text above for suggested modifications when preparing library pools with less samples.

Component	Volume per reaction (μ l)	Mastermix (example for 96)*	
Tagmentation buffer (2x)**	5	520	
Tagmentation Enzyme***	0.45	47	
Water*	1.55-1.7*	177	
Tagmentation Mastermix		744	7
DNA (1.2 ng/ μ l)****	3		3
Total reaction volume	10		10

*Prepare 10% extra to compensate for dead volume in batch pipetting. Slightly more water can be added for extra volume. For 96 we calculated with 104 samples and 1.7 μ l water per reaction.

**Use commercial 2x Tagmentation Buffer (Illumina) or make the 2x buffer yourself: 20 mM Tris (pH 8.4-9), 10 mM MgCl₂

***Aim for 0.10 to 0.16 μ l of enzyme per ng of genomic DNA

****Adjust the DNA amount per sample depending on the total number of samples. See text above.

- Distribute 7 μ l of Mastermix per reaction/well into a clean 96-well plate (reaction plate) according to your sample layout. Tip: Use an 8-channel pipette and distribute from a strip-of-8.
- With a multichannel pipette, add 3 μ l of sample DNA (~approx. 3.6 ng) per well from your sample plate to the reaction mix in the reaction plate and mix by gentle pipetting up and down (once or twice).
- Place reaction plate in a PCR machine and incubate at 55 C for 10 min. Use a heated lid at 65°C. Suggested program for the PCR machine:

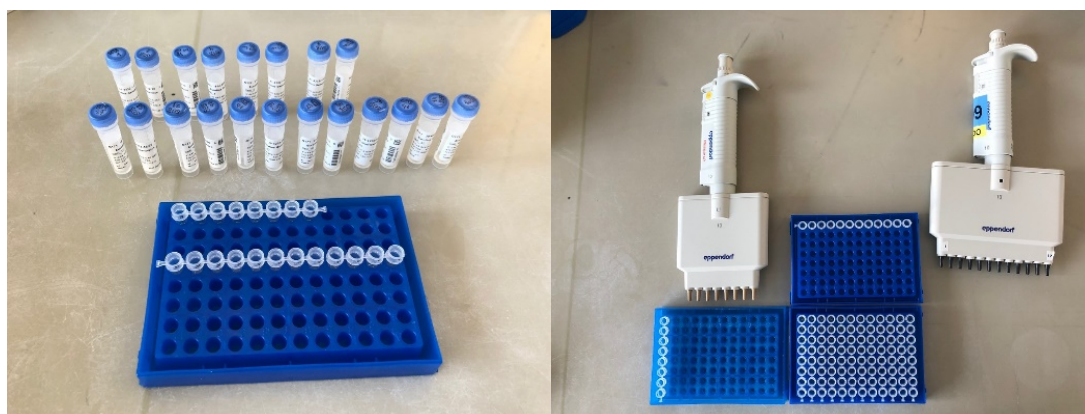
Temperature	Time
22° C	0:30
55° C	10:00
15° C	forever

- Proceed to preparing the Barcoding PCR. Retrieve the reaction plate from the PCR machine when needed for the Barcoding PCR step.

3.3 BARCODING PCR

The barcoding PCR achieves sample enrichment, attachment of sequencing technology-specific binding sites, and combinatorial barcoding through index sequences. The barcoding primers fit the adaptors that were added by the Tagmentation reaction. Each Tagmentation product will be amplified with a pair of indexed primers. The primer combinations should be chosen such that the combinatorial barcoding is unique for each sample at least within the planned sequencing run. I.e., a set of 8 forward and 12 reverse primers allow for 96 combinations (8x12). We provide 24 FWD and 36 REV primer sequences with a combinatorial barcoding capacity for up to 864 samples (9x 96-well plates). Performing the Barcoding PCR comprises the following steps: 1) preparing arrays of PCR primer dilutions, 2) preparing the PCR Mastermix, 3) setting up the PCR reactions (distribute mastermix, add primers, add templates), and 4) running a PCR of about 30 min duration.

The Barcoding Primers are given as i5 and i7. Each sample in an experiment needs to be assigned a unique combination of one i5 and one i7 primer. When working in plates it is practical to assign one i5 primer per row and one i7 primer per column and array them in a way that allows for distribution into the 96-well plate with multichannel pipettes. 8-channel for the columns and 12-channel for the rows. We use PCR tubes in strips-of-8. See pictures below.



Left: Dilute i5 and i7 oligos (to 3 μ M) from stock (100 μ M) into 2 arrays. One array of eight oligos for the columns, and one array of twelve oligos for the rows. **Right:** Distribution into rows and columns of the 96-well PCR plate with multichannel pipettes (8- and 12-channel) is then straight-forward.

3.3.1 Preparing arrays of PCR primer dilutions

Dilute each oligo, from 100 μ M stock to 3 μ M, into the respective tube (eight i5 and twelve i7 oligos) to 3 μ M for example like so:

	H2O	100 μ M oligo stock
i5 oligos (rows)	40 μ l	1.2 μ l
i7 oligos (columns)	30 μ l	1.0 μ l

3.3.2 Preparing the PCR Mastermix

Prepare the PCR mastermix according to the table below. We have good experience with Q5 DNA Polymerase (NEB). For other polymerases the details will vary. It will obviously need to be a proof-reading enzyme and the number of cycles should be kept to a minimum. Note that the first step in the PCR program is an elongation step at 72C which precludes the use of HotStart enzymes. Do NOT use HotStart Polymerases!

Component	Volume per reaction (µl)	PCR Mastermix example (96)*	
Nuclease-Free Water	0	0	
Q5 reaction buffer (5x)	3.05	336	
Q5 High GC Enhancer (5x)	2.5	275	
dNTP (10 mM)	0.3	33	
Q5 High-fidelity DNA Polymerase	0.15	16.5	
PCR Mastermix		660	6
Forward primer (3 µM)	2.5		+2.5
Reverse primer (3 µM)	2.5		+2.5
Template DNA (=Tagmentation)	6		+6
Total reaction volume	17		17

*Prepare about 10% extra to compensate for dead volume when batch pipetting. For 96 we calculate with 110 samples.

3.3.3 Preparing the PCR reaction

- Distribute 6 µl of PCR Mastermix per reaction/well into a clean 96-well plate (PCR plate) according to your sample layout. Tip: Use an 8-channel pipette and distribute from a strip-of-8, where each tube contains 80 µl of PCR Mastermix.
- With an 8-channel pipette add 2.5 µl of the i5-primers column-wise to the PCR reaction mix so that the same i5-primer is used across a row.
- With a 12-channel pipette add 2.5 µl of the i7-primers row-wise to the PCR reaction mix so that the same i7-primer is used across a column.
- Transfer 6 µl of each Tagmentation product into the respective well of the PCR plate using a multichannel pipette. Mix by gently pipetting up and down (once or twice).
- Seal the plate and run a PCR with below program (duration approx. 30 min).

3.3.4 PCR program

Temperature	Time	
72 C	3:00	
98 C	0:30	
98 C	0:10	8-10 cycles
63 C	0:30	
72 C	1:00	
10 C	inf	

3.4 POOLING OF INDIVIDUAL LIBRARIES

We found it not necessary to quantify the individual libraries at this step, and hence recommend to simply pool the libraries using the same volume of each library. How much to use will need to be guided by the number of samples and desired total amount of DNA needed for sequencing. We found 6 µl per library a good number. It leaves enough volume behind should an additional pooling be required at a later stage. Tip: Pool column-wise into a strip-of-8 PCR tubes with a multichannel pipette, and then combine into a 1.5 ml Eppendorf tube. The libraries are PCR products and can be kept at -20°C for extended periods of time.

3.5 LIBRARY CLEAN UP AND SIZE SELECTION

Standard procedures for Illumina library size selection and clean-up can be followed.

Note: The library pool is a pool of PCR reactions and as such contains unincorporated primers and dNTPs, primer dimers, enzymes, etc. In addition, the size distribution of the Nextera library fragments is usually broad at this stage ranging from about 200 to 2,000+ bp. Clean-up and size selection can be achieved in several ways, for example by cutting the desired size range from an agarose gel, as is common practice in Illumina library preparations. Initial volume reduction can be achieved through a column-based clean-up, e.g., Qiagen PCR reaction cleanup or MinElute Kit.

We have good experience with an initial clean-up through Qiagen PCR reaction cleanup columns, followed by a 2-sided bead clean-ups with Agencourt SPRI beads, thus purging undesired too large and too small fragments. For a 150bp PE sequencing run we aim at a minimum fragment length of 380 bp to minimize read trough. For the clean-up procedures including 2-sided size selection please consult the respective manuals. It will require adjustments for your specific needs.

3.6 QUALITY CONTROL

Sequencing service providers will have minimum requirements with respect to DNA amount, volume and molarity. We recommend that the average fragment size in the library is determined with a Fragment Analyzer (Bioanalyzer or equivalent) and the amount of dsDNA in ng by fluorescence-based DNA quantification (Qubit or equivalent). Molarity can then be calculated using the formula below and, in our experience, sequencing data output is good if the sequencing service providers use this number.

$$\frac{\text{concentration} \left(\frac{\text{ng}}{\mu\text{l}} \right) * 10^6}{660 * \text{Average fragment length}} = \text{Molarity} \left(\frac{\text{nmol}}{\text{l}} \right)$$

<https://bitesizebio.com/23105/quantifying-your-ngs-libraries/>

Illumina has published a technical note on the quantification of Nextera Libraries of similar content: https://www.illumina.com/documents/products/technotes/technote_nextera_library_validation.pdf

3.7 OPTIONAL/IF REQUIRED: ADDITIONAL LIBRARY ENRICHMENT

Should, after QC, the size be correct but the DNA amount too little for the intended platform, an attempt can be made to amplify the final library. We have had good experience with 2 and 4 cycles of PCR using Q5 DNA Polymerase (NEB). See primers and protocol below. To reduce the impact of PCR errors, perform several parallel PCRs and combine PCR products. Clean-up and size select as appropriate.

Enrichment PCR primers		
Universal forward primer [i5]	AATGATACGGCGACCACCGAGA	T _m =66.2
Universal reverse primer [i7]	CAAGCAGAAGACGGCATAACGAGA	T _m =64.4

PCR Reaction Mix	25 µl reaction
5X Q5 Reaction Buffer	5
10 mM dNTPs	0.5
5 µM Forward Primer	2.5
5 µM Reverse Primer	2.5
Template DNA	10
Q5 High-Fidelity DNA Polymerase	0.2
5X Q5 High GC Enhancer (optional)	3
Nuclease-Free Water	1.3

PCR program

Temperature	Time	
98	30s	
98	10s	2x, 4x
63	15s	
72	30s	
10	forever	

4 APPENDIX 1 – SPECIFIC CONSUMABLES

DNA Quantification

Quant-iT™ dsDNA Assay Kit, high sensitivity, ThermoFisher Scientific, Catalogue number: Q33120

Tagmentation

TDE1, TAGMENT DNA ENZYME, 24 RXN, Illumina catalogue number 15027865, EUR 1,688

TD, TAGMENT DNA BUFFER (2x), Illumina catalogue number 15027866 EUR 192

Alternatively, this buffer can be used (2x): 20 mM Tris (pH 8.4-9), 10 mM MgCl₂

Barcoding PCR, DNA polymerase

Q5®High-Fidelity DNA Polymerase, NEB, catalogue numbers M0491S, M0491L

QC, Agilent Bioanalyzer tape station 2200 consumables.

5067-5584 High Sensitivity D1000 Screen Tape, 112 samples (EUR 280)

5067-5585 High Sensitivity D1000 Reagents, 112 samples, (EUR 100)

5 APPENDIX 2 – INDEX PRIMERS FOR BARCODING AND ENRICHMENT

During the Tagmentation step, the Tn5 transposase cuts the sample DNA and adds the following sequences at either end of each fragment:

5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG -3

5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG -3

The index PCR then adds the P5 and P7 termini that bind to the flow cell and also the dual 8-bp index tags (barcodes), denoted by the i5 and i7 in the generic primer sequences below.

5' AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC -3'

5' CAAGCAGAAGACGGCATAACGAGAT[i7]GTCTCGTGGGCTCGG -3

For in-silico trimming of adapter sequences from Nextera runs (with cutadapt, trimmomatic, etc.) specify as custom adaptor CTGTCTCTTATACACATCT instead of the default AGATCGGAAGAGC. Most Illumina platforms require specifying the index sequences in sense orientation for demultiplexing.

List of index/oligo sequences (developed by Norman Warthmann, PBGL)

i5-indexes (rows)		
Name	Index	Primer (5'-3')
Seib_1	AACCATGC	AATGATACGGCGACCACCGAGATCTACAC AACCATGC TCGTCGGCAGCGT*C
Seib_2	AGAGCCTG	AATGATACGGCGACCACCGAGATCTACACAGAGCCTGTCGTCGGCAGCGT*C
Seib_3	ATAGCCAT	AATGATACGGCGACCACCGAGATCTACACATAGCCATTCGTCGGCAGCGT*C
Seib_4	CCGTAACC	AATGATACGGCGACCACCGAGATCTACACCCGTAACCTCGTCGGCAGCGT*C
Seib_5	GATCTTAA	AATGATACGGCGACCACCGAGATCTACACGATCTTAATCGTCGGCAGCGT*C
Seib_6	GGTTGAGG	AATGATACGGCGACCACCGAGATCTACACGGTTGAGGTCGTCGGCAGCGT*C
Seib_7	GTCAGGCT	AATGATACGGCGACCACCGAGATCTACACGTCAGGCTTCGTCGGCAGCGT*C
Seib_8	TCGCCGTA	AATGATACGGCGACCACCGAGATCTACACTCGCCGTATCGTCGGCAGCGT*C
Seib_9	ACCAAGAT	AATGATACGGCGACCACCGAGATCTACACACCAAGATTCGTCGGCAGCGT*C
Seib_10	CAAGGAAG	AATGATACGGCGACCACCGAGATCTACACCAAGGAAGTCGTCGGCAGCGT*C
Seib_11	CCGCCAGC	AATGATACGGCGACCACCGAGATCTACACCCGCCAGCTCGTCGGCAGCGT*C
Seib_12	CGTAACGT	AATGATACGGCGACCACCGAGATCTACACCGTAACGTTTCGTCGGCAGCGT*C
Seib_13	GGTCTGAG	AATGATACGGCGACCACCGAGATCTACACGGTCTGAGTCGTCGGCAGCGT*C
Seib_14	TACGTTCA	AATGATACGGCGACCACCGAGATCTACACTACGTTTCATCGTCGGCAGCGT*C
Seib_15	TTATTCTC	AATGATACGGCGACCACCGAGATCTACACTTATTCTCTCGTCGGCAGCGT*C
Seib_16	TTGAGGTA	AATGATACGGCGACCACCGAGATCTACACTTGAGGTATCGTCGGCAGCGT*C
Seib_17	AACCAACG	AATGATACGGCGACCACCGAGATCTACACAACCAACGTCGTCGGCAGCGT*C
Seib_18	AATTCTAG	AATGATACGGCGACCACCGAGATCTACACAATTCTAGTCGTCGGCAGCGT*C
Seib_19	AGAACCGA	AATGATACGGCGACCACCGAGATCTACACAGAACCGATCGTCGGCAGCGT*C
Seib_20	CCGAATGC	AATGATACGGCGACCACCGAGATCTACACCCGAATGTCGTCGGCAGCGT*C
Seib_21	CCTGCCTT	AATGATACGGCGACCACCGAGATCTACACCCTGCCTTTCGTCGGCAGCGT*C
Seib_22	GGATGGTA	AATGATACGGCGACCACCGAGATCTACACGGATGGTATCGTCGGCAGCGT*C
Seib_23	GGCGTACT	AATGATACGGCGACCACCGAGATCTACACGGCGTACTTCGTCGGCAGCGT*C
Seib_24	TCGATTCC	AATGATACGGCGACCACCGAGATCTACACTCGATTCCCTCGTCGGCAGCGT*C

i7-indexes (columns)		
Name	Index	Primer (5'-3', note that the index is reverse complement)
Seib_25	AACCATAA	CAAGCAGAAGACGGCATAACGAGATTTATGGTTGTCTCGTGGGCTCG*G
Seib_26	ACCAGCAA	CAAGCAGAAGACGGCATAACGAGATTTGCTGGTGTCTCGTGGGCTCG*G
Seib_27	ACGCCGTT	CAAGCAGAAGACGGCATAACGAGATAACGGCGTGTCTCGTGGGCTCG*G
Seib_28	CAATATCC	CAAGCAGAAGACGGCATAACGAGATGGATATTGGTCTCGTGGGCTCG*G
Seib_29	CCGCCGCA	CAAGCAGAAGACGGCATAACGAGATTGCGGGCGGGTCTCGTGGGCTCG*G
Seib_30	CGATTCGG	CAAGCAGAAGACGGCATAACGAGATCCGAATCGGTCTCGTGGGCTCG*G
Seib_31	GAAGAACG	CAAGCAGAAGACGGCATAACGAGATCGTTCTTCGTCTCGTGGGCTCG*G
Seib_32	GTCTCGTT	CAAGCAGAAGACGGCATAACGAGATAACGAGACGTCTCGTGGGCTCG*G
Seib_33	GTTAGCGG	CAAGCAGAAGACGGCATAACGAGATCCGCTAACGTCTCGTGGGCTCG*G
Seib_34	GTTGGAAT	CAAGCAGAAGACGGCATAACGAGATATTCCAACGTCTCGTGGGCTCG*G
Seib_35	TGCGTATC	CAAGCAGAAGACGGCATAACGAGATGATACGCAGTCTCGTGGGCTCG*G
Seib_36	TTGCTTGC	CAAGCAGAAGACGGCATAACGAGATGCAAGCAAGTCTCGTGGGCTCG*G
Seib_37	AACCAGGT	CAAGCAGAAGACGGCATAACGAGATACCTGGTTGTCTCGTGGGCTCG*G
Seib_38	AAGAACCG	CAAGCAGAAGACGGCATAACGAGATCGGTTCTTGTCTCGTGGGCTCG*G
Seib_39	AAGACCGC	CAAGCAGAAGACGGCATAACGAGATGCGGTCTTGTCTCGTGGGCTCG*G
Seib_40	CCTAGATA	CAAGCAGAAGACGGCATAACGAGATTATCTAGGGTCTCGTGGGCTCG*G
Seib_41	CGTTCCAT	CAAGCAGAAGACGGCATAACGAGATATGGAACGGTCTCGTGGGCTCG*G
Seib_42	CTGGTTCG	CAAGCAGAAGACGGCATAACGAGATCGAACCAGGTCTCGTGGGCTCG*G
Seib_43	GGATTACC	CAAGCAGAAGACGGCATAACGAGATGGTAATCCGTCTCGTGGGCTCG*G
Seib_44	GGTATCTA	CAAGCAGAAGACGGCATAACGAGATTAGATACCGTCTCGTGGGCTCG*G
Seib_45	GTCGCTAG	CAAGCAGAAGACGGCATAACGAGATCTAGCGACGTCTCGTGGGCTCG*G
Seib_46	TCAGAGAT	CAAGCAGAAGACGGCATAACGAGATATCTCTGAGTCTCGTGGGCTCG*G
Seib_47	TCTATGGA	CAAGCAGAAGACGGCATAACGAGATTCCATAGAGTCTCGTGGGCTCG*G
Seib_48	TGAGGTTC	CAAGCAGAAGACGGCATAACGAGATGAACCTCAGTCTCGTGGGCTCG*G
Seib_49	AACCGAAC	CAAGCAGAAGACGGCATAACGAGATGTTGCGTTGTCTCGTGGGCTCG*G
Seib_50	AACCTATA	CAAGCAGAAGACGGCATAACGAGATTATAGGTTGTCTCGTGGGCTCG*G
Seib_51	ACCAAGTT	CAAGCAGAAGACGGCATAACGAGATAACTTGGTGTCTCGTGGGCTCG*G
Seib_52	ATACCTAC	CAAGCAGAAGACGGCATAACGAGATGTAGGTATGTCTCGTGGGCTCG*G
Seib_53	CGGTAGGT	CAAGCAGAAGACGGCATAACGAGATACCTACCGGTCTCGTGGGCTCG*G
Seib_54	CTAGTACC	CAAGCAGAAGACGGCATAACGAGATGGTACTAGGTCTCGTGGGCTCG*G
Seib_55	GGATGCTA	CAAGCAGAAGACGGCATAACGAGATTAGCATCCGTCTCGTGGGCTCG*G
Seib_56	GGTTCTGA	CAAGCAGAAGACGGCATAACGAGATTCAGAACCGTCTCGTGGGCTCG*G
Seib_57	TATCCTCG	CAAGCAGAAGACGGCATAACGAGATCGAGGATAGTCTCGTGGGCTCG*G
Seib_58	TCGAGAAG	CAAGCAGAAGACGGCATAACGAGATCTTCTCGAGTCTCGTGGGCTCG*G
Seib_59	TCTGGCGG	CAAGCAGAAGACGGCATAACGAGATCCGCCAGAGTCTCGTGGGCTCG*G
Seib_60	TCTTAGCT	CAAGCAGAAGACGGCATAACGAGATAGCTAAGAGTCTCGTGGGCTCG*G