

DNA Extraction Protocol & IIbRAD library Preparation

June 2014, updated Aug 2015, updated July 20, 2016.

Protocol: DNA Isolation

Reagents:

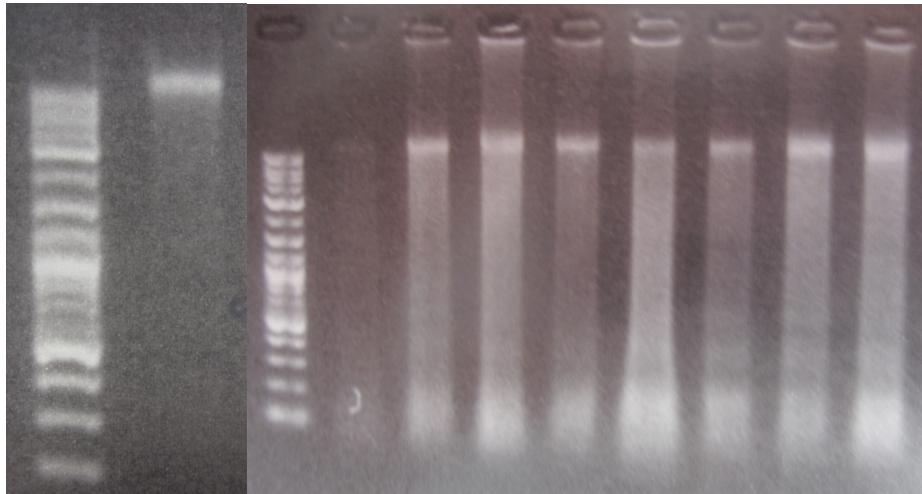
- A. DNA Dispersion Buffer for DNA extraction
- B. RNase A (1000 U/uL)
- C. PCA: 25:24:1 buffer-saturated phenol, chloroform, and isoamyl alcohol stored 4°C fridge
- D. 3M NaOAc
- E. 100% EtOH
- F. 80% EtOH
- G. Fresh milliQ water
- H. ZYMO RESEARCH - D4011 Genomic DNA Clean and Concentrator Kit

Steps (Time estimate: ~3.5 hrs.):

1. Samples should be stored in -20°C in >80% ethanol.
2. Use small petri dish. Get a sample, dry it briefly on tissue, place in petri dish and add 1mL of DNA Dispersion Buffer. Using forceps and razor blade scrape the tissue from the skeleton into the dispersion buffer and transfer to a labeled 1.5mL tube.
3. Repeat for all samples. Vortex all samples 1-2 seconds.
4. Incubate for 1 hour at room temp. Vortex several seconds after 30 minutes.
***** Note: While samples are lysing, prepare centrifuge for 4°C spin.
5. Spin samples at max speed at RT for 1 min to sediment any debris.
6. Transfer 750µl supernatant to a new sterile, labeled 1.5ml tube and put on ice.
7. In the fume hood add 750µl of PCA and ensure cap is on tight. Be careful PCA will burn skin so be sure to wear gloves and if PCA gets on them, switch them!
8. Vortex samples several seconds or shake tube and place on ice for 1 min.
9. Spin at max speed for 20 minutes at 4°C.
10. Pipette 550µl of the upper aqueous phase (Take care to avoid the interphase as it contains inhibiting proteins and can be very viscous! If you touch the interphase, re-spin for 5 minutes and aim for less) and put in a sterile, labeled tube. This tube will be the final tube so label clearly. Discard PCA waste in waste container.
11. Add 55µl (10%) 3M NaOAc and 1300µl (2.5x) 100% EtOH, vortex samples several seconds. Alternatively add the same volume of isopropanol (no sodium acetate) to precipitate the DNA.
12. Spin at max speed for 30 minutes at 4°C (this will pellet the DNA).
13. Pour off supernatant into liquid waste under the hood.

14. Add 500 μ l 80% EtOH and ensure lid is on tight. **Gently** wash EtOH around tube.
15. Spin at max speed for 2 minutes at 4°C.
16. Pour off ethanol supernatant and place upside down on Kimwipe.
17. Dry tubes for ~15 mins until no liquid can be seen in tube.
18. Leave tubes upright in tube holder covered in kimwipe for 5 additional minutes to ensure all EtOH is evaporated.
19. Re-suspend pellet in 50 μ l milliQ water.
20. RNase DNA samples with 0.5 μ l of RNaseA for one hour.
21. Purify DNA extractions using ZymoClean and Concentrate Kit according to manufacturer's protocol (ZYMO RESEARCH Genomic DNA Clean and Concentrator Kit Cat N D4011). That step should clean out RNase as well.
22. Load 1 μ l of each sample onto a 1.5 % agarose gel to check integrity. Genomic band with/without smear should be detected; if not DNA isolation was not successful.
23. Quantify the purified DNA using Quanti iT Picogreen dsDNA kit. It's extremely important to equalize the amount of DNA going into the first step. We usually use from 100 to 200ng of DNA per sample. Whatever number chosen, it should be the same value for all samples.
24. Prepare the 96 well plate with DNA. Total sample volume should be 4 μ l.

Sample DNA images



Protocol: DNA digest

Note: For best results, proceed only with 100-200 ng intact, high-quality genomic DNA in 4 uL (as little as 25 ng/uL). This concentration can be accomplished by vacuum drying or ethanol precipitation. We have successfully prepared samples from as little as 50 ng of DNA.

- ❖ Materials: BcgI, NEB Buffer #3, 150 uM SAM
 - ❖ Equipment: Heat block or thermocycler
1. Prepare the digestion master mix. Mix buffer and SAM. Add Bcg I restriction enzyme individually to the wells/tubes. The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra).

	1 rxn (uL)
NEB Buffer #3	0.6
150 uM SAM	0.4
BcgI (1 U/uL)	1

**SAM concentrations vary by vendor. Adjust your amount accordingly.

*** Don't add BcgI to the mix of buffer and SAM, you will add it separately to each reaction latter on

Master mix preparation tips:

- ✓ Add reagents to master mix in order of least expensive to most expensive to minimize the cost of making a mistake.
 - ✓ Make sure your total volume will fit in the tube you plan to mix it in BEFORE you start adding reagents. You may have to use two tubes.
 - ✓ Check off your reagents as you go to help keep track of what has been added.
2. Combine 1 uL master mix with each 4 uL (150ng) DNA sample (5 uL final volume). After buffer added to water/DNA mix add 1 ul of BcgI enzyme for each reaction. *Avoid bubbles at this step! Don't pipet to the second stop.*
 3. Incubate at 37C for 1 hour
 4. If using a heat-inactivated enzyme (e.g., BcgI, Alfl), inactivate the enzyme at 65C for 20 min.
 5. Return samples to ice

Optional: Load 1 uL of digested DNA on a 1% agarose gel alongside a comparable amount of intact DNA from the same sample to verify the effectiveness of the digest. Often you will not see much of a difference because the signs of an effective enzyme digest are subtle. Expect to see a slight downward shift in the high molecular weight DNA band and a subtle smear trailing downward from that band.

Protocol: DNA ligation

- ❖ Materials: 10 mM ATP, 10X T4 ligase buffer, 10 uM 5' Illumina NNRW adaptor, 10 uM anti-III RW adaptor, 10 uM 3' Illumina BC_{ii} adaptor, 10 uM antiBC_{ii} adaptor, T4 DNA ligase (NEB), nuclease-free water
- ❖ Equipment: Heat block or thermocycler

1. Prepare double-stranded adaptors by combining each pair of adaptors.
For Adaptor 1, mix 5III-NNRW (10 uM) with the same volume of anti-III RW (10 uM) and **three volume of water (1:1:3)**
Every sample gets this same adaptor 1.

For Adaptor 2, mix 3III-BC(1-12) (10 uM) with the same volume of anti-BC(1-12) (10 uM) and **three volumes of water (1:1:3)**

There are 12 different ligation 3III-barcodes for different samples

Incubate in thermocycler: 75°C 2', 75°C 1' – decrease 2 degree per cycle, repeat 25 cycles.

*This program takes ~40 mins. Plan to start this step 40-50 mins prior to the end of the gDNA digest process so both sample and adaptors are ready at the same time for the following step.

2. Prepare a ligation master mix for each barcoded 3' adaptor (up to 12). The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra). **Avoid bubbles!!** Don't pipet to the second stop.

	1 rxn (uL)
10 uM ATP	0.5
10X T4 ligase buffer	2
Adaptor 1	1
Adaptor 2	1
T4 DNA ligase	1
Nuclease-free H2O	13.5

3. Combine 19μl master mix with the remaining 6μl of digested DNA sample (25μl final volume)

4. Incubate at 4C overnight.

Note: Try to keep it around 12 hours to avoid over-ligating.

5. Heat inactivate at 65C for 10 minutes.

Protocol: Pooling ligations into groups

The purpose of this step is to equally pool samples with different 3' barcode primers into groups of (up to) 12. You can have up to 8 groups of 12 on a 96 well plate. Each group serves as an individual sample in the following Amplification and Purification steps.

Ideally, if the same amount of DNA went into the digest and ligation, ligation can be pooled together in equal proportions. Don't pool all the material! Mix 2µl of each ligation with **different 3' barcodes (!)** into one tube (usually one row on the 96 well plate). Seal the rest of the plate and store at -20C in case you will need to redo some samples.

Optionally, perform pcr or/and Qpcr to numerically equalize ligation before pooling (amendment)

Ligations always stay on ice when not in the -20C! NEVER keep them at room temperature!

Protocol: DNA amplification

Now you are operating with ligations mixed together as new samples. From this point on in this protocol, samples refer to groups of pooled ligations.

- ❖ Materials: Nuclease-free water, 2.5 mM dNTPs, 10 uL IC1-P5, 10 uL IC2-P7, 10 uM Mpx primer, 10X HF buffer, Phusion polymerase (NEB)
- ❖ Equipment: Thermocycler

1. Prepare the PCR master mix. The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra).

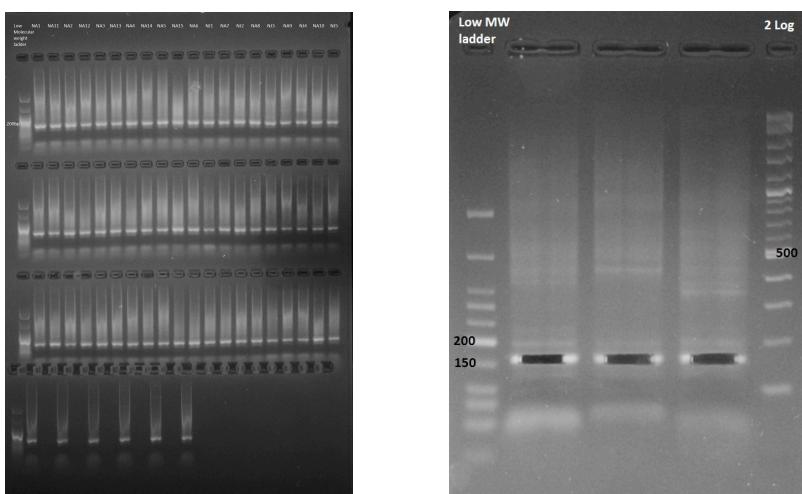
	1 rxn (uL)
Nuclease-free H2O	20.5
2.5 mM dNTP	6
10 uM Mpx primer	0.44
10 uM IC1-P5	0.6
10 uM IC2-P7	0.6
5X HF buffer	12
Phusion polymerase	0.6

2. Combine 40.6μl master mix with 15μl of pooled ligations
3. Add 4.4μl of 1 μM Ill-Rad-BC (barcoding-bearing) primer to each reaction
4. Amplify in a thermocycler using the following profile:

70C 30 sec (92C 15 sec, 65C 15sec, 72C 15 sec) for **optimum # of cycles**

Run 12-14 cycles first and check 3-5 uL on gel. If needed add 1-2 cycles. In our experience 12-16 cycles is usually the right number.

Note: You may also see a 130 bp band. This is an artifact from the excess of adaptors in the previous ligation. Do not confuse it with the 170 bp band.



Protocol: DNA purification

The product of interest's size is 170bp. There are different ways of extraction of that band. The cheapest is gel-electrophoresis, but other methods could be used.

- ❖ Materials: Agarose, gel buffer, SYBR Green I nucleic acid stain, low molecular weight ladder, 6X loading dye, nuclease-free water
 - ❖ Equipment: Gel box, UV light, balance, blue light
1. Add ~12 uL of 6X loading dye to each tube/well after PCR is complete and samples have cooled.
 2. Centrifuge the tubes/plate briefly
 3. Mix the samples with the loading dye by aspirating and dispensing the sample a few times before loading the well.
 4. Run 50 uL of each reaction + ~12 uL loading dye on a large 2% agarose gel with SYBR Green nucleic acid stain.
 5. Run the gel slowly at 100 V for 90 minutes or until the bands between 150 - 200 bp are clearly resolved
 6. Use a blue light gel illuminator to safely cut the 170 bp band with a clean razor blade
Note: Be careful to cut only the 170 bp band. There might be another band at approximately 130 bp. This is a result of excess primers and should be avoided.
 7. Transfer the cut band to a clean 1.5 mL centrifuge tube
 8. Add 15 uL of nuclease-free water to each tube. Make sure the water and gel pieces are in contact and incubate overnight at 4C to let DNA diffuse into the water from the gel.
 9. The following day, centrifuge each tube for 2 minutes at max speed.
 10. Remove as much liquid as possible and dispense it into fresh strip tubes/96 well plate
 11. Alternatively, use a gel-extraction kit like QIAGEN QIAquick Gel Extraction Kit CatN-28704 for example. Elute in 15 uL.
 12. Run Quant-IT picogreen DS DNA assay (Life Technologies P7589) to determine the final concentrations of the eluted product in order to mix libraries in equal proportions.
 13. Store barcoded and size-selected DNA at -20C until further use

Picogreen assay Protocol: (Life Technologies P7589)

- 1) Place 100ul 1X TE into all first column_wells except B1
- 2) Add 150ul of stock curve (@ 2ug/ml) into B1
- 3) Serially dilute standards by taking 50ul of B1, mixing into C1, taking 50ul of C1, mixing into D1, and so on until taking 50ul from H1 and throwing it out.
- 4) To all sample wells, add 98ul of 1X TE.
- 5) Add 2ul sample DNA to sample wells.
- 6) Mix Pico Green Master mix: 99.5ul 1XTE + 0.5ul PicoGreen for one sample. Multiply accordingly (plus 8 wells for DNA standard).
- 7) Add 100ul of master mix to all standard and sample wells, bringing up final volumes in each well to 200.
- 8) Turn on the plate reader (SpectraMaxM2 in our case).
- 9) Open software (SoftMaxPro V5 in our case).
- 10) Select the premade program and run it. Read the fluorescence (excitation 480nm, emission 520nm).
- 11) Save the data into txt file, assemble the results in Excel in two-column form – well, reading - save it as comma-delimited (.csv) file. The file must contain all A1-H1 wells (blank and calibrators) plus an arbitrary number of sample wells, in any order.
- 12) Use picogreen.R script to calculate sample concentrations (ng/ul in the original sample).

Protocol: Quality check PCR

- ❖ Materials: milliQ water, 2.5 mM dNTPs, 10X PCR buffer, 10 uM IC2-P7 primer, 10 uM IC1-P5 primer, Titanium Taq, agarose, gel buffer, GelRed, 6X loading dye, 2log ladder
- ❖ Equipment: Thermocycler, gel box, balance, UV light box, microwave

The quality check PCR ensures that the correct size fragments were selected. The P7 and P5 primers will amplify from each end of the DNA to ensure that the intervening fragment is of the correct size (400-500 bp).

1. Prepare a quality check PCR reaction master mix. This master mix is calculated for 1 uL uL of template, so adjust the volume of water accordingly if you use more DNA.

	1 rxn (uL)
H2O	6.6
2.5 mM dNTPs	1
10X PCR buffer	1
10 uM IC2-P7 primer	0.1
10 uM IC1-P5 primer	0.1
Titanium Taq	0.2

2. Pipet 9 uL of master mix into each tube/well
3. Pipet 1 uL of gel-extracted template DNA into each tube/well
4. Amplify in a thermocycler using the following profile:
95C 5 min (95C 40 sec, 63C 1 min, 72C 30 sec) for 10-12 cycles
5. Run 5 uL of product on a 1.5 % agarose gel. The size of the product should match the size of the cut band (~170 bp) and brightness should be in agreement with picogreen assay results.

Protocol (optional): equalizing and pooling ligation into groups

- ❖ Materials: nuclease-free water, 2.5 mM dNTPs, 10 uM IC1-P5, 10 uM IC2-P7, 10 uM Mpx primer, 5X HF buffer, Phusion polymerase, agarose, gel buffer, GelRed, 2log ladder, 6X loading dye; For qPCR: nuclease-free water, SYBR Green Master Mix, 10 uM Mpx primer, any Ill-RAD-BC primer, 10 uM IC1-P5, 10 uM IC2-P7
- ❖ Equipment: Thermocycler, balance, microwave, gel boxes, UV light box; For qPCR: Roche 480 LightCycler

Use the following procedures (i and ii) to prepare a test-scale PCR to compare ligation efficiencies. Do the qPCR (ii) first and compare results with the gel (i). Ideally these results would match, but if they differ and you must choose pick the gel results.

Ligations always stay on ice when not in the -20C! NEVER keep them at room temperature!

- (i) Prepare a standard PCR as described in the “Amplification” step but smaller volume (15 or 20ul). Use 14 cycles and then determine the relative efficiency of amplification by examining the relative brightness of the bands run on a 1.5% agarose gel. Mix 5, 10, 15 or 20 uL of each ligation in groups of 12 depending on how bright (brightest = 5 uL) or dim (dimmest = 20 uL) the bands were. If all bands look the same – pool 12 ligations (with different BC ligated adapters) in equal proportion.
- (ii) Set up a qPCR and analyze Critical Thresholds.
 - a) Prepare the master mix below. The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra). Do two replicas for each sample.

	1 rxn (uL)
H ₂ O	5.53
SYBR Green Mix	7.5
10 uM Mpx	0.07
1 uM any Ill-BC primer	0.7
10 uM IC1-P5	0.1
10 uM PC2-P7	0.1
 - b) Add 14 uL of master mix to each well that will receive template + a few no template controls. AVOID BUBBLES
 - c) Add 1 uL of each ligation reaction to the appropriate wells in the qPCR plate. AVOID BUBBLES
 - d) Place a Roche cover film on the plate and seal it very carefully and thoroughly
Seal around the edges carefully.
Avoid touching the film with your fingers to prevent oils from sticking to the film.
Press out any bubbles in the film.

- e) Briefly centrifuge the plate
- f) Turn on the qPCR machine and open
- f) LightCycler 480 software
- g) Select “New Experiment from Template”
- h) Change the “Reaction Volume” to 15 uL
- i) Under “amplification” change the settings to match the settings below:

# Cycles	Step	Temperature	Acquisition	Time
1x	Pre-Incubation	95C	None	5 min
35x	Amplification	95C	None	40 sec
		65C	None	1 min
		72C	Single	30 sec
1x	Melting Curve	95C	None	5 sec
		65C	None	1 min
		97C	Continuous	
1x	Cooling	40C	None	10 sec

- j) Change amplification cycles to 35
- k) Select your wells under “Subset Editor”. Add a new subset using the “+” button and highlight the appropriate wells.
- l) Save your experiment by clicking the save button that looks like a floppy disk. Save your file in a meaningful folder and give it a very specific name.
- m) Run the PCR program on the qPCR machine. Samples should start to amplify around 10-15 cycles. No template controls may start to amplify around 30 cycles.
- n) Export your data onto a flash drive and move it to your working computer into a directory that contains the mix_illumina_qpcr.R file
- o) Arrange the data in Excel in the form of a table with four columns: sam (sample name), lane (intended HiSeq lane), conc and ct (qPCR result for this sam-conc combination).
- p) Save the data from Excel as comma-separated values (.csv) into the working directory
- q) Open the script mix_illumina_qpcr.R in R
- r) Follow the instructions given in comments within the script
- s) Mix samples according to the final mixing table (output of R program)

Protocol: Making Dispersion Buffer

Recipe for dispersion buffer comes from Generation of cDNA Libraries page 106

*Handle all reagents under fume hood

- ❖ Materials: Guanadine thiocyanate (light sensitive, stored in flame cabinet, may be wrapped in foil), sodium citrate dihydrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$; pH of a solution at 25.0°C 7.0-9.0), beta-mercaptoethanol (stored in flame cabinet), milliQ water.
- ❖ Equipment: Plate with stirring rod, beaker for mixing, glass bottle for storage.

Buffer Contents:

Reagent	Target Concentration	Molecular Mass
Guanadine thiocyanate	4 M	118.16
Sodium Citrate dihydrate	30 mM	294.10
B-mercaptoethanol	30 mM	Stock concentration = 14.3 M

Recipe for making 50 ml of buffer:

Sodium citrate dihydrate: 0.441 g

Guanadine thiocyanate: 23.632 g

β -mercaptoethanol: 105 μl

1. Set up stirring plate under hood
2. Set 50 ml of milli Q water stirring
3. Add reagents slowly to stirring liquid
4. transfer dispersion buffer to labeled storage container
5. Store at 4°C protected from light

Oligonucleotide sequences for Illumina HiSeq

Name	Sequence (5' – 3')
5ILL-NNRW	CTACACGACGCTCTTCCGATCTNNRWCCNN
Anti5ill-NNRW	GGWYNNAGATCGG/3InvdT ¹ /
3ILL-NN	CAGACGTGTGCTCTTCCGATCTNN
anti-ILL	AGATCGGAAGAGGC/3InvdT ¹ /
5ILL-NG ²	CTACACGACGCTCTTCCGATCTNNRWCCNG
3ILL-NG ²	CAGACGTGTGCTCTTCCGATCTNG
ILL-Mpx ³	AATGATACTGGCACCACCGAGATCTACACTCTTCCCTACACGACGCTTCCGAT
ILL-RAD-bc ⁴	CAAGCAGAAGACGGCATACGAGAT [barcode] ⁵ GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
IC1-P5	AATGATACTGGCACCACCGA
IC2-P7	CAAGCAGAAGACGGCATACGA

1 InvdT: inverted dT to prevent extension by DNA polymerase.

2 These two adaptors can be used to reduce representation of the 2b-RAD tags down to approximately 1/16th of the total number. This is a useful cost-lowering trick for applications such as basic population genetics, relatedness analysis, or QTL mapping, the power of which would max out already at a few hundred polymorphic markers.

3 This is a standard Illumina “universal” primer in TrueSeq v.3 (configuration 5' P5-index2-R1primingSite 3')

4 “bc” stands for “barcode”, and is typically replaced by the barcode number (or other barcode-specific identifier) in the actual name of the primer. This primer can be substituted for a standard TruSeq v.3 barcoded oligo of the configuration 5' P7-Index1-R2primingSite 3'.

5 barcode: a 6-base sequence easily distinguishable from other sequences on that same sequencing run. A list of good working barcodes can be found here:

<https://wikis.utexas.edu/display/GSAF/Illumina+-+all+flavors>

BUT NOTE that the barcode sequences listed at that site are in fact REVERSE COMPLEMENTS relative to what needs to be written in the ILL-RAD-bc oligo (we had so many fun moments because of that)

Secondary-barcoded adapters for Ligation (to be used instead of 3ILL-NN and anti-ILL)

3illBC1	CAGACGTGTGCTCTTCCGATCT ACAC NN
3illBC2	CAGACGTGTGCTCTTCCGATCT GTCT NN
3illBC3	CAGACGTGTGCTCTTCCGATCT TGGT NN
3illBC4	CAGACGTGTGCTCTTCCGATCT CACT NN
3illBC5	CAGACGTGTGCTCTTCCGATCT GATG NN
3illBC6	CAGACGTGTGCTCTTCCGATCT TCAC NN
3illBC7	CAGACGTGTGCTCTTCCGATCT CTGA NN
3illBC8	CAGACGTGTGCTCTTCCGATCT AAGC NN
3illBC9	CAGACGTGTGCTCTTCCGATCT GTAG NN
3illBC10	CAGACGTGTGCTCTTCCGATCT GACA NN
3illBC11	CAGACGTGTGCTCTTCCGATCT GTGA NN
3illBC12	CAGACGTGTGCTCTTCCGATCT AGTC NN
antiBC1	GTGT AGATCGGA/3InvdT/
antiBC2	AGAC AGATCGGA/3InvdT/

antiBC3	ACCA AGATCGGA/3InvdT/
antiBC4	AGTG AGATCGGA/3InvdT/
antiBC5	CATC AGATCGGA/3InvdT/
antiBC6	GTGA AGATCGGA/3InvdT/
antiBC7	TCAG AGATCGGA/3InvdT/
antiBC8	GCTT AGATCGGA/3InvdT/
antiBC9	CTAC AGATCGGA/3InvdT/
antiBC10	TGTC AGATCGGA/3InvdT/
antiBC11	TCAC AGATCGGA/3InvdT/
antiBC12	GACT AGATCGGA/3InvdT/

Barcoded PCR primers (can be substituted for standard TruSeq v.3 oligos: 5' P7-**Index1**-R2primer 3')

ILL-RAD01	CAA GCA GAA GAC GGC ATA CGA GAT CGT GAT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD02	CAA GCA GAA GAC GGC ATA CGA GAT ACA TCG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD03	CAA GCA GAA GAC GGC ATA CGA GAT GCC TAA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD04	CAA GCA GAA GAC GGC ATA CGA GAT TGG TCA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD05	CAA GCA GAA GAC GGC ATA CGA GAT CAC TGT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD06	CAA GCA GAA GAC GGC ATA CGA GAT ATT GGC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD07	CAA GCA GAA GAC GGC ATA CGA GAT GAT CTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD08	CAA GCA GAA GAC GGC ATA CGA GAT TCA AGT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD09	CAA GCA GAA GAC GGC ATA CGA GAT CTG ATC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD10	CAA GCA GAA GAC GGC ATA CGA GAT AAG CTA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD11	CAA GCA GAA GAC GGC ATA CGA GAT GTA GCC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD12	CAA GCA GAA GAC GGC ATA CGA GAT TAC AAG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD13	CAA GCA GAA GAC GGC ATA CGA GAT TTG ACT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD14	CAA GCA GAA GAC GGC ATA CGA GAT GGA ACT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD15	CAA GCA GAA GAC GGC ATA CGA GAT TGA CAT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD16	CAA GCA GAA GAC GGC ATA CGA GAT GGA CGG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD17	CAA GCA GAA GAC GGC ATA CGA GAT CTC TAC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD18	CAA GCA GAA GAC GGC ATA CGA GAT GCG GAC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD19	CAA GCA GAA GAC GGC ATA CGA GAT TTT CAC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD20	CAA GCA GAA GAC GGC ATA CGA GAT GGC CAC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD21	CAA GCA GAA GAC GGC ATA CGA GAT CGA AAC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD22	CAA GCA GAA GAC GGC ATA CGA GAT CGT ACG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD23	CAA GCA GAA GAC GGC ATA CGA GAT CCA CTC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD24	CAA GCA GAA GAC GGC ATA CGA GAT GCT ACC GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD25	CAA GCA GAA GAC GGC ATA CGA GAT ATC AGT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD26	CAA GCA GAA GAC GGC ATA CGA GAT GCT CAT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD27	CAA GCA GAA GAC GGC ATA CGA GAT AGG AAT GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD28	CAA GCA GAA GAC GGC ATA CGA GAT CTT TTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD29	CAA GCA GAA GAC GGC ATA CGA GAT TAG TTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD30	CAA GCA GAA GAC GGC ATA CGA GAT CCG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD31	CAA GCA GAA GAC GGC ATA CGA GAT ATC GTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD32	CAA GCA GAA GAC GGC ATA CGA GAT TGA GTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD33	CAA GCA GAA GAC GGC ATA CGA GAT CGC CTG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD34	CAA GCA GAA GAC GGC ATA CGA GAT GCC ATG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD35	CAA GCA GAA GAC GGC ATA CGA GAT AAA ATG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD36	CAA GCA GAA GAC GGC ATA CGA GAT TGT TGG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD37	CAA GCA GAA GAC GGC ATA CGA GAT ATT CCG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT
ILL-RAD38	CAA GCA GAA GAC GGC ATA CGA GAT AGC TAG GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT

ILL-RAD94 CAA GCA GAA GAC GGC ATA CGA GAT ATA GAA GTG ACT GGA GTT CAG ACG TGT GCT CTT CCG AT