Protocol for population-based barcode amplification by deep sequencing

This protocol is used to amplify the barcodes from fresh or frozen experimental samples and prepare them for multiplex DNA sequencing. This protocol is also used for amplification of the plasmid pool for a reference library.

General tips before you start:

- Link to the lysis buffer Viagen http://www.viagenbiotech.com/index.php/directpcr-lysis-reagents/cell/directpcr-lysis-reagent-cell.html
- Run a positive control (using 10ng of plasmid DNA)
- We find that these plates are the best to allow for the large PCR volumes (they can hold 300 uL). The reactions are too close to the top of the wells in other plates we have used, increasing the risk of contamination. https://www.fishersci.com/shop/products/thermo-scientific-96-well-non-skirted-plates-17/ab0600
- We seal all of our PCR plates with metal foil for long term storage to minimize evaporation loss.

The protocol entails broad steps;

1. Lysis in Viagen buffer
2. 1st round PCR with common primer sets
3. 2nd round PCR with a different primer for every sample
4. Pooling for DNA sequencing

1. Lysis in Viagen buffer.

Conduct this step in a ultra clean Pre-PCR room

- Cell pellets (of up to 0.5 x 10^6 cells) should be spun down into PCR tubes or plates.
- One sample every 2 wells, to allow for technical replicate splitting (see next). Thus, for a full 96 well plate, there are 48 samples. See template below.

		1	2	3	4	5	6	7	8	9	10	11	12
A	SAMPLES												
B	Empty												
C	SAMPLES												
D	Empty												
E	SAMPLES												
F	Empty												
G	SAMPLES												
H	Empty												

- Each sample must first be resuspended in 40µL Lysis buffer which includes
    - 40 µL of Viagen lysis buffer for cells
    - 2ul of 20mg/ml proteinase K
    - (make a master mix for Viagen and proteinase K with 5% extra for losses)
- Try and include a negative control at this stage. Meaning, a well with no cells, but that will undergo a mock lysis, PCR, etc. This will control for any contamination of reagents or at some point during the entire process.

IMPORTANT: *For the next steps, ensure there is no spill or cross-contamination between wells.

- Dispense the lysis buffer into a clean disposable multichannel tray
- Using a multichannel with 40 µL capacity and 200 µL tips, resuspend the cells thoroughly (10 rounds of the pipette) and dispose the tips
- Repeat until all of the samples have been resuspsended
- Place a seal on the PCR plate.
- Run the plates on the 'Viagen'protocol. (55°C for 2 hrs, then 85°C for 30 min, then 95°C for 5 min).
- The samples can either be stored at -20°C at this stage.
- Alternatively, the samples can be used directly for the next step of the protocol.

2. 1st round PCR with common primer sets

Conduct this step in the hood in the Pre-PCR room

In this step, a PCR master mix (160 µL per sample) is added to each 40 µL of viagen/cell lysis mixture. We mix each sample, then transfer 100 µL to the below neighbouring rows,

effectively splitting them in two prior to the PCR. This ensures separate products for our "self-self" PCR comparisons later. We only include barcodes present in both technical replicates in the final analysis.

Based on the number of samples, calculate the amount of reagent that is needed and make 5% more. Make a master mix based on the amounts per sample below.

Add 160 uL of PCR mix to add to 40 µL of Viagen/cell lysis mix. (total final = 200 µL)

	Per sample
Water	100
Buffer (5x)	40
dNTPs (10 mM each)	4
PCR1 FWD stagger (10 µM)	5
PCR1 REV stagger (10 µM)	5
Q5 polymerase	2
Total	160 µL

*Keep on ice until you are ready to add to the samples.

- Thaw the viagen/cell lysis pellets if frozen.
- Give the samples a quick spin to ensure minimal liquid in the lids
- Keep the samples on ice
- Remove the sticker with care
- Take a fresh multi-well channel reagent tray and add the PCR mix
- *Use filter tips for the 200 µL multichannel pipette
- Dispense 160 µL of PCR mix to the first wells of the experiment, then mix well.
- Without changing tips, change the setting to 100 µL of the multichannel and aliquot 100µL of the mix to the neighbouring empty well.
- Discard the tips
- Repeat this for each row/column on the sample until every well contains 100 µL of lysate/PCR mix and has been 'split' in two.
- Reapply the lids/stickers of the well ready for PCR

PCR program

98°C	3 min		
98°C	15 sec	\	
68°C	15 sec	}	25 cycles
72°C	30 sec	/	
72°C	5 min		
4°C	for ever		

3. 2nd round PCR with indexed primers

Add all reagents together in the NO DNA room before DNA is added at the bench.

Broad steps:

1. Create diluted stock of index primers (see last page of protocol).
2. Prepare a master PCR mix and aliquot into multi-well plates
3. Aliquot index primers into wells
4. Add the template from the 1st PCR product to each well and seal.
5. Run PCR2
6. Run on gel
7. Pool samples for deep sequencing

1. Master Mix

Based on the number of samples, calculate the amount of reagent that is needed and make 5% more. Make a master mix based on the amounts per sample below and keep on ice. Do this in the NO DNA room.

	Per sample
Water	16.75
Buffer (5x)	5
dNTPs (10 mM each)	0.5
Q5 DNA polymerase	0.25
Total	22.50ul

2. Aliquot index primers into wells

- Take a fresh multiwell channel reagent tray and add the master mix
- *Use filter tips for the 200 µL multichannel pipette
- Aliquot 22.5µl of the master mix into 96 well PCR plates
- Aliquot 1.5µL from the Dual Index Plate using a multichannel. A1 in this plate should go to A1 on the sample plate and so on. It is critical to change your tips between

samples as not to contaminate indexes. *(see end of protocol for primer plate layout, plate contains both F and R primers)

- Each well needs a separate index, even if it is the split from the same sample for "Self-self" comparisons. NTC also need separate indices.
- **Take plate out of the NO DNA room and to your bench to add the DNA from PCR1.**

3. Add template into wells.

- Thaw the PCR product from the 1st PCR at room temp.
- Aliquot 1 µL into each well of the plates, with a multichannel. Make sure you remember which index primer correlates to which sample.
- Replace with a fresh PCR lid.

4. PCR incorporating sequencing primers

PCR program 2nd round

98°C	3min		
98°C	15 sec	\	
68°C	15 sec	}	20 cycli
72°C	30 sec	/	
72°C	5 min		
4°C	for ever		

Run all products on a gel

- 2% agarose with SYBRSafe
- 100 bp ladder
- 280-300bp size

Important notes for sequencing

- Check agarose to see if every reaction worked. There are a few non-specific bands that are a higher molecular weight than the barcode. Ignore these, they are a PCR artifact

resulting from a PCR "bubble" and do not affect sequencing. If all samples worked, pool at equal volumes (5 uL per sample). Repeat PCR2 again for any samples that failed.

- The no template control will have a strong primer dimer band in it that is 200 bp in length. This will help understand if samples have failed as they will also have this band present in them.
- Clean up pooled barcode samples using 1X bead clean up with Agencourt Ampure XP DNA beads.
- Submit to sequencing core. If they run a tapestation, they may get weird bands appearing in places that do not correspond to the barcode amplicon. Again, this is a PCR "bubble" and will denature upon preparation for sequencing. However, make sure the sequencing core are aware of this as they need to account for all of the peaks present on the tapestation when they are calculating their final loading concentration. If they use qPCR to quantify then this is fine.
- As a general rule, we sequence each sample at 2 million reads. So a plate of 96 samples we would sequence at ~200M 75bp single end reads .

Primer Sequences:

PCR1: same primers for every sample

PCR1 FWD stagger is a mixture of the following 4 primers at a final conc of 10uM:

ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGGATCctgaccatgtacgattg
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNCGGATCctgaccatgtacgattg
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCGGATCctgaccatgtacgattg
ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNCGGATCctgaccatgtacgattg

PCR1 REV stagger is a mixture of the following 4 primers at a final conc of 10uM:

ACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCGCTAGCCTAGTCAGTACGCAT
ACTGGAGTTCAGACGTGTGCTCTTCCGATCTNGCGCTAGCCTAGTCAGTACGCAT
ACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNGCGCTAGCCTAGTCAGTACGCAT
ACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNGCGCTAGCCTAGTCAGTACGCAT
ACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNGCGCTAGCCTAGTCAGTACGCAT

PCR2: introducing an index

	FWD	INDEX READ MISEQ	INDEX READ NEXTSEQ
PCR2 FWD i501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACG	TATAGCCT	AGGCTATA
PCR2 FWD i502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACG	ATAGAGGC	GCCTCTAT
PCR2 FWD i503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACG	CCTATCCT	AGGATAGG
PCR2 FWD i504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACG	GGCTCTGA	TCAGAGCC
PCR2 FWD i505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACG	AGGCGAAG	CTTCGCCT
PCR2 FWD i506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACG	TAATCTTA	TAAGATTA
PCR2 FWD i507	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACG	CAGGACGT	ACGTCCTG
PCR2 FWD i508	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACG	GTACTGAC	GTCAGTAC

	REV	INDEX READ
PCR2 REV i701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGC	ATTACTCG
PCR2 REV i702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGC	TCCGGAGA
PCR2 REV i703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGC	CGCTCATT
PCR2 REV i704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGC	GAGATTCC
PCR2 REV i705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGC	ATTCAGAA
PCR2 REV i706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGC	GAATTCGT
PCR2 REV i707	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGC	CTGAAGCT
PCR2 REV i708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGC	TAATGCGC
PCR2 REV i709	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGC	CGGCTATG
PCR2 REV i710	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGC	TCCGCGAA
PCR2 REV i711	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGC	TCTCGCGC
PCR2 REV i712	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGC	AGCGATAG

Dual Index Primer plate layout.

Make several plates containing 50 uL total of FWD and REV PCR2 primers at a final 10 uM concentration. Store at -20 degrees.

		i701	**i702**	**i703**	**i704**	**i705**	**i706**	**i707**	**i708**	**i709**	**i710**	**i711**	**i712**
		1	2	3	4	5	6	7	8	9	10	11	12
i501	A	i701/i501	i702/i501	i703/i501	i704/i501	i705/i501	i706/i501	i707/i501	i708/i501	i709/i501	i710/i501	i711/i501	i712/i501
i502	B	i701/i502	i702/i502	i703/i502	i704/i502	i705/i502	i706/i502	i707/i502	i708/i502	i709/i502	i710/i502	i711/i502	i712/i502
i503	C	i701/i503	i702/i503	i703/i503	i704/i503	i705/i503	i706/i503	i707/i503	i708/i503	i709/i503	i710/i503	i711/i503	i712/i503
i504	D	i701/i504	i702/i504	i703/i504	i704/i504	i705/i504	i706/i504	i707/i504	i708/i504	i709/i504	i710/i504	i711/i504	i712/i504
i505	E	i701/i505	i702/i505	i703/i505	i704/i505	i705/i505	i706/i505	i707/i505	i708/i505	i709/i505	i710/i505	i711/i505	i712/i505
i506	F	i701/i506	i702/i506	i703/i506	i704/i506	i705/i506	i706/i506	i707/i506	i708/i506	i709/i506	i710/i506	i711/i506	i712/i506
i507	G	i701/i507	i702/i507	i703/i507	i704/i507	i705/i507	i706/i507	i707/i507	i708/i507	i709/i507	i710/i507	i711/i507	i712/i507
i508	H	i701/i508	i702/i508	i703/i508	i704/i508	i705/i508	i706/i508	i707/i508	i708/i508	i709/i508	i710/i508	i711/i508	i712/i508