**CircleSeq from aged cells**

Use LoBind tubes and siliconized (orange) tips throughout this prep. All incubations are in a PCR machine with the lid set 10º higher

Starting material is gDNA from one 125ml ageing culture (24-48 hours) purified by standard low cell gDNA prep and re-suspended overnight in 20μl 1xTE

To 20μl gDNA, add 15.5μl CutSMART

 0.75μl RNase T1 (Fermentas)

 118.75μl water

 flick to mix

Incubate 15 min at RT then split into 3x 49μl and 1x 3μl in 0.5ml tubes

To the 3μl aliquot, add 13μl 0.1x TE and store at -30º

Each 49μl aliquot gets a different restriction enzyme. These must all cut the rDNA and 2 micron plasmid, but otherwise can be varied

a) 1μl EagI-HF (NEB)

b) 1μl PvuI-HF (NEB)

c) 0.5μl PvuII-HF (NEB) – add 0.5μl of this each hour as enzyme is unstable

Incubate these digests 4 hours at 37º, adding enzyme every hour for unstable enzymes

To each tube, add 6μl 10mM ATP[[1]](#footnote-1)

 1μl CutSMART

 1μl RecBCD (NEB)

 1μl ExoI (NEB)

Incubate overnight at 37º

Extract digests with 60μl phenol:chloroform pH8

Add 1μl GlycoBlue

 6μl 3M NaOAc pH5.2

 150μl ethanol

Incubate 1 hour at -30º (no longer), spin 15min top speed 4ºC, wash 70% ethanol

Dry pellets for 10min at RT, dissolve 10min in 45μl 0.1x TE

Add 6μl CutSMART

 6μl 10mM ATP

 1μl RecBCD

 1μl ExoI

 1μl same restriction enzyme as previous day

Incubate overnight at 37º

Extract with phenol chloroform and precipitate as above (addition of more GlycoBlue is not necessary), dissolve pellet in 33μl 0.1xTE[[2]](#footnote-2)

Combine the three tubes, add 10μl 3M NaOAc pH5.2, 250μl ethanol, 1 hour at -30º spin, wash and dissolve pellet in 16μl 0.1x TE

To the digested DNA and also the input material saved from the first day, add:

 2μl Fragmentase buffer

 2μl NEBNext fragmentase AFTER vortexing enzyme 3s

Incubate 37º for 45min EXACTLY

Add 5μl 0.5M EDTA then 25μl water

AMPure clean with 50μl beads, elute with 26.5μl 0.1xTE to get 25.5μl back

Add 3.5μl NEBNext Ultra II end repair buffer

 1.5μl NEBNext Ultra II end repair enzyme

Pipette to mix and incubate 30 min at 20º then 30 min at 65º

 spin briefly

Add 1.25μl diluted NEBNext adaptor (1:10 for input and 48 hour old digested samples, 1:25 for 24 hour old digested samples[[3]](#footnote-3))

 0.5μl ligation enhancer

 15μl NEBNext Ultra II ligation mix

Pipette to mix and incubate 15 min at 20º

Add 1.5μl USER enzyme and incubate 15 min at 37º

Clean with 44μl AMPure beads, elute with 31μl 0.1xTE for 30μl yield

Clean again with 27μl AMPure beads, elute with 23.5μl 0.1xTE for 22.5μl yield

Run test PCRs [use these to work out the actual cycle number needed to give minimal amplification. This is a good idea as it is hard to predict and these libraries are prone to over-amplification due to high cycle numbers required]

In PCR tubes, mix 1.25μl library

 0.4μl NEBNext index oligo

 0.4μl NEBNext universal oligo

 5μl NEBNext Ultra II PCR mix

 2.95μl water

98º 30s

98º 10s \

65º 75s / x N cycles (suggested N: 8 for inputs, 16 for 48 hour, 18 for 24 hour)

65º 5 min

4º hold

Clean with 9μl AMPure beads, elute in 2.5μl 0.1x TE to get 1.5μl back

Run undiluted sample directly on Bioanalyzer

Calculate ideal cycle number for each library (you can try to keep pairs of control vs experimental with the same cycle number if not too different)

The aim is for a yield in the range 1.2-2nM. This is very low compared to most libraries but anything above 1nM can be sequenced and our facility can go lower than this even

The tests will yield the same concentration (roughly) as your final 1:4 dilution, in other words if the concentration of the test is 0.3-0.5nM, you are spot on

The circle libraries always give a higher molecular weight than the inputs, probably due to low complexity

Amplify libraries:

In PCR tubes, mix 21μl library

 2μl NEBNext index oligo

 2μl NEBNext universal oligo

 25μl NEBNext Ultra II PCR mix

98º 30s

98º 10s \

65º 75s / x N cycles

65º 5 min

4º hold

Clean with 45μl AMPure beads, elute with 26μl 0.1xTE for 25μl yield

Clean again with 22.5μl AMPure beads, elute with 11μl 0.1xTE for 10.5μl yield

Dilute 0.5μl with 1.5μl water for Bioanalyzer

1. ATP solution should be stored at -80º for long term, split the 1ml aliquots into 100μl aliquots for short term storage at -30º [↑](#footnote-ref-1)
2. The pellet is often large and white at this point, sometimes on the previous day too. This should disappear on re-precipitation. [↑](#footnote-ref-2)
3. The yield of circles is much higher from highly aged cells – 24 to 48 hours is a big difference, and beware of ageing conditions that slow the ageing process [↑](#footnote-ref-3)