PCR amplicon next generation sequencing V.3

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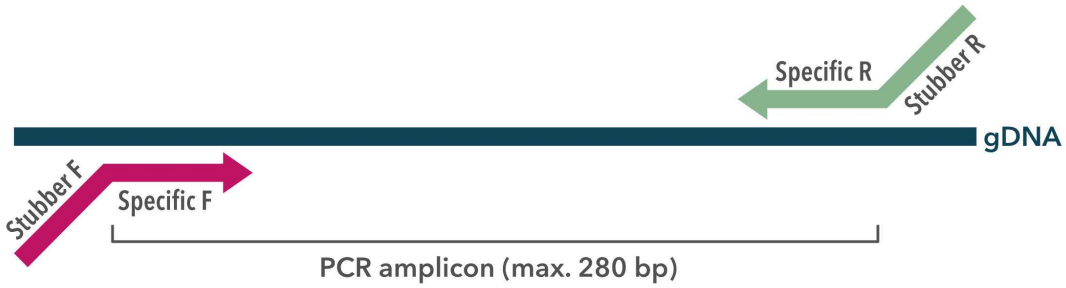
ABSTRACT

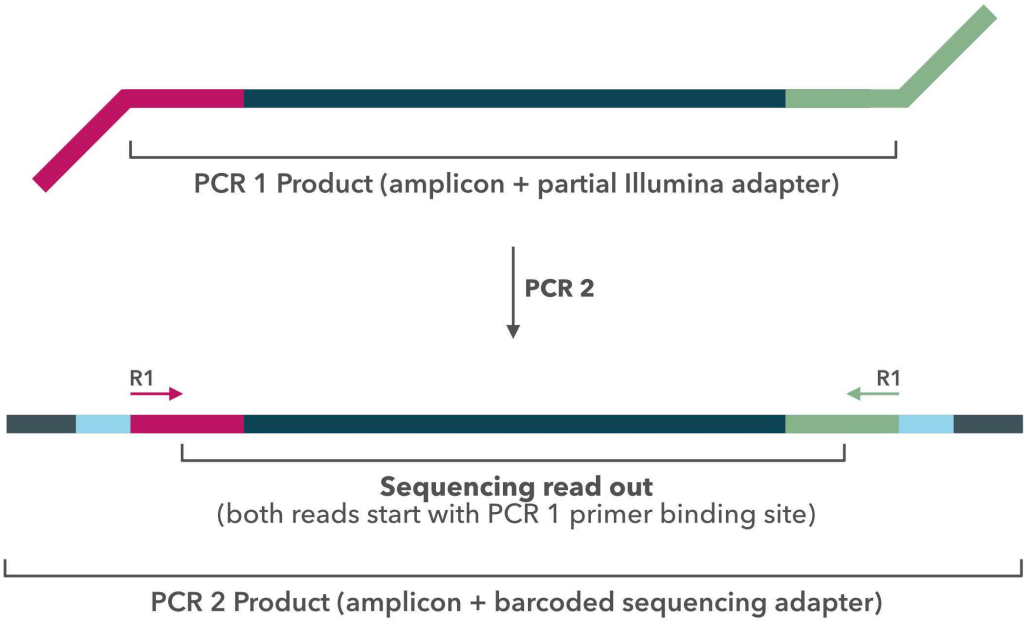
Preparation of Amplicons for deep sequencing is based on two PCR steps. The genomic loci of interest is enriched in a first PCR including a stubber sequence to both ends of the molecule. For the subsequent barcoding PCR the stubber sequence is targeted and the full NGS sequencing adapter including dual barcodes are introduced to the molecule ends, enabling Illumina sequencing on all instruments.

By default we recommend a 150 PE sequencing workflow and amplicon size including the specific primer binding sites should not extend 280bp.

Schematic overview



PCR 1: The specific PCR primer surround the genomic loci of interest and include a "*stubber*" sequence which carry the partial TruSeq Illumina adapter sequence.



PCR 2: Universal dual barcoded primers are used to finalise the amplicons for sequencing on Illumina instruments.

ATTACHMENTS

Amplicons for Deep Sequencing.xlsx

GUIDELINES

See attached excel document for notes and guidelines for preparing deep sequencing libraries from edited cells. It includes the primer design strategy, indexing barcodes for PCR 2 (order, setup and sample sheet entries) and a pooling guideline/calculation.

MATERIALS

STEP MATERIALS

Epicentre QuickExtract™ DNA Extraction Solution **Epicentre Catalog #QE0905**

Q5 Hot Start High-Fidelity DNA Polymerase - 100 units **New England Biolabs Catalog #M0493S**

SPRIselect reagent kit **Beckman Coulter Catalog #B23317**

Alternatively, prepare homemade beads using SeraMag Speed Beads

Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **GE Healthcare Catalog #44152105050350**

LINK PROTOCOL

NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) - 24 rxns **New England Biolabs Catalog #E7335S**

Alternatively, order multiplexing oligos with a manufaturing company and premix entire barcoding plates for multichannel pipette usage.

Qubit® 3.0 Fluorometer **Thermo Fisher Scientific Catalog #Q33216**

Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200) **Contribute by users**

MiSeq Reagent Kit V2 (300-cycles) **Illumina, Inc. Catalog #MS-102-2002**

Extract genomic DNA from edited cells using QuickExtract s..

1 Resuspend cell pellet to ≥2,500 cells/µL in QuickExtract solution. Vortex or pipette-mix to resuspend thoroughly.

Epicentre QuickExtract™ DNA Extraction Solution **Epicentre Catalog #QE09050**

Make sure to check whether QuickExtract is appropriate for your sample and application. Take a look at

for a more extensive list of gDNA extraction methods available in the lab.

2 Place on 68 °C heat block or thermocycler. Incubate for 00:30:00. 45m

Place on 98 °C heat block or thermocycler. Incubate for 00:15:00.

NOTE: Extract genomic DNA in a pre-PCR area to prevent amplicon carry over.

Prepare first PCR (genomic DNA PCR)

3 Prepare PCR 1 Mastermix with **100ng input gDNA** per sample (adjust primer concentrations and annealing temperature according to your setup).

Recipe for one rxn:

Reagent	Volume [µl]
5X Q5 Reaction Buffer	10
dNTPs [10 mM]	1
Q5 High-Fidelity DNA Polymerase [2U/µl]	0.5
Forward primer + Stubber [10µM]	1.5
Reverse primer + Stubber [10µM]	1.5
gDNA (100ng)	10
Nuclease-Free Water	25.5
total	50

PCR 1 Mastermix

Safety information

Prepare PCR 1 reaction in a pre-PCR area and transfer the reactions to a cycler in post-PCR. **Don't run PCRs in the pre-PCR area!**

Q5 Hot Start High-Fidelity DNA Polymerase - 100 units **New England Biolabs Catalog #M0493S**

Thermal Cycling protocol:

Step	Temperature °C	Time [sec]
Initial Denaturation	98	30
Denaturation	98	10
Annealing	variable *	15
Extension	65-72	60
Final Extension	65-72	300
Hold	12	hold
Lid 105°C		
Cycles: 30-36 | *use NEB calculator		

NEB Tm calculator https://tmcalculator.neb.com/#!/main

Purify first genomic PCR using SPRI beads

40m

4 40m

Safety information

Important! Change to post-PCR area of your lab for all subsequent steps.

Purify 50 µL PCR with 0.8X SPRI beads, eluting in 30 µL water.

Note

For reference, a 0.8X SPRI bead selection picks up any DNA fragments equal or larger than 250bp.
Please refer to the user manual at https://www.cytivalifesciences.com/en/us/shop/molecular-biology/pcr-and-amplification/kits-and-ready-to-go-beads/sera-mag-select-p-10218#related-documents for a complete look at which ratio to use for which size of DNA.

Sera-Mag Select **GE Healthcare Catalog #29343052** or homemade beads

4.1 1. Add 40 µL SPRI beads to each 50 µL PCR (0.8X ratio), mix thoroughly, and incubate for 5-8 minutes
2. Place on magnetic stand, wait for solution to clear (~1-2 minutes).
3. Remove clear supernatant.
4. Wash 2x with 80% Ethanol:
Add 200 µL ethanol solution (wait 30 seconds), remove, add 200 µL ethanol solution (wait 30

seconds), remove.
5. Remove residual ethanol with a P20 pipette. Remove plate from magnetic stand.
6. Allow sample to visibly dry, 3-5 minutes. Do not over-dry the samples as this can affect the yield.
7. Resuspend beads in 30 µL PCR-grade water. Place on magnetic stand.
8. Allow solution to clear. Remove 27 µL of clear supernatant (contains DNA) and place in a new 96 well plate.

QC the first PCR

1h

5 Confirm proper amplification by running a 2% agarose gel with 5 µL of purified product. Alternatively, you can run the gel on the PCR, before purification. 1h

The first PCR product is often impure, especially when amplifying more complex samples (e.g. mouse samples). Confirm presence of the desired band before proceeding with the second PCR.

Measure concentration of first PCR with the Qubit Spectrophotometer.

For qubit, use 2 µL Purified PCR per sample, and the '1x DNA HS kit'. Follow manufacturer's instructions.

Note

NOTE: The qubit assay can be readily adapted to use with a plate reader, for higher throughput.

Qubit® 3.0 Fluorometer **Thermo Fisher Scientific Catalog #Q33216**

Prepare the second PCR from 10 ng of product from the firs..

1h 30m

6 Place 10 ng of first PCR product into a second 50 µL Q5 polymerase master mix with the Illumina PCR primers containing dual 8nt barcodes. This PCR completes the Illumina adapter to be sequenced on a Illumina MiSeq sequencer. 1h 30m

Note

Choose unique barcode combinations to enable sample multiplexing during sequencing. Check our Google Drive Amplicon Sheet for the available indeces: https://docs.google.com/spreadsheets/d/1P4r4A3eduDalBilpqQwZdQAjaZHgOk08oSbkK3sNMJQ/edit#gid=653138277

Recipe for one rxn:

Reagent	*Volume [µl]*
5X Q5 Reaction Buffer	10
dNTPs [10 mM]	1
Q5 High-Fidelity DNA Polymerase [2U/µl]	0.5
Illumina i7 (10µM)	5
Illumina i5 [10µM]	5
PCR 1 product (10-20ng)	25
Nuclease-Free Water	3.5
total	50

PCR 2 Mastermix

Thermal Cycling protocol:

Step	*Temperature °C*	*Time [sec]*
Initial Denaturation	98	120
Denaturation	98	10
Annealing	65	15
Extension	72	60
Final Extension	72	300
Hold	12	hold
Lid 105°C		
Cycles: 6-8		

Confirm proper amplification by running a 2% agarose gel with 5 µL of purified product. Primer dimers will be removed in the following bead purification.

Purify the Second PCR using SPRI beads

40m

7 Purify 50 µL PCR with 0.8X SPRI beads, eluting in 30 µL water. 40m

Sera-Mag Select **GE Healthcare Catalog #29343052** or homemade beads

Use SPRI beads at RT.

1. Add 40 µL SPRI beads to each 50 µL PCR (0.8X ratio), mix thoroughly, and incubate for 5-8 minutes
2. Place on magnetic stand, wait for solution to clear (~1-2 minutes).
3. Remove clear supernatant.
4. Wash 2x with 80% Ethanol: Add 200 µL ethanol solution (wait 30 seconds), remove, add 200 µL ethanol solution (wait 30 seconds), remove.
5. Remove residual ethanol with a P20 pipette. Remove plate from magnetic stand.
6. Allow sample to visibly dry, 3-5 minutes. Do not over-dry the samples as this can affect the

yield.

7. Re-suspend beads in 30 µL PCR-grade water. Place on magnetic stand.
8. Allow solution to clear. Remove 27 µL of clear supernatant (contains DNA) and place in a new 96 well plate.

QC the second PCR and pooling

8

1. Measure the concentration of your purified PCR 2 product using the Qubit Spectrophotometer.
2. Calculate the molar concentration of your samples using the fragment length of your PCR 2 product. **Molar concentratron [nM] = (PCR 2 concentration [ng/µl] / 660 [g/mol] * {fragment length} [bp]) * 10^6**
3. Pool the samples equimolar to a final concentration of 10nM.
4. Measure the Pool with a Tapestation D1000 measurement. Tapestation Quick Guide: https://www.agilent.com/cs/library/usermanuals/public/ScreenTape_D1000_QG.pdf

NOTE: Calculation of molar concentration and pooling is provided in the attached excel file.

NOTE: General recommendations for sequencing depth:

	Sample type	Read depth (*10^6)
	Amplicons for editing check	0.2
	Clone genotyping	0.02
	ChIP	20

Illumina sequencing

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Sequencing recommendations

- We recommend a read length of 151bp from both sides as well as two 8bp barcode reads for amplicon sequencing (151 I 8 I 8 I 151)
- Add PhiX PhiX Control v3 **Illumina, Inc. Catalog #FC-110-3001** as amplicons are considered low-diversity libraries.

Illumina MiSeq

- Add 10% of PhiX to ensure sequencing variability.

- Start the run with a final loading concentration of 8-9pM

Sequencing kit options:

- Sequencing read output 15M reads

MiSeq Reagent Kit V2 (300-cycles) **Illumina, Inc. Catalog #MS-102-2002**

- Sequencing read output 4M reads

Miseq v2 Micro Sequencing Reagent Kit (300 cycles) **Illumina, Inc. Catalog #MS-103-100**

Illumina NextSeq2000

- Add 15-20% of PhiX
- Start the run with a final loading concentration of 650pM

Sequencing kit options:

- Sequencing read output 100M reads

NextSeq 1000/2000 P1 Reagents (300 Cycles) **Illumina, Inc. Catalog #20050264**