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Preparation of PCR amplicons from edited cells for deep sequencing V.2

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We use this protocol and it's working

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Abstract

This protocol describes how to extract and amplify DNA from genome-edited cells, and sequence the resulting amplicons using Illumina MiSeq NGS.

Attachments

XLSX
Primestar GXL worksh...
10KB

DOCX
PREPARING PCR AMPLIC...
930KB

Guidelines

See attached word document for notes and guidelines for preparing deep sequencing libraries from edited cells. See attached excel document for a worksheet that can be used to formulate PCR master mixes for amplification with PrimeStar GXL.

Materials

STEP MATERIALS

- Epicentre QuickExtract™ DNA Extraction Solution **Epicentre** **Catalog** #QE09050
- Qubit® 3.0 Fluorometer **Thermo Fisher Scientific** **Catalog** #Q33216
- TruSeq Nano DNA HT Library Preparation Kit (96 indexes in plate format, 96 samples) **Catalog** #FC-121-4003
- NEBNext Library Quant Kit for Illumina - 500 rxns **New England Biolabs** **Catalog** #E7630L
- PrimeSTAR GXL DNA Polymerase **Catalog** #R050A

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Troubleshooting

Extract genomic DNA from edited cells using QuickExtract solution

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

2 Place on 68ºC heat block or thermocycler. Incubate for 30 minutes.

3 Vortex or pipette-mix for 5 seconds.

4 Place on 98ºC heat block or thermocycler. Incubate for 15 minutes.

Prepare first PCR (genomic DNA PCR)

5 Prepare the following Master Mix using the initial PCR primers (annealing outside the HDR template region, see guidelines for design requirements), and the **PrimeStar GXL** polymerase kit from Clontech. Include a master mix-only control.

Master mix (per reaction):
    0.15 µL 100 µM forward primer
    0.15 µL 100 µM reverse primer
    4 µL Takara dNTP solution (2.5 mM each, or 1 µL 10 mM each)
    1 µL GXL polymerase
    30.7 µL H_20
    10 µL 5X Buffer

Consider making 10-15% extra master mix to account for pipette error.

DOCX PREPARING PCR AMPLICONS FOR ...

XLSX Primestar GXL worksheet.xlsx

PrimeSTAR GXL DNA Polymerase **Catalog** #R050A

6 Mix 4 µL of genomic DNA extract with 46 µL of Master mix in a 96-well PCR plate. Add 46 µL master mix to an empty well as a master mix only control. Mix thoroughly with pipette. Seal plate with tape.

7

8 Purify 50 µL PCR with 1.8X SPRI beads, eluting in 30 µL water.

1. Add 90 µL SPRI beads to each 50 µL PCR (1.8X ratio), and mix thoroughly.

Purify first genomic PCR using SPRI beads

9 2. Place on magnetic stand, wait for solution to clear (~1-2 minutes).

10 3. Remove clear supernatant.

11 4. Wash 2x with 80% Ethanol in water: Add 200 µL ethanol solution, remove, add 200 µL ethanol solution, remove.

12 5. Remove residual ethanol with a P20 pipette. Remove plate from magnetic stand.

13 6. Allow sample to visibly dry, 3-5 minutes. Do not over-dry the samples as this can affect the yield.

Purify first genomic PCR using SPRI beads

14 7. Resuspend beads in 30 µL PCR-grade water. Place on magnetic stand.

15 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

16 9. Measure concentration of first PCR using Nanodrop (A260) or Qubit Spectrophotometer.

For qubit, use 2 µL Purified PCR per sample, and the '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

17 2. 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.

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.

Prepare the second PCR from 50 ng of product from the first PCR

18 Place 50 ng of first PCR DNA into a second 100 µL GXL Primestar master mix with the second PCR primers. This produces an amplicon short enough to be sequenced on a MiSeq. Neither primer can anneal to the HDR template. See attached Word document for more design instructions.

0.3 µL 100 µM forward primer 2
0.3 µL 100 µM reverse primer 2
8 µL Takara dNTP solution (2.5 mM each, or 1 µL 10 mM each)
2 µL GXL polymerase
XX µL H20 (final volume: 100 µL)
20 µL 5X Buffer
XX µL PCR1 product (50 ng)

DOCX PREPARING PCR AMPLICONS FOR ...

XLSX Primestar GXL worksheet.xlsx

19 Cycle using the following parameters:

7 cycles of:
    98ºC 10 sec
    60ºC 15 sec
    68ºC 30 sec

20

Purify the Second PCR using SPRI beads

21 2. Place PCR plate on magentic stand, allow solution to clear (2-3 minutes).

22 3. Remove clear supernatant.

23 4. Add 180 µL of 80% ethanol to wash. Remove, and repeat for a total of 2 washes.

24 5. Remove residual ethanol with a P20 pipette.

Purify the Second PCR using SPRI beads

25 6. Remove plate from magnetic stand, let pellet air dry for 3-5 minutes. Do not overdry.

26 7. Resuspend pellet in 30 µL of PCR-grade water. You can also use Illumina RSB.

27 8. Place plate on magnetic stand, allow solution to clear.

28 9. Remove 27 µL of clear supernatant and transfer to a new plate. Try to avoid bringing any SPRI beads with the sample.

29 9. Measure concentration of first PCR using Nanodrop (A260) or Qubit Spectrophotometer.

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

QC the second PCR.

30 2. 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.

The second PCR product should be extremely pure, as only the targeted fragment should re-amplify.

Prepare NGS amplicons for sequencing using the Illumina Truseq Nano HT kit

31 You now have purified, blunt, PCR product of the appropriate length, ready for adaptor ligation using off-the-shelf NGS kits. To use these kits, start with the "Adenylation step" to A-tail the blunt DNA, and then follow manufacturer's instructions.

We will outline the next steps using the Illumina Truseq Nano HT kit (our preference) in the following steps.

1. Begin at the "Adenylate 5' ends" step of the protocol. Mix 100 ng of purified PCR product with Illumina RSB (supplied) to a final volume of 17.5 µL.
2. Add ATL mix and A-tail according to manufacturer's instruction.

TruSeq Nano DNA HT Library Preparation Kit (96 indexes in plate format, 96 samples) **Catalog #**FC-121-4003

32 3. Ligate with Illumina Truseq Nano HT adaptors (provided with the kit). Double purify with SPRI (included) as instructed.

NOTE 1: We use home-made SPRI beads for PCR cleanup, but NOT for this step. Use the SPRI beads included with the kit ONLY.

NOTE: Illumina includes >2X more adaptors than you need in a 96 sample kit. Save unused adaptors for later use!

33 4. Enrich DNA fragments with Illumina proprietary amplification mix/primers.

NOTE: The primer cocktail is proprietary to Illumina, and is the limiting reagent in the 96 sample kit.

QC NGS libraries

34 We QC our libraries by three methods:

1. Run a 2% agarose gel of your final enriched NGS libraries.

Look for an absence of adaptor dimer at ~100 bp. Small amounts of residual adpator can severely affect downstream sequencing!

35 2. Quantify libraries using the Qubit Spectrofluorometer.

Use the "DNA HS" kit and manufacturer's instruction.

36 3. Pool libraries based upon qubit and gel result.

Ask your sequencing facility for pooling requirements. If all the libraries are the same size, you can pool on mass. If not, convert qubit data to molar concentration using an online calculator and pool using that value. Make sure the pool contains an equal molar amount of each library Use a manageable volume for the most concentrated sample, and put an equal mass of each library into the pool. We shoot for a pooled concentration of ~2-5 ng/µL.

37 NEBNext Library Quant Kit for Illumina - 500 rxns **New England Biolabs Catalog** #E7630L

Sequence NGS libraries

38 Sequence libraries for Illumina MiSeq, 2×250 paired end read. Our sequencing facility performs this step.