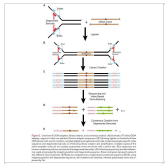

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DIDA-Seq Custom Capture cfDNA Library Preparation

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ABSTRACT

Dual Index Degenerate Adaptor Sequencing, or **DIDA-Seq**, an ultra-sensitive cell-free DNA sequencing approach for the detection and quantification of circulating tumor DNA in peripheral blood plasma at fractions below the background error-rate of traditional next-generation sequencing (NGS). DIDA-Seq combines unique molecular identifiers (UMIs) and patient-specific custom capture panels to routinely detect ctDNA with mutation allele frequencies at or below 0.02% (or 1 tumor derived DNA fragment in 5k normal fragments at a given genomic locus) with error-rates below 1 base in 10kb-50kb (Butler et al 2019, doi:10.1101/mcs.a003772; Boniface et al 2021, doi:10.3390/diagnostics11010073). This protocol describes the preparation of DIDA-Seq libraries using cell-free DNA, custom DIDA-seq library adaptors and blocking oligonucleotides, and custom hybridization capture panels. It uses reagents developed and manufactured by Roche, LTD and Integrated DNA Technologies, Inc with minimally modified versions of their methods.

ATTACHMENTS

Butler_etal_2019.pdf Boniface_etal_2021_diagnostics-11-00073.pdf

DOI

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PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Boniface C, Deig C, Halsey C, et al. The feasibility of patient-specific circulating tumor DNA monitoring throughout multi-modality therapy for locally advanced esophageal and rectal cancer: A potential biomarker for early detection of subclinical disease. Diagnostics (Basel). 2021;11(1). doi: 10.3390/diagnostics11010073.

KEYWORDS

cell-free DNA, cfDNA, ctDNA, circulating tumor DNA, NGS

IMAGE ATTRIBUTION

Butler TM, Boniface CT, Johnson-Camacho K, et al. Circulating tumor DNA dynamics using patient-customized assays are associated with outcome in neoadjuvantly treated breast cancer. Cold Spring Harb Mol Case Stud. 2019;5(2). doi: 10.1101/mcs.a003772.

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MATERIALS TEXT

NucleoSnap DNA Plasma Kit (Macherey-Nagel, Cat. # 740300.10)

AgenCourt AMPure XP Beads (Beckman-Coulter, Cat. #A63880)

Digital Electrophoresis Chips and Kits for Bio-Analyzer, High Sensitivity DNA Kit (Agilent, Cat. #5067-4626)

KAPA Hyper-Prep Kit, Illumina Platforms (Roche, KR8500)

KAPA HiFi Hotstart PCR master mix (Roche)

Qubit 3 HS DNA quantification reagents

xGen Lockdown Probe Pools (IDTDNA.com - designed using patient-specific genomic loci derived from WES mutation calls)

DIDA-Seq Adaptor Oligos, PAGE purified (n=fixed index sequencing; N=degenerate/random base):
I/P5 DIDA Adapter Oligo (8bp index):
5'-AATGATACGGCGACCACCGAGATCTACACnnnnNNNNACACTCTTTCCCTACACGACGCTCTTCCGATC*T–3'
I/P7 DIDA Adapter Oligo (8bp index):
5'-/5Phos/GATCGGAAGAGCACACGTCTGAACTCCAGTCACnnnnNNNNATCTCGTATGCCGTCTTCTGCTTG-3'

DIDA-Seq Hybridization Capture Blocking Oligos PAGE purified (i=inosine)
I/P5 Blocker:
5'-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTiiiiiiiiGTGTAGATCTCGGTGGTCGCCGTATCATT-3'
I/P7 Blocker:
5'-CAAGCAGAAGACGGCATACGAGATiiiiiiiiGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-3'

Illumina Library Amplification primers, Standard Desalting purification:
Forward (p5):
5'-AATGATACGGCGACCACCGA-3'
Reverse (p7):
5'-CAAGCAGAAGACGGCATACGA-3'

cfDNA Isolation 2h

1 Using 3-10 mL of plasma (as needed, see note below) and NucleoSnap DNA Plasma Kit (Macherey-Nagel), follow manufacturer's protocol to isolate cfDNA and elute with 50 ul nH2O or TE-low. Store at 4C for several days or freeze for long-term storage. 1h 30m

Yield is highly subject to the physiological state and health of a patient where tumor-burden, treatment type and schedule, as well as physical activity, can greatly influence cfDNA levels. A healthy person might range from 5-30ng cfDNA/mL plasma, and a person with metastatic disease undergoing treatment might have 15-200ng cfDNA/mL plasma. The optimal input amount of cfDNA is >=100ng for the custom capture protocol described here, however a minimum input for library prep can be as low as ~20-30ng (~5-10k haploid genome copies). Lower input quantities require more pre-capture PCR and will therefore result in **less than optimal, lower-complexity libraries where assay sensitivity is limited by total number of input genomes**. Ligation efficiency is also dependent on input concentration and may suffer with lower input levels (<50) leading to lower-complexity libraries and assay sensitivity.

2 Quantify cfDNA using either Qubit 3.0 (Thermo-Fisher), qPCR using KAPA gDNA Quantification Kit (using 44bp primers 30m

Citation: Christopher T T Boniface (07/19/2021). DIDA-Seq Custom Capture cfDNA Library Preparation. https://dx.doi.org/10.17504/protocols.io.bwm8pc9w

only), or by Agilent 2100 BioAnalyzer using HS DNA Kit. Total DNA can range between 15-500+ ng for <10mL of plasma input. Avoid samples showing high molecular wright species as this is an indication of gDNA contamination from lysed lymphocytes, etc (i.e., non-cfDNA).

End-repair, A-tailing and Ligation of DIDA-Seq Adaptors 20h

3 Using 20-100 ng of cfDNA from step 2 and KAPA HyperPrep Kit, prepare and carry out end-repair and A-tailing reaction per manufacturer's protocol (https://rochesequencingstore.com/wp-content/uploads/2017/10/KAPA-HyperPrep-Kit_KR0961-%E2%80%93-v7.19.pdf) 1h 30m

Note: cfDNA does not require sonication as it is naturally fragmented into ~165bp molecules.

4 Prepare dual-indexed, degenerate-tagged, adaptor (DIDA) to an appropriate stock concentration (100uM) by combining, in equal parts, i5 and i7 custom adaptor oligonucleotides such that i5 and i7 oligos contain matching index sequences. Anneal the single-stranded oligos by heating the mixture to 95C for 5 min and letting cool to RT for 30-60 minutes. 1h

5 On ice, dilute above annealed adaptors such that 5 uL gives a 200:1, [adaptor]:[cfDNA], molar excess and combine with above end-repair and A-tailing reaction, ligase, and ligation buffer per manufacturer's protocol. Be careful not to allow above reagents and/or ligation reaction to exceed 4-16C prior to next incubation step. 30m

6 Ligate overnight (12-18hrs) at 16C in a thermocycler (lid heat "off"). 16h

7 Bead-purify ligation reaction: Add 88uL (0.8X vol.) well-mixed, room-temperature SPRI Ampure XP Bead slurry (Beckman-Coulter) and mix thoroughly by pipetting or vortexing. Incubate at room temperature for 10 minutes, separate supernatant from beads using magnet and discard supernatant. Immediately add 200 uL of 80% EtOH to wash, remove EtOH and repeat wash. After removing second EtOH wash, very briefly spin-down and quickly remove excess EtOH.* Immediately resuspend beads in 25 ul of nH2O. 30m

***Note:** It's very important not to let beads dry after removal of second EtOH wash. Proceed immediately to spin step, quickly remove remaining EtOH by pipet and resuspend in 25 uL nH2O. It is not critical that every last bit of EtOH is removed - the risk of reduced yield from over-drying greatly outweighs any issues associated with a small amount of EtOH carry-over.

Pre-Capture Library Amplification 2h 30m

8 Prepare 50 ul PCR reaction using 25ul KAPA HiFi Hotstart 2x MasterMix and Illumina Library Amplification Primers (10 uM [final]), 20 ul of above post-ligation reaction (from step 7), and 5 uL of nH2O + primers. 30m

9 Amplify library using KAPA HiFi Hotstart temperature protocol for 8-12 cycles (60C annealing temperature). 30m

10 Bead purify using 50ul bead solution (1X vol.) washing and eluting as above (step 7). 30m

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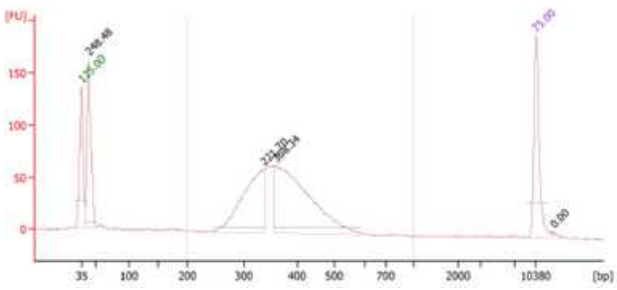
11 Quantify using BioAnalyzer 2100 (more accurate quantification can be done with qPCR via KAPA Library Quantification Kit but DNA electrophoresis is necessary to assess molecular size distribution). 1h

4. Custom Oligo Capture Hybridization (per IDT xGen Lockdown Protocol) 1d

12 Follow IDT xGen Lockdown "Tube" Protocol (version 4, pages 15-30, see link) using DIDA-Seq blocking oligos. 1d

(manufacturer's protocol: https://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/protocol/xgen-hybridization-capture-of-dna-libraries.pdf?sfvrsn=ab880a07_16)

Please note the following changes to the manufacturer's protocol:
- Samples may be multiplexed prior to drying down with a speed-vac system (pg 23, step 3).
- When drying samples down, use 1 uL 100 uM adaptor i7 **custom blocker** and 1 uL 100 uM adaptor i5 **custom blocker** (2 ul total)
- Hybridization capture baits were purchased through Integrated DNA Technologies, Inc (https://www.idtdna.com/) as xGen Lockdown Probe Pools
- Incubate hybridization reaction overnight (~16hrs) at 65C.
- Post-library amplification can be done with 12 cycles of PCR using Illumina library amplification primers (step 9, above)



Representative BioAnalyzer 2100 trace of an amplified, post-capture DIDA-Seq library using the Agilent High Sensitivity DNA Kit.

13 Amplified, post-capture DIDA-Seq libraries are now ready for dilution and sequencing using an Illumina NGS platform.

Raw sequencing data (fastq files) alignment and DIDA-Seq error-correction software pipeline is available here: https://github.com/ohsu-cedar-comp-hub/DIDA-Seq

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