Mar 22, 2019

Working

DASH Protocol v2.5

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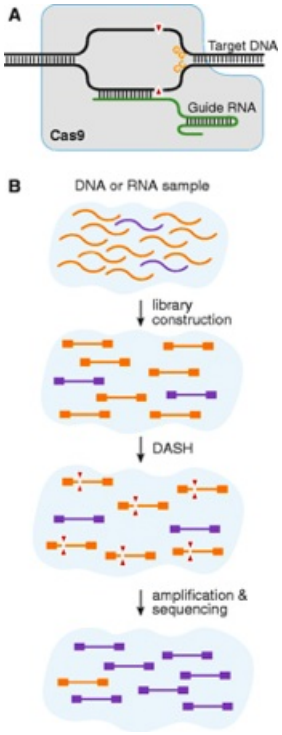
ABSTRACT

This protocol is for performing Depletion of Abundant Sequences by Hybridization (DASH) after preparing sequencing libraries and pooling together.

DASH is most useful for RNA-seq of human metagenomic samples, where abundant species such as human mitochondrial ribosomal RNAs (rRNAs) occupy a majority of the sequencing space available, leaving a minor fraction for regions of interest.

DASH treatment is performed after ligation of adapters and unique barcoding of the RNA-seq library. It employs CRISPR-Cas9 complexed to a set of guide RNAs (gRNAs) targeted to the abundant regions to be depleted in a given library. These abundant regions in the library are then cleaved, leaving only the fragments with intact adapters on both ends to be further amplified and sequenced.



PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

This protocol describes DASH for an RNA library prepared using the NEBNext Ultra II RNA Library Prep Kit. Other standard library preparation kits may be used instead, as long as DASH is applied after ligation of adapters.

MATERIALS

NAME	CATALOG #	VENDOR

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USER Enzyme - 250 units	M5505L	New England Biolabs
NEBNext Adaptor for Illumina	E7337 in Kits E7335, E7500, E771	New England Biolabs
Thermocycler		
NEBNext Ultra II RNA Library Prep Kit for Illumina - 96 rxns	E7770L	New England Biolabs
Nuclease-free water	AM9932	Ambion
Qubit dsDNA HS kit	Q32851	Life Technologies
Bioanalyzer chips and reagents (DNA High Sensitivity kit)		Agilent Technologies
TruSeq i7/i5 Indexing Primers - Custom (or NEBNext® Multiplex Oligos for Illumina)	E7500L	New England Biolabs
Cas9 40µM		
Dual guide RNAs (40µM - targeted to genes or regions to be depleted - crisprRNA and tracr RNA - quantified by RNA Qubit)		
10X Cas9 Activity Buffer (500nM Tris pH 8.0 100nM MgCl2 10nM TCEP)		
Proteinase K	P8107S	New England Biolabs
SPRI beads (homemade) or Ampure XP beads	View	
Kapa HiFi Real-Time Amplification Kit	KK2702	Kapa Biosystems
Zymo DNA Clean & Concentrator - 5	D4014	Zymo Research
Magnetic rack for PCR strips	View	
qPCR machine	View	

STEPS MATERIALS

NAME	CATALOG #	VENDOR
NEBNext Ultra II RNA Library Prep Kit for Illumina - 96 rxns	E7770L	New England Biolabs
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific
Bioanalyzer chips and reagents (DNA High Sensitivity kit)		Agilent Technologies
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Qubit dsDNA HS kit	Q32851	Life Technologies
Kapa HiFi Real-Time Amplification Kit	KK2702	Kapa Biosystems
SPRI beads (homemade) or Ampure XP beads	View	
Qubit dsDNA HS Assay kit	Q32854	Thermo Fisher Scientific
Bioanalyzer chips and reagents (DNA High Sensitivity kit)		Agilent Technologies

SAFETY WARNINGS

BEFORE STARTING

Prepare your sequencing libraries, and make sure you have gRNAs for DASH. We used the *DASHit* software to generate an optimized set of guide RNA sequences based on a dataset of unwanted genes. (For *DASHit* software, visit: https://github.com/czbiohub/guide_design_tools/blob/master/dashit/dashit-reads/dashit-reads.org). You can buy the gRNAs or buy DNA templates and transcribe them. For an IVT protocol for crispr RNA and tracr RNA, please contact emily.crawford@czbiohub.org or amy.lyden@czbiohub.org)

Prepare indexed RNA-seq library

1 Follow all steps using NEB Ultra II RNA library preparation kit. Use an input RNA volume of 25ng if available, or less if not available. Perform 12-18 cycles of indexing PCR.

NEBNext Ultra II RNA Library Prep Kit for Illumina - 96 rxns
by New England Biolabs
Catalog #: E7770L

2 **Choose one option:**

a. Pooled DASH: If there are multiple samples, you may pool them at normalized concentrations of DNA between 200 – 1000 bp by Qubit or BioA/Tapestation/Fragment Analyzer, or using a preliminary low-depth sequencing run (such as an iSeq or MiSeq) of an equivolume pool to determine pooling ratios. Alternatively, if you suspect that your samples may vary drastically in DASHable material, you can pool equivolumes of your samples and DASH, then run an iSeq or MiSeq sequencing. Then pool normalized concentrations based on these values. This pooled library will go into a single DASH reaction.

b. Single Sample DASH: Additionally if you suspect your samples may vary in DASHable material or have a high amount of DASHable material, you may DASH each sample individually and pool them after. In this case, each sample will have its own DASH reaction, and the following steps will be performed on each individual library.

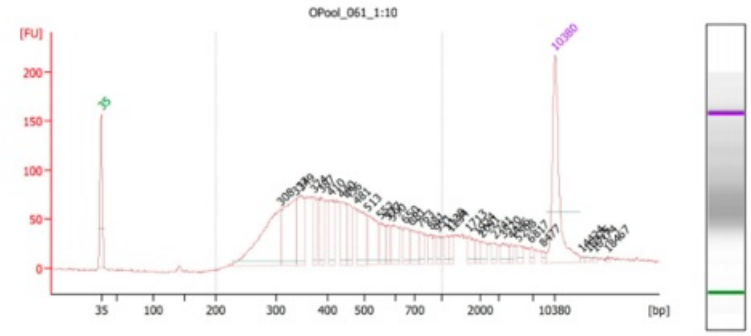
3 Quantify by HS DNA Qubit.

Qubit dsDNA HS Assay Kit
by Thermo Fisher Scientific
Catalog #: Q32851

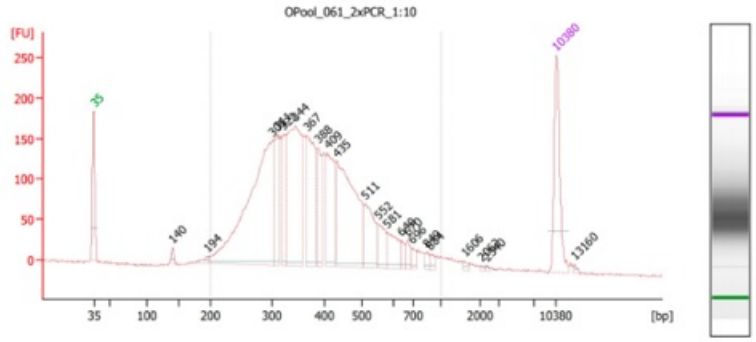
4 Perform a HS DNA Bioanalyzer (or other fragment analysis) and ensure for your library(ies):

1) that adapter dimers have been removed. Several SPRI bead clean-ups at a sample:bead volume ratio of 1:1 may be necessary.
2) there is sufficient sample in between the 200 – 1000 bp range.
3) There is no 'PCR bubble' (appears as secondary bump in fragment analysis trace at higher bp regions, see trace below). PCR bubble will be eliminated with 1-2 extra PCR cycles. We recommend.

- 100ng of DNA in 23µL H20
- 25µL Kapa HiFi Real-Time Amplification Master Mix
- 2µL Illumina primers at 5µM.



Library pre-PCR cycles with DNA in 2000-10000bp region. This library may be over-amplified and cannot be used in DASH.



Library post 2xPCR cycles with no DNA in 2000-10000bp region. This confirms library previously had PCR bubble. This library can be used in DASH.

Bioanalyzer chips and reagents (DNA High Sensitivity kit)
by Agilent Technologies

5 Each DASH reaction requires at least 10 µL of 2.8 nM DNA library (approximately 0.83 ng/µL for an average 450 bp library). You may concentrate your sample by SPRI bead clean-up if necessary.

DASH

6 Thaw necessary reagents and let come to room temperature before use. If you will not be using for more than 10 minutes, put on ice and take off 5 minutes prior to mixing.

Cas9 40µM

10X Cas9 Activity Buffer (500nM Tris pH 8.0 100nM MgCl2 10nM TCEP)

Dual guide RNAs (40µM - targeted to genes or regions to be depleted - crisprRNA and tracr RNA - quantified by RNA Qubit)

7 Prepare master mix (MM) of Cas9 and gRNAs:

Ensure that the dual-guide RNAs have been annealed at 95°C for 30 seconds and then cooled to room temperature. No additional heating is recommended if pairs were annealed prior to storage.

Cas9-gRNAs MM will be replenished once in the protocol. Ensure you have enough reagents for 2X MM for each sample.

Reagent	Stock Concentration	Final Concentration	1X cas9-gRNA MM
10X cas9 buffer	10X	1.25X	2.5µL
cas9	40µM	5µM	2.5µL
dual-guide RNAs	40µM	10µM	5µL
			10µL total

DASH Master Mix

8 Mix well by gently pipetting and tapping and incubate this mixture at 37 °C for 00:05:00 .

9 Add 10 µl of a 2.8 nM barcoded DNA library (pooled or not) to every 10 µl of the above Cas9-gRNA mix.

o This reaction may be scaled up from the original 1X (20 µl DASH reaction)

o Calculator for µg moles: https://www.promega.com/a/apps/biomath/

Reagent	Stock Concentration	Final Concentration	1X cas9-gRNA MM
10X cas9 buffer	10X	1.25X	2.5µL
cas9	40µM	5µM	2.5µL
dual-guide RNAs	40µM	10µM	5µL
DNA library	2.8nM	1.4nM	10µL
			20µL total

Final DASH reaction (1X, 20µL)

10 Incubate the DASH reaction at 37 °C for 00:30:00 .

11 Before your incubation is up, prepare a second batch of 1X Cas9-gRNA MM for all your samples and incubate mixture at 37 °C for

00:05:00 .

12 After incubation, perform a column clean up. Use the Zymo DNA Clean & Concentrate - 5 kit to purify your reaction. Follow the kit instructions for PCR product (100 µl of binding buffer to 20 µl of DNA in DASH reaction). Elute in 10.5 µl H20, and then reload all 10.5 µl onto the column, and elute again.

Zymo DNA Clean & Concentrator - 5
by Zymo Research
Catalog #: D4014

13 Add 10 µl of your eluted DNA to 10 µl of 1X Cas9-gRNA MM and mix by pipette.

14 Incubate the DASH reaction at 37 °C for 01:30:00 .

Total incubation of DNA library in Cas9-gRNA MM should be 02:00:00 . Column clean-up timing has not been optimized, and could be split to be 37 °C for 01:00:00 , column clean, and then 37 °C for 01:00:00 .

15 Add 1 µl proteinase K to each 20 µl reaction and mix well by gently tapping or pipetting.

Proteinase K
by New England Biolabs
Catalog #: P8107S

16 Incubate at 50 °C for 00:15:00 for cas9 inactivation.

Clean up DASH reaction

17 Prepare to do a SPRI selection of sample:bead 1:0.9 (equivalent to Ampure 0.7X)

This goal of this step is to purify your target library away from the DASH reagents (buffers, Cas9, gRNAs) and the DASH-digested fragments. Depending on DASH efficiency and the size of the expected digested fragments, this ratio may be altered to best remove the fragments.

Other magnetic beads such as AmpureXP may be used instead of SPRI. However, please take note of the different size cut-offs for sample:bead ratios, as they may vary from the homebrew SPRI beads used in this protocol.

18 Equilibrate SPRI beads to room temperature and vortex well to mix.

SPRI beads (homemade) or Ampure XP beads
View

19 Add beads equivalent to 0.9X the sample volume to each sample tube (for **21 µl** of sample, add **18.9 µl** beads).

20 Mix well by pipetting or tapping the tubes and spin down briefly.

21 Incubate for **00:05:00** at room temperature, then put the tubes on the magnetic rack. Allow beads to separate on the magnet for 3-5 minutes, or until the supernatant is clear.

22 Keeping the tubes on the magnet, carefully remove and discard the supernatant.

23 Add **200 µl** 80% ethanol (prepared fresh). Incubate beads for **00:01:00** and then remove the ethanol.

24 Repeat the above ethanol wash step.

25 Allow the beads to air dry for approximately **00:05:00** . Do not over-dry. Dry beads should appear matte (rather than glossy) but should not have a cracked appearance. Overdried beads may not resuspend or elute well.

26 Remove tubes from magnet and add **27 µl** nuclease-free H2O.

27 Resuspend well by vortexing, tapping the tubes, and spinning down briefly.

28 Allow **00:02:00** for DNA to elute from beads, then transfer tubes back to magnet

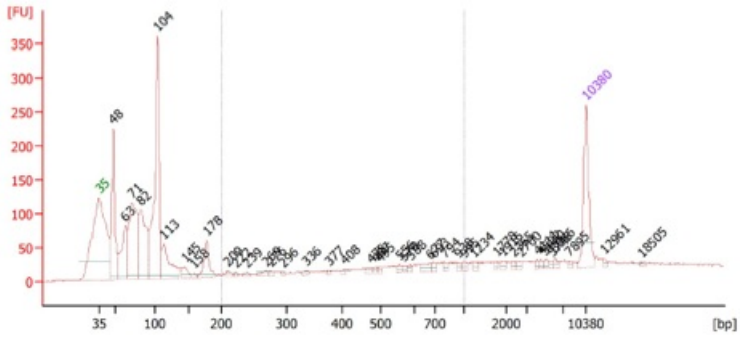
29 Allow the beads to separate on magnet.

30 Collect 25 µl of supernatant from each sample and transfer it to a clean PCR tube.

31 Run a fragment analysis, such as with the HS dsDNA Bioanalyzer kit or the Agilent HS D5000 Tapestation kit.

Bioanalyzer chips and reagents (DNA High Sensitivity kit)
by Agilent Technologies

If DASH is successful, the trace may be characterized by the presence of small peaks between 30 – 100 bp. The size of these peaks will depend on the proportion of expected depleted product over target product, and how closely spaced together the target guide RNAs were designed.



DASHed sample showing the digested fragments and gRNAs (30-140), an adapter dimer (178), and low concentration of target library (sloping from 300 – 1000+). Bioanalyzer internal markers in green (35) and purple (10380). This sample needs additional cleanup and amplification.

32 If desired, also quantify by HS DNA Qubit.

Qubit dsDNA HS kit
by Life Technologies
Catalog #: Q32851

33 As needed, perform additional clean-up steps to remove the digested fragments and adapter dimers in your sample. Proceed to amplification of target library if your concentration in the 200-1000bp region is very low, or looks nonexistent.

Amplification of Target Library

34 Perform this Real-Time PCR amplification step if after cleaning out the digested fragments and adapter dimers your library concentration is too low for loading onto a sequencer.

This step could be optional, if your DASHable material is low and your concentration after DASH is high. However, we have found it to be almost always necessary.

35 Thaw Kapa HiFi Real-Time Amplification Master Mix and Standard 2 on ice

Kapa HiFi Real-Time Amplification Kit
by Kapa Biosystems
Catalog #: KK2702

36 Prepare the following reaction for each library:

Reagent	Volume (1X)
2X Kapa HiFi Real-time Amplification Master Mix	25µL
5µM Universal Illumina Primers (5sol-20&21)	2µL
DNA (bring up volume with H20 if needed)	23µL
	50µL

37 Place each sample in a tube or strip of tubes that is physically separated from the others, so that they can be removed one at a time.

38 Put **50 µl** of Standard 2 in a PCR tube

39 Cycle with the following conditions in an RT-PCR machine. Make sure that if your machine does a baseline correction, that you remove it.

Temperature	Time	Cycles
98C	45 sec	1
98C	15 sec	25
63C	30 sec	
72C	1m 45 sec	
Plate read		
72C		

Cycling conditions for PCR

40 Watch the qPCR graph during the plate read. When a sample reaches the fluorescence threshold given by standard 2, remove that sample **DURING the 72°C for 20 second timeframe** after the plate read.

41 Perform a SPRI cleanup as described in `Clean up DASH reaction` section above at a sample:bead ratio of 1:0.9 to remove amplification reagents, and elute in a suitable volume of water. go to step #17

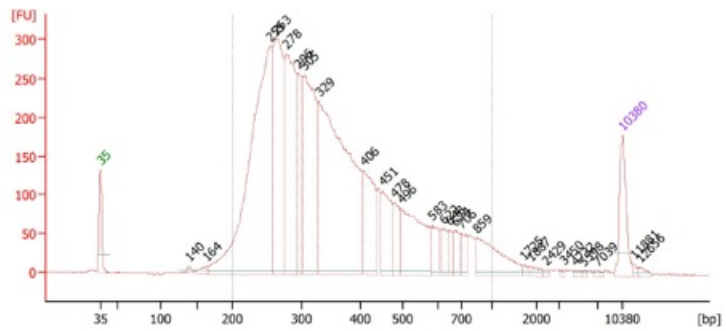
SPRI beads (homemade) or Ampure XP beads
View

42 Quantify and analyze your library by HS DNA Qubit and HS dsDNA Bioanalyzer.

Qubit dsDNA HS Assay kit
by Thermo Fisher Scientific
Catalog #: Q32854

Bioanalyzer chips and reagents (DNA High Sensitivity kit)
by Agilent Technologies

This trace should resemble a regular library with a majority of the library sized between 200 – 1000bp:



Same DASHed sample from above.SPRI cleaned at sample:bead ratio 1:0.7, 2 amplification cycles. Small primer dimer at 140bp.

43 Perform more SPRI clean-ups or amplification if necessary.

44 Submit your library for sequencing!