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Tn5 Library Prep for Deep Sequencing Loci of CRISPR/Cas9 Edited Cells. Single gene specific primer amplification (UDiTaS protocol with alterations)

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1 Works for me dx.doi.org/10.17504/protocols.io.7k2hkye

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ABSTRACT

This is the Tn5 gDNA prep published in the supplementary file of the UDiTaS paper (https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-4561-9).

Usage: measuring chromosome fusions, InDels, mRNA splicing, structural variants, large resection, off target integration. -> Some of these DNA repair outcomes are demonstrated using such a sample preparation in this paper (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6455975/).

My protocol is a modified version of UDiTaS but with a few tweaks like using nested primers. You will need a protein facility to produce the recombinant Tn5.

Software for analysis is on https://github.com/ericdanner
or https://github.com/editasmedicine/uditas

MATERIALS

NAME	CATALOG #	VENDOR
PrimeSTAR GXL DNA Polymerase	R050A	
Qubit® 3.0 Fluorometer	Q33216	Thermo Fisher Scientific
pTXB1-Tn5	60240	addgene
Ampure XP beads	A63881	Beckman Coulter
Magnesium chloride hexahydrate	M2670	Sigma Aldrich
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific
2100 Electrophoresis Bioanalyzer Instrument	G2939AA	Agilent Technologies
Tris-HCl		Sigma Aldrich
KAPA Library Quantification Kit for Illumina® Platforms	KK4835	Kapa Biosystems
NN-Dimethylformamide	D4551	Sigma Aldrich
TAPS	T5130	Sigma Aldrich

MATERIALS TEXT

Citation: Eric Danner (04/17/2020). Tn5 Library Prep for Deep Sequencing Loci of CRISPR/Cas9 Edited Cells. Single gene specific primer amplification (UDiTaS protocol with alterations). https://dx.doi.org/10.17504/protocols.io.7k2hkye

Crucial is to have your core facility or someone produce hyperactive Tn5 using the Addgene plasmid - Picelli, S., Sa, Å., Bj, K., Orklund, €, Orn Reinius, B. €, Sagasser, S., … Sandberg, R. (2014). Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. *Genome Research*, *24*(12), 2033–2040. https://doi.org/10.1101/gr.177881.114

BEFORE STARTING

Make hyperactive Tn5 in core facility as buying it is extremely expensive.

Make sure you wash the gDNA. I used gDNA extract from Fred Alt's protocol (LAM-HGTGT paper) and for some reason it inhibited Tn5 tagmentation. So as a rule- wash the gDNA using SPRI beads.

Reagent Preparation

1 **Order the correct DNA oligos**

WorkflowA.U.i5.N501UMITn5	AATGATACGGCGACCACCGAGATCTACACTAGATCGCNNNNNNNNNTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
WorkflowA.U.i5.N(S)502UMITn5	AATGATACGGCGACCACCGAGATCTACACCTCTCTATNNNNNNNNNTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
WorkflowA.U.i5_N(S)503UMITn5	AATGATACGGCGACCACCGAGATCTACACTATCCTCTNNNNNNNNNTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
WorkflowB.U.i5.N501UMITn5	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNNNTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
WorkflowB.U.i5.N(S)502UMITn5	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNNNCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

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WorkflowB.U.i5_N(S)503UMITn5	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNNNTATCCTCTTCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
U.Tn5-Abottom	[Phos]CTGTCTCTTATACA[ddC]
U.P5.i5	AATGATACGGCGACCACCGAGATCTACAC
Nested gene specific primer with overhang for i7 index addition:	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus- specific sequence]
i7_N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATG
i7_N702	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATG

"WorkflowA and workflowB" are shown in pictures below and relate to which sequencing machine you choose. This only shifts the location of the UMI based on the i5 index reading direction.

For i5 Side Primers/ DNA Oligos:
WorkflowA.U.i5.N501UMITn5:
*AATGATACGGCGACCACCGAGATCTACAC****TAGATCGC***(NNNNNNNNNN)**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**

bold italics **=** 5xx index
(N*10) = umi
Italicized = se= i5 indexing primer binding sequence for workflow B steups and read 1 primer
bold underline = i5 indexing primer binding sequence for workflow B steups and read 1 primer always

WorkflowB.U.i5.N501UMITn5:
AATGATACGGCGACCACCGAGATCTACAC(NNNNNNNNNN)***TAGATCGC*****TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**

For i7 Side DNA Oligos:
gene primer overhang: *GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG*-[locus- specific sequence]
Make your locus specific primer 22-27nt (25ish) if possible.

i7_N701 example*:*
*CAAGCAGAAGACGGCATACGAGAT****TCGCCTTA***GTCTCGTGGGCTCGGAGATG

italics sequence= this is the portion of the amplicon that the indexing primer i7 and read 2 amplify from regardless of WorkflowA or B. It is also the binding sequence for putting on the i7 indexing primer.
italics underline: = this is what binds to the flow cell
bold italics = this is the 70X indexing sequence

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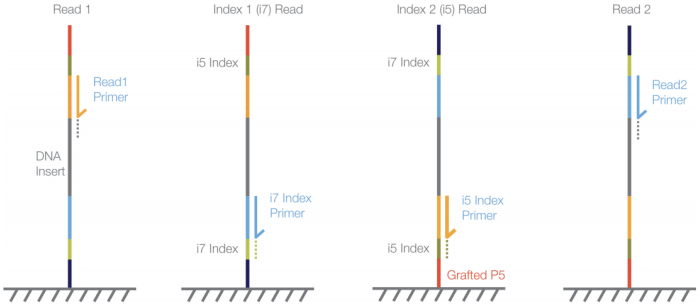
normal font= this is the overhang that binds to the locus specific primer sequnce during indexing.

You can of course make all your own i7 and i5 nextera primers for much cheaper than illumina for just switching out the index sequences.

Figure 3 Dual-Indexed Sequencing on a Paired-End Flow Cell (Workflow B)



1 **Read 1**—Read 1 follows the standard Read 1 sequencing protocol using SBS reagents. The Read 1 sequencing primer is annealed to the template strand during the cluster generation step.

2 **Index Read preparation**—The Read 1 product is removed and the Index 1 (i7) sequencing primer is annealed to the same template strand.

3 **Index 1 (i7) Read**—Following Index Read preparation, the Index 1 (i7) Read performs eight cycles of sequencing.

4 **Read 2 resynthesis**—The Index 1 Read product is removed and the original template strand is used to regenerate the complementary strand. Then the original template strand is removed to allow hybridization of the Index 2 (i5) sequencing primer.

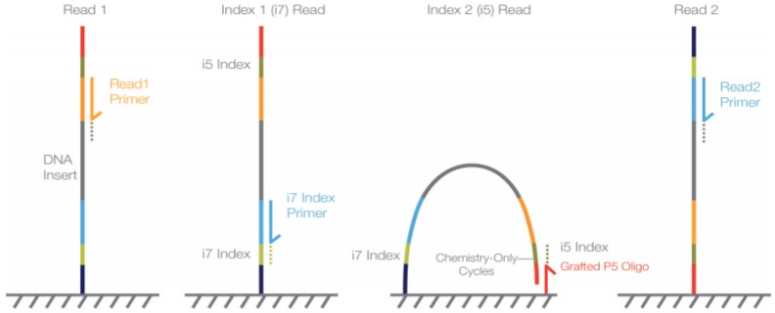
5 **Index 2 (i5) Read**—Following Read 2 resynthesis, the Index 2 (i5) Read performs eight cycles of sequencing.

This for the miniseq- the sequencer we used in our paper.

Forward Strand Workflow

The chemistry applied to the Index 2 Read during a paired-end, dual-indexed run on the NovaSeq 6000, MiSeq, HiSeq 2500, or HiSeq 2000 System is specific to the paired-end flow cell. Reading the i5 index requires seven additional chemistry-only cycles. This step uses the resynthesis mix, a paired-end reagent, during the Index 2 Read process.

Figure 2 Dual-Indexed Sequencing on a Paired-End Flow Cell (Forward Strand)



This is workflow A

Here is an example amplicon with all of the correct ends ready for illumina sequencing.

n704mcherryfwd-tn5-n501.gb

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2 **Make TAPS buffer**

5X TAPS-DMF: 50mM TAPS-NaOH @pH8.5, 25 mM MgCl2, 50% DMF

- -DMF- Sigma = D4551-250ML
- TAPS- Sigma = T5130-25G Mw- 243.2
- MgCl2 - mw 95.22

DMF is unstable so make 10x and then dilute 1:1 on day of use. For 50ml (100mM TAPS, 50mM MgCl2 pH 8.5) 1.22g TAPS + 0.24g MgCl2. Raise to 40 ml. Titrate to pH8.5 w/ NaOH. Raise to 50ml. If it smells fishy discard.
I have used it for 2 weeks without any issue after mixing.

3 **Adapter Oligo Annealing**

Re-suspend at 100uM in 10mM Tris-Hcl pH8, 0.5mM EDTA (edta inhibits Tn5 Mg2+ but it is so low in final working solution that it shouldn't matter). But might help stabilize DNA that is baked for 12 hrs during annealing not degrade from nucleases. I have had issues with the DNA I resus in only Tris not working as well and I have no idea why- but maybe due to this.
the final Mg2+ conc. is 5mM so even 0.5mM EDTA shouldn't hurt it massively

Take 50ul of each primer (i5-UMI-TMI and Tn5A-bottom) and hybridize 12 hr. 95 C to cold ˜ 8min/1C

4 **Complex annealed oligos with Tn5** (Tn5 1.85mg/ml in 2x Tn5 dialysis buffer)

100ul ul rxn: 85.7ul Tn5 + 14.3ul primer (original paper)
116.6 ul rxn : 100ul Tn5 + 16.8ul primer (scaled)
58.3 ul rxn: 50ul Tn5 + 8.4 ul primer

23C on shaker (230rpm) for 60min. *shaking is key for consistency
** Mixing it by hand and leaving it on bench top produced variation of tagmentation quality. This variationtook me AGES to figure out. Such a bad use of time. **

Best to do larger 100ul rxn if possible. You need to do a titration exp to figure out reactivity and then you are left with a known working stock.

1 hr incubation at RT is what was used in the original Piscilli paper with this primer ratio. According to https://www.biorxiv.org/content/biorxiv/early/2019/03/06/568915.full.pdf*the Tn5-ProteinA fusion has the same hypermutated Tn5 protein, and is stable after primer complexing for 10 days at RT.*

Tagment DNA

5 **Clean up DNA before Tagmentation**
Important - I found Tn5 reaction inhibited by my gDNA extract. Not sure what was causing the inhibition but it goes away when you clean with SPRI beads.

1. Remove RNA. The Tn5 can't react with RNA so probably don't need to remove it. But can easily add ul of 10mg/ml Rnase to 100ul DNA (i did 1ug/ul) in the TE buffer. Digest 10 min 37C shaker. Then the Nanodrop is more accurate.
2. Wash with 1:1 SPRI beads to remove RNAse and any contamination. I washed 2x. S*uspended in Tris-Cl and not EDTA. (or 0.1mM EDTA). EDTA inhibits the Tn5.*

6 **Measure gDNA concentration.**

Use Qubit as the nanodrop is not accurate. RNA contamination will make it seem like there is a lot more DNA than there actually is. However if you use Rnase, the Nanodrop can be accurate (check the 260/280 is 1.8).

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7 **Tagment gDNA**

You can scale this and do a larger set if you have multiple genes to check on the same gDNA. Each tagmentation puts on its particular N50X index. But there are dozens of i7XX indexes so you can use a single i5 index, tagment it all together, and then use aliquots for the gene specific primer. Then for each sample give it a different i7 index.

Reagents	1 reaction
Nuclease free water	4 µL
5x TAPS-DMF	4 µL
Genomic DNA (10 ng/µL)	10 µL
Assembled transposome	2 µL
Total reaction (µL)	20 µL

First mix everything together except gDNA. Then add the gDNA second. Always pipette very gently. I have also added the Tn5 last and not noticed a difference.

Tagment 55C for 7 min.

8 **Inhibit the Binding of Tn5 protein to tagmented DNA**

There are 3 ways to proceed to remove the Tn5 connected to the DNA:

1. Add 10ug (0.5ul of 20ug/ul) of Protinase K and incubate 10 minutes 55C. This is just for running on gel or bioanalzyer. Don't want protiinase K in the PCR reaction

2. *Fast but I found inconsistent:* Use Znymo DNA conc columns. Don't use the MN columns as they require a volume too big for elution and also don't release the DNA as well. Add 100ul of DNA binding buffer. Then load on column.
wash 2x with the wash buffer (200ul) and then elute. No need to spin dry. Follow instructions in kit.

3. ***Best:*** Use SPRI beads. 1:0.8 ratio (0,9X beads for 1 vol DNA). Quench the 25ul reaction with 5ul of 0.2% SDS and put back on 55C for 7 min. Then increase vol to 100ul with water. Add 90ul beads (1: 0.9). Elute in 22ul Tris-Hcl 10mM (pH8). You can scale up if you are going to do multiple PCRs off a prep

9 **Test that tagmentation worked:**

1. Check by run on DS DNA High Sensitivity Bioanalzyer Chip.HS-DNA chip. It's hard to see this accurately on a gel. You can see smears on a gel of 50-70ng tagmented gDNA (use reduced ladder amount if using gel as its very dim). **You want 2ul of Tn5 to tagment 50ng of gDNA to about 2-3kb peak size.**

Bioanalyzer often fails. Make sure it has been recently cleaned. Scrub the electrode with DI water and a toothbrush. You want tagmented peak around 2-3kb. and that while pressurizing the chip the syrninge should spring back to 0.6-0.7 < .

2. Test your tagmentation functionally (w/ PCR). Take a tagmented 50ng gDNA SPRI cleaned and split it into 2 or 3 volumes and do PCR. i5 only to check background, i5 + primer1, and i5 + primer2. Then you can get an idea of the background tagmented gDNA. It's important to do an only i5 control as if you over-tagment or if the i5 concentration is too high you get i5-i5 amplicons of the size that overlaps with your desired library size.

Normally i5-i5 background amplicons are not made smaller than 1kb due to the suppresion PCR effect.

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PCR 1

10 **PCR1 amplify the target region**

Amplify using gene specific primer. This is just a 20-25 nt primer to create amplicons. This does not have the long 5' of the primer that binding of the i7 indexing primer. We will then do a nested PCR from these amplicons.

The original Uditas protocol did not do a Nested PCR but any mis-priming event results in an amplicon as the i5 is universal.

SPRI beads effect PCR so be sure to have no beads remaining in eluent. Even trace beads can effect PCR

Reagents	1 reaction	
	25 µl reaction	50 µl reaction
5X PrimeSTAR Buffer	5	10
TMAC (0.5M)	1.5	3
10 mM dNTPs (#N0447)	2	4
Gene Primer (10uM)	0.5	1
i5 Primer (10uM) prE359	0.25	0.5
OneTaq DNA Polymerase	0.5	1
Template DNA	10	20
Nuclease-free water	5.25	10.5

You can use TMAC or not. The TMAC did not improve specificity but TMAC did seem to improve the reaction yield of all events.

Use a low concentration of i5 primer.
You need to do control for i5 primer only to see where the* suppression *PCR size limit is. Do this for every tagemented sampled. *You do not want the i5-i5 amplicons to contaminated your specific PCRs.*

GXL Tm primer calculator:
Tm (°C) = 2(NA + NT) + 4(NC + NG) - 5 (Ta (annealing) is usually lower than Tm (melting)) When the Tm value (calculated by the following formula) is greater than 55°C, set the annealing temperature to 60°C. When the Tm value is 55°C or less, set the annealing temperature to 55°C. : Tm value calculation method: Tm (°C) = 2(NA + NT) + 4(NC + NG) - 5 where N represents the number of nucleotides in the primer having the specified identity (A, T, C, or G)

the i5 primer is predicted to have a 69C annealing with GXL. So any temp should work from that side.

STEP	TEMP	TIME		
Initial Denaturation	98°C	30 seconds		
12x	98°C 60 ° C68°C	10 seconds 15 seconds 60 sec	4000x fold amp	
Final Extension	72°C	3 minutes		
Hold	10C			

11 **Cleanup With Ampure XP SPRI beads**

1. Use (1:1 vol). Can initially add 50 ul water to make the volumes bigger.
2. Elute in nuclease free water (25 ul) or TE.

Can also use 0.5x to get ride of >1kb bands before doing the normal clean up to remove the tagmented gDNA. The two figures shown below illustrate this idea.

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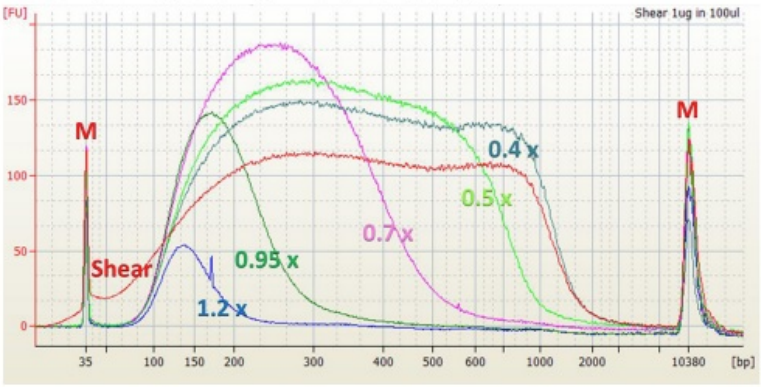
Figure 3 Agilent High Sensitivity DNA chip Electropherogram.



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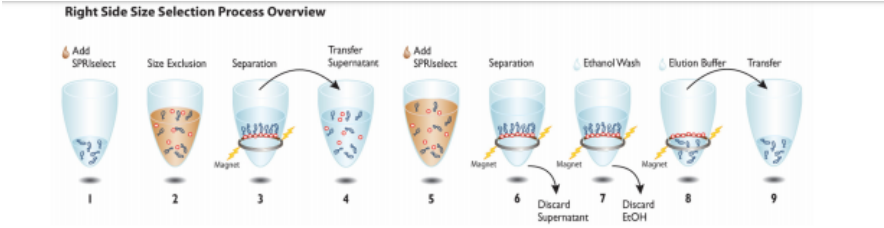

1. Thoroughly shake the SPRIselect bottle to resuspend the SPRI beads. Following the trend depicted in Figure 3, add the required volume of **SPRIselect** for the desired ratio to the sample.

   TIP Volume of sample * ratio = volume of **SPRIselect**.
   Example: 50 µL * 0.7x ratio = 35 µL of **SPRIselect**

2. Mix the total reaction volume by pipetting 10 times and incubate at RT for 1 minute
   OR

1-5

I Size Selection
ize Selection

Vortex for 1 minute at an appropriate speed until homogenous (depending on labware and total volume).

NOTE Insufficient mixing of sample and SPRIselect will lead to inconsistent size selection results. Make sure to mix well.

3. Place the reaction vessel on an appropriate magnetic stand or plate and allow the SPRI beads to settle to the magnet. Settle times will vary; a higher initial sample volume, higher **SPRIselect** ratio or weaker magnets will require a longer settle time.
4. Transfer the clear supernatant, which contains the Right Side Size Selected sample, to a new reaction vessel. The reaction vessel with the remaining beads can be discarded.

   NOTE Care should be taken not to aspirate more than a trace amount of beads during this step as the undesired larger fragment sizes are associated with the beads. Significant bead transfer will cause tailing into the larger size range.

5. Add the required volume of **SPRIselect**, using the calculation below, to the supernatant from Step 4 above. This will bind the fragments in the supernatant to the new SPRI beads.

   TIP Sample Volume µL * (1.8x – the initial ratio) = volume of SPRIselect
   Example: 50 µL * (1.8 – 0.7) = 55 µL of **SPRIselect**

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PCR 2 Nested

12 **PCR2. Nested PCR**

This is the nested PCR amplifying the amplicon you want to sequence. The gene specific primer contains a 5' sequence on it with a i7 adapter sequence.

Reagents	1 reaction			
		50 µl reaction		
5X PrimeSTAR Buffer		10		
10 mM dNTPs (#N0447)		4		
TMAC (0.5M)		3		
GSP primer (10uM)		1		
i5 Primer (10uM) prE359		0.5		
OneTaq DNA Polymerase		1		
Round 1 product		25		
Nuclease-free water		5.5		

You can add TMAC or not. I found that it enhances PCR efficiency but not specificity.

STEP	TEMP	TIME		
Initial Denaturation	98°C	30seconds		
12x	98°C 60C C68°C	10 seconds 15 seconds 60 sec		
Hold	10C			

Clean up with Ampure 1:0.9 SPRI
Elute 25ul with 10mM Tris, 0.1 mM EDTA

PCR- i7 indexing

13

PCR-3: i7 indexing

This adds the i7 indexing sequence. The primers can be from the nextera kit or you can synthesize them yourself.

Reagents	1 reaction		
		50 µl reaction	
5X PrimeSTAR Buffer		10	
10 mM dNTPs (#N0447)		4	

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i7 primer (10uM) Tm = 67 so use 60C for Ta		2	Uditas protocol stated crazy high conc but this is closer to what Illumina uses (Illumina is likely around 3ul of 10uM)
i5 Primer (10uM) prE359		1	
OneTaq DNA Polymerase		1	
Round 1 product		25	
Nuclease-free water		7	

For Nanodrop Nextera XT Index Kit V2. The Nextera Kit says to use 5ul of their adapter for a 50ul reaction. Online I found someone saying the Nextera kits primers are about 5uM and you add 5 ul. So this is <1/20 of the concentration the UDITAS kit says.

The Overhang sequence between the gene specific primer and the i7 index is predicted 67C with GXL. The i5 primer is predicted 69C. So one can do the PCR as a 2step at 68C or 3 step at 65C.

STEP	TEMP	TIME		
Initial Denaturation	98°C	30seconds		
10x - 12x	98°C 65C C68°C	10 seconds 15 seconds 60 sec	I have used 12 and don't see why not to use it as it maxes out nucleotides	
Hold	10C			

Clean up with Ampure 1:1 SPRI

14 Check with TOPO cloning

If this is a new protocol, TOPO clone some of your samples and align to finished sequences on the computer. Make sure all parts of your primers are there. Check where your sequencing primers will bind and make sure that the indexing primers read the i5 index before the i5 attached UMI's. Otherwise masking becomes complicated as your UMI is trapped between two indexing

example amplicon with correct ends from my work.

n704mcherryfwd-tn5-n501.gb

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15 **QPCR check**

This is the best way to quantify your amplicons and also show that they will functionally amplify in the sequencer flow cell.

Kappa Quantification Kit . This will give a functional assay/quantification of your library. It uses the P5/P7 primers and you can then see how well your library amplifies, which will represent how it amplifies on the flow cell.
I have found that my concentrations using size on Bioanalzyer/Qubit conc over estimates by 3-5x the functional concentration as measured by Qubit. And then when I sequence I see my samples are under represented compared to the PhiX

Can also skip and just use bioanalyzer and QuBit but that is not recommended.

16 **Pool and Concentrate Samples**

Pooling and SPRI cleaned amplicons.
1. Run your samples on a gel and cut out the size you want to sequence. Also run your i5 control on the gel to make sure your amplicons are not from background.

The i5 added transposon adds 80bp. The overhangs/indexes off my gene specific primer for i7 are 66bp . So there is an extra of 146bp beyond the amplicon to be sequenced. If you want 200bp genomic read then sequence 346bp amplicon.

2A. The most accurate way to measure concentration is qPCR using Kappa Quantification kit. This amplifies all amplicons that recieved the correct P5/P7 sequences. These are the sequences on the flow cell that will bind and amplify during sequencing.
2B. Also could calculate the molar amount of each product based on the concentration from Qubit and the size from the bioanalyzer.

3. Pool equimolar amounts of each product into a 1 mL Eppendorf tube.
4. Clean and concentrate pool using Agencourt Ampure XP SPRI beads (0.9x) Elute with 35 µL of 1x low TE

17 **Sequencing**

You need to know the nM concentration of your sample. You will need to make 100ul of 1nM so you really don't need much.
10ng of 500bp is 32 fmol. So this would have to be diluted 32x to get the 1nM
10ng of 400bp is 40 fmol. We would have to dilute this 40x
10ng of 300bp is 54 fmol.
1pmol/ul = 1uM.

1 fmol/ul = 1nM

Need to change the amount of indexing cycles from the normal 8 to 18.
Read order: 1) Read 1 (from i5 index side do 141 bp read). 2) Index 1 - i7 index 8bp read. 3) index 2 read i5 side 18 bp. 8bp i5+ 10bp UMI. 4) r*ead 2* primer goes from the i7 (do 151 bp). The gene specific primer was on the i7 side.

18 **Check your run**

Check your run BCL files with Sequence Analysis Viewer software from Illumina. Check the amount of aligned reads (your phiX reads). When you demultiplex you see a large 'undetermined' with indexes GGGGGGGG+AGATCTCG. This is phiX

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19 **BCL to Fastq conversion**

For normal sequencing you can use the BCL2FASTQ software. But we added the UMIs in the indexing sequence so it alters the normal masking. Also the Uditas software has a pipeline for demultiplexing built in.

If you want to just move the UMIs into the beginning of read sequence 2 (not Uditas pipeline)
change the readinfo.xml
or -use-bases-mask=y151,I8,I8,y151 command while running the BCL2FASTQ software

BCL to unsorted FastQ

this is what we use in our pipeline/ Uditas pipeline

```
bcl2fastq \
--runfolder-dir /input_directory
--no-lane-splitting \
--barcode-mismatches 1 \
--use-bases-mask=Y151,I8,I18,Y141 \   #Y151,I8,I8,Y151 pushes UMI onto read2
--create-fastq-for-index-reads \
-p 8 \
--output-dir /direcotry \
--stats-dir /directory \
--reports-dir /directory
```

20 **Software for analysis**

https://github.com/editasmedicine/uditas
https://github.com/ericdanner/uditas-replace

Required files:

for running uditas:

Bowtie Index:

One may compile your own bowtie2 index. But if your using only human or mouse then you can download the pre compiled and save a few hours.
Download the .X.2bt files for your respective genome: http://bowtie-bio.sourceforge.net/bowtie2/index.shtml
hg38.1.bt2, hg38.2.bt2, etc (Make sure they are renamed to hg38.X.bt2 etc)

Set enviornmental variable:
bash command: export BOWTIE2_INDEXES=/file location

2Bit genomes:

Download hg38.2bit https://hgdownload.cse.ucsc.edu/goldenPath/hg38/bigZips/hg38.2bit
Set environmental variable:
bash command: export GENOMES_2BIT=/file location

Citation: Eric Danner (04/17/2020). Tn5 Library Prep for Deep Sequencing Loci of CRISPR/Cas9 Edited Cells. Single gene specific primer amplification (UDiTaS protocol with alterations). https://dx.doi.org/10.17504/protocols.io.7k2hkye