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LAM-HGTGTS (Linear Amplification-mediated high-throughput genome-wide translocation sequencing) Our Working Protocol. V.2

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We use this protocol and it's working

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Abstract

LAM-HTGTS = linear amplification mediated high-throughput genomic translocations sequencing

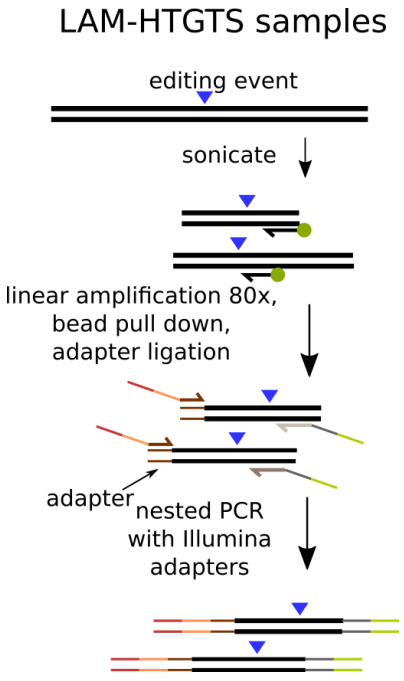
The presented version is our working version of the Frederick Alt and Richard Frock paper "Detecting DNA double-stranded breaks in mammalian genomes by linear amplification–mediated high-throughput genome-wide translocation sequencing" published in Nature Protocols in 2016.

The main application of the method is sequencing of the unknown sequences that flank known regions. It can be used in two ways:
1. inside-out (in genomic engineering it can be used to characterize the off-targets, for example).
2. outside-in (in the same setting of genomic engineering it can be used to measure the on-target efficiency and characterize the on-target events).

Main advantage of the method is its unbiased nature: the template genomic DNA is sheared by sonication which is presumably sequence-independent.

Original protocol is here:
Hu, J., Meyers, R. M., Dong, J., Panchakshari, R. A., Alt, F. W., & Frock, R. L. (2016). Detecting DNA double-stranded breaks in mammalian genomes by linear amplification-mediated high-throughput genome-wide translocation sequencing. *Nature Protocols*, *11*(5), 853–871. https://doi.org/10.1038/nprot.2016.043

These are the changes that made it work in our hands.



overview of LAM-HGTGTS

Guidelines

When we worked on a sheared control plasmid it failed repeatedly. It has worked consistantly on gDNA.

The original protocol stated that the beads do not affect PCR. This was not the case in our hands. We lowered bead concentration and it works well now.

› 7-8 hours of hands-on time
› 2-3 days total completion time

Materials

MATERIALS

- PrimeSTAR GXL DNA Polymerase **Catalog #**R050A
- NaCl **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**53014
- T4 DNA Ligase, 500u **Promega** **Catalog #**M1804
- Tris-HCl **Merck MilliporeSigma (Sigma-Aldrich)**
- Ultrasonicator
- EDTA **Fisher Scientific** **Catalog #**16 004Y
- Dynabeads™ MyOne™ Streptavidin C1 **Thermo Fisher** **Catalog #**65002
- Magnetic Stand-96 **Thermo Fisher** **Catalog #**AM10027
- Hexammine cobalt(III) chloride **Merck MilliporeSigma (Sigma-Aldrich)**
- Poly(ethylene glycol) 8000 **Merck MilliporeSigma (Sigma-Aldrich)**
- T4 DNA Ligase Buffer 10x **Promega**

Before start

Template: should be more than 5 ug of gDNA to provide enough material for the first step.

Prepare Reagents

1 **Lysis Buffer Preparation**

- 5M NaCl Dissolve 292.5 g of NaCl in H2O and bring to 1 liter. Autoclave, store at room temperature (RT; 20–25 °C) for up to 1 year.
- 0.5 M EDTA (pH 8.0) Dissolve 186.12 g of EDTA–Na2·2H2O in H2O, adjust the pH to 8.0 using 2.5 N NaOH and then adjust the total volume to 1 liter. Autoclave the solution and store it at RT for up to 1 year. Needs to be pH8 to dissolve
- 1 M Tris-HCl (pH 7.4) Dissolve 121.14 g of Tris base in H2O, adjust the pH to 7.4 using HCl, and then bring the total volume to 1 liter. Autoclave the solution and store it at RT for up to 1 year. Needs a lot of HCl- use 10N
- Cell lysis buffer Cell lysis buffer is 200 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM EDTA (pH 8.0) and 0.2% (wt/vol) SDS; store it at RT for up to 6 months. Proteinase K is added (final concentration at 200 ng/ml) before use. Can use frozen aliquots of Proteinase K.

Hexaamminecobalt(III) chloride (Sigma, #481521-25G). Dissolve it in water to produce 20 mM working solution.
PEG8000. Dissolve it in water to produce 50% solution.
2x B&W buffer: 2 M NaCl, 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA (pH 8.0). Dilute it with H2O to make 1x B&W buffer
TE buffer: 10 mM Tris-HCl (pH 7.4) and 0.5 mM EDTA (pH 8.0)

TE buffer for gDNA/primers

- TE buffer is 10 mM Tris-HCl (pH 7.4) and 0.5 mM EDTA (pH 8.0); store it at RT for up to 6 months.
- For 10ml : 10ml water, 100µl Tris-HCl stock, 10µl EDTA stock

Annealing Buffer:
25 mM NaCl, 10 mM Tris-HCl (pH 7.4) and 0.5 mM EDTA (pH 8.0). Store it at RT for up to 1 year.

Purchase Primers:

- Purchase 5' biotinylated primers

	Bridge adapter -upper	AdUp	5'GCGACTATAGGGCACGCGTGGNNNNNN-NH2-3'
	Bridge adapter -lower	AdLo	/5-Phosphorylation/CCACGCGTGCCCTATAGTCGC-NH2-3'
	Nested Gene Specific Primer_with-Nextera_Adapter_overhang	GSNest-overhang	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-NNNNN-gene_specific_sequence(20-25nt)-3'
	Nextera-adapterSequence-on_Bridge_adapter	Adapter-overhang	5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNGACTATAGGGCACGCGTGG-3'
	Indexing-N5xx (index is lowercase)	i5-N504	5'AATGATA

			CGG CGAC CACC GAGA TCTA CACa gagta gaTC GTCG GCA GCGT C-3'
	Indexing-N7xx (index is lowercase)	i7-N705	5'CA AGCA GAAG ACG GCAT ACGA GATa ggagt ccGT CTCG TGGG CTCG G-3'
	gene-specific biotinylated primer	GSBio	5'-biotylanted-gene_specific_20-25nt-3'

Explanation of the primers is below

primer explanation:
https://teichlab.github.io/scg_lib_structs/methods_html/SMART-seq_family.html

i5 /P5 side Oligos:

5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**-NNNNN-Gene-specific-sequence(20-25nt)-3'
bold = Nextera adapter seq
NNNNN = 5 nucleotides to help with cluster generation in flowcell

Indexing Primer-P5-i5xx-primer
5'-***AATGATACGGCGACCACCGAGATCTACAC-****TCTTTCCC*-TCGTCGGCAGCGTC-3'
Bold italics = this is the P5 flowcell binding sequence
italics = index (will vary)
normal font = sequence that binds to the Nextera adapter

For i7 Side DNA Oligos:

Adapter primer with 5' Trueseq overhangs
GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-NNNNN-*GACTATAGGGCACGCGTGG*
bold italics = Nextera primer binding site
NNNNN = 5 random nucleotides to assist in cluster generation in flowcell
italics = "Bridge adapter" sequence used to ligate onto the end of the sheared DNA

Indexing primer i7_N701 primer breakdown:
*CAAGCAGAAGACGGCATACGAGAT***TCGCCTTA**GTCTCGTGGGCTCGG
italics sequence= this is the portion of the amplicon that the indexing primer i7 and read 2 amplify from regardless of
italics underline: = this is what binds to the flow cell
bold = this is the N701 indexing sequence
normal font= this is the overhang that binds to the adapter overhangs

By changing out the N701 or i50x sequences above for other indexes you can snythesize you own Nextera library for much cheaper.

Here is a annotated amplicon with the Illumina adapters added for clarity:

n706-example-lam-n505-correct.gb

2 **gDNA extraction w/ Lysis buffer** (1 day)

1. Re-suspend up to 1*10^7 cells in 500 µl of cell lysis buffer and incubate them at 56 °C overnight (or 10-16hr). average 6pg.gDNA/cell → 60 ug for 10 million.
2. Add 500 µl of isopropanol directly into the microtube, and mix it immediately by inverting the microtube until the genomic DNA can be seen to form a pellet.
3. Use a pipette to transfer the DNA pellet to a new microtube containing 1 ml of 70% (vol/vol) ethanol. Centrifuge it at 13,000g for 5 min at 4 °C.
4. Discard the supernatant completely, and dissolve the pellet in 200 µl of TE at 56 °C for at least 2 h.
5. Check the concentration of a 1-µl aliquot with a NanoDrop (A260/280 >1.8). Make 100ng/µl

3 **Sonication** (1 hr)
Do in amplicon free environment.

1. In 1.5 ml microtube dilute 5 ug of gDNA down to 100 ng/µl with water (final volume - 50 µl). Original protocol says 20-100ug in 200µl so this can be scaled and is flexible.

2. If the number of samples is lower than 6, prepare the 6-N tubes with 50 µl of water (N - number of samples).
3. Insert 6 tubes in the small metal holder, close it with the metal lid-knob and place it in the Bioruptor.
4. Switch on the device, select the LOW intensity of the ultrasound.
5. Sonicate the DNA for 8 cycles of 30s ON and 90s OFF (This point is subject to change, cause it produced the products too short for the MinION sequencing). Cold bath @4C/ Bioruptor system at 4 °C
6. Load 500 ng (5 µl) of gDNA on 0.8% agarose gel along with the 1 kb Ladder. The peak of the length distribution should be around 2 kb. If it is higher, add 2 cycles of the sonication and repeat the analysis. If it is lower, redo the sonication from the non-sonicated material.

	Energy output	Low
	Working time	30 s
	Resting time	90 s
	Sonication cycles	8 cycles

Every sonicator and setup is different so you need to do some tests to figure out what shears it to the right setting

4 **LAM (linear amplification)-PCR**

Use GXL-hotstart. Alt paper recommends 5µg in each pcr tube so this could be altered. And 20-100µg of gDNa processed when wanting deep examination of translocations and they use the rule of thumb of 1:300 cells has translocation. For off target integration of the donor vector we can use 500ng.

GXL Tm primer calculator (from company):
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℃, set the annealing temperature to 60℃. When the Tm value is 55℃ or less, set the annealing temperature to 55℃.

	Component	Volume, µl	
	5x GXL buffer	10	
	GXL dNTPs 2.5 mM each	4	
	GSBio-primer (1uM)	2	0.04 uM Final
	GXL-enzyme	0.5	
	sonicated gDNA	5	500ng
	water	28.5	
	total vol	50	

Perform the amplification using this program: 98° 5 min --> [98° 30s --> Ta 60°-65° 30s --> 68° 90s] x 80 -->
68° 2 min

∗Clean up the PCR amplicon as quick as possible as some polymerases chew up ssDNA∗

Important: For the first use of new primers, check 5 µl of the reaction on the 0.8% agarose gel to ensure there was no exponential amplification.

5 **Biotinylated products capture (3hr)**

We believes that these capture most of the DNA.

Wash (N+1)∗(Volume of rxn/25) µl of StrepBeads in B&W two times, resuspend the beads in mQ and mix the
reactions as follows

Clean beads/ prep before adding them to add to linear PCR mix:
a) vortex the beads 30 sec
b) transfer 20 µl Dynabeads C1 Streptavidin (400µg)(10µg/µl) to a microtube. Add 600µl 1x B&W buffer. Pipette.
c) capture beads on magnet stand for 1 min
d) discard supernatant.

e) wash in 600µl B&W buffer 1x, bind to beads 1 min, discard supernatant. (2x washing total).
f) beads are ready to go

Bind amplicons to beads:
1. Re-suspend beads in PCR the recently completed PCR solution contained biotinylated linear amplicons.
2. Put bead+PCR solution on mixer for at least 2 hr. ∗2 hr is ok, but 4 hr recommended if possible* Can mix overnight
3. Capture the DNA-beads complex on the magnet stand, and wash the DNA-beads complex with 200 µl of 1× B&W buffer (3x wash)
4. Re-suspend the beads in 1 ml of H2O, capture the beads on the magnet stand for 1 min and discard the supernatant.
5. Re-suspend the beads in 9 µl of H2O for ligation reaction (next step_.

	Component	V, µl	If you took 5 ul for gel: ------>	V, µl
	LAM-PCR product	50		45
	5M NaCl	14		14
	0.5 M EDTA	0.7		0.7
	Washed Strep Beads	2		1.8
	water (mQ)	3.3		8.5
	Total	70		70

Incubate the reactions on the roller for at least 2h (4h is recommended, the reaction can be rolled
overnight).

6 **On-Bead Adapter Ligation (5hr)**

This ligates the adapter sequence onto the 3' end of the Linear Amplicon from the last step. This will allow for PCR.

Anneal your Bridge adapter upper+lower: dissolved oligos in the annealing buffer to 400 uM, mixed 1:1. Put mixture in a beaker filled with boiling water, kept 100 degrees for five

minutes and allowed it to cool to RT. Diluted the annealed oligos to 50 uM with water and use aliquots as the working solution.

1. Capture the DNA-beads complex on the magnetic stand and wash the beads with 150 µl of B&W three times. When working with StrepBeads, you need to re-suspend the beads by pipetting on each washing step.
2. Wash the DNA-beads complex with 150 µl mQ one time and re-suspend the beads in 9 µl of mQ.
3. Mix the reagents as in the table below, add the beads-DNA complex and then add 50% PEG8000 with cut tips. Mix the reaction thoroughly by pipetting.

critical step: To improve the ligation efficiency, re-suspend the mixture after 2 h of incubation. Do not spin the mixture before incubation, as the settling of DNA beads greatly reduces the ligation efficiency.

pause point: The ligation reactions can be optionally incubated at 16 °C overnight instead of 1 h.

	Component	Vol, µl
	Bead-DNA	9
	10x T4 lig buffer	2
	Bridge Adapt (50 µM)	1
	T4 DNA lig	1
	Hexa Cobalt	1
	50% PEG8000	6
	Total	20

Put on thermocycler. 22° 1h --> 16° 1h --> 14° 1h --> 10° 1h --> stored at +4

7 On-beads Adapter PCR

This uses a primer that is nested from the Linear Amplification primer and the adapter sequence.

Wash Ligation product
A) Add 20µl of 2x B%W buffer.
B) Capture beads on magnet
C) Wash 2x. Each time take off of magnet and let beads re-suspend in solution
D) wash in 200µl water off magnet.
E) Capture beads on magnet. Re-suspend in 50µl water/TE buffer
Pause Point. Can store at -20C in preparation for PCR

Run PCR:
We found that the beads inhibit PCR (though the bead manual says bead do not affect PCR). 4µg/50µl rxn is maximum. The beads are 10µg/µl. This protocol has been using 20µg beads/50µl linear PCR. So that means after ligation use 2µl of the ligation product or 1/10. In this we hope to capture the products from all 500ng gDNA alleles as we got the 80x cycles.

Run a PCR using nested primers with a Illumina adapter overhang (primer: GSNest-overhang). Uses an adapter primer with a Illumina adapter overhang (primer: Adapter-overhang).

	5x GXL buffer	10	1x	
	GXL dNTPs	4		
	GSNest-overhang (10uM)	2		
	Adapter-overhang (10uM)	2		
	GXL-hotstart	0.5		
	sonicated DNA	5		
	water	28.5		
	total vol	50		

Program for Adapter PCR: 98° 1 min --> [98° 10s --> Ta° 15s --> 68° 60s] x 30 --> 68° 2 min

Purify the reactions with 1.0x AMPure XP beads and elute it in 25µl 1x TE.
SPRI beads can inhibit PCR so work to remove them as best as possible.

8 **Nextera Indexing PCR**
This adds the indexing sequences and flow cell binding sequences needed for Illumina.

Use the (Indexing-N5xx primer) and (Indexing-N7xx primer)

	5x GXL buffer	10		
	GXL dNTPs	4		
	Indexing-N5xx-primer (10uM)	2		
	Indexing-N7xx-primer (10uM)	2		
	GXL-hotstart	1		
	Adapter PCR eluate	25		
	water	6		
	total vol	50		

Program for Indexing PCR: 98° 1 min --> [98° 10s --> 55° 15s --> 68° 60s] x 5 --> 68° 2 min

Load on gel (see next step)

9 **Check size distribution, quantify and pool samples**

Goal: measure your amplicons for concentration and ready them for pooling

Method 1:

1. Load the products on 2% agarose gel along with 100 bp ladder. Cut out the desired size for sequencing and gel extract.
2. Take a small amount and quantify amplicons using Kappa Quantification Kit. This uses the same primer binding as the flow cell and so measures functional amplicons.
3. Pool samples in ratio desired.

Method 2:

1. Load a few µl of PCR products on 2% agarose gel along with 100 bp ladder. Analyze the gel by GelAnalyzer or any
other gel analysis software. Determine the peak length of the library and calculate the mass of the samples using the Area Under Curve method. Using these data with density of the ladder as control, calculate the molarity of your samples.
2. Pool samples with equal molarity.
3. Run on gel and gel extract band of desired size. (Could also use BluePipen)
4. Check with QuBit for measured concentration.
5. Pool samples as desired.

Send the library for sequencing or store it at -20°

10 **Dumultiplexing and Informatics**

BCL to Fastq conversion: you can use the BCL2FASTQ software with the conventional .csv file method.

Check for mispriming by filtering for reads with the expected sequence infront of your primer binding site.