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SSLib v2.0 (Gansauge et al. 2017)

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ABSTRACT

This bench protocol is based on the work of Gansauge and Meyer (2013) and Gansauge et al (2017), for preparing shotgun libraries from single-stranded DNA, typically for ancient and degraded DNA.

ATTACHMENTS

Gansauge_etal_2017.pdf | Gansauge-2013-Single-stranded DNA.pdf | Gansauge_etal_2017__Supp.pdf | SSLIBV2.0_Sequencing_expectations.pdf

GUIDELINES

Use at your own risk. You are responsible for double-checking that everything is correct!

Familiarise yourself with the relevent literature (Gansauge and Meyer 2013, and Gansauge et al. 2017) before attempting!

MATERIALS

MATERIALS

- T4 RNA Ligase Reaction Buffer - 3.0 ml **New England Biolabs Catalog #B0216L**
- Tris-HCl, pH 8.0 (UltraPure) **Thermo Fisher Scientific Catalog #15568025**
- 5 M Sodium chloride (NaCl) **Sigma Aldrich Catalog #S5150-1L**
- DMSO **Sigma Catalog #D8418**
- Dynabeads™ MyOne™ Streptavidin C1 **Thermo Fisher Scientific Catalog #65001**
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977023**

Protocol status: Working
We use this protocol and it's working

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Last Modified: Sep 25, 2023

PROTOCOL integer ID: 34124

- AmpliTaq Gold™ DNA Polymerase with Gold Buffer and MgCl2 **Thermo Fisher Catalog #4311806**
- SDS, 20% Solution, RNase-free **Thermo Fisher Catalog #AM9820**
- FastAP Thermosensitive Alkaline Phosphatase (1 U/µL) **Thermo Fisher Catalog #EF0651**
- T4 Polynucleotide Kinase (10 U/µL) **Thermo Fisher Catalog #EK0031**
- T4 DNA Ligase (5 U/µL) **Thermo Fisher Catalog #EL0011**
- T4 DNA Ligase, HC (30 U/µL) **Thermo Fisher Catalog #EL0013**
- Klenow Fragment (10 U/µL) **Thermo Fisher Catalog #EP0051**
- SYBR™ Green I Nucleic Acid Gel Stain - 10,000X concentrate in DMSO **Thermo Fisher Catalog #S7563**
- UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #15575020**
- T4 DNA ligase buffer 10 X **Thermo Fisher Scientific Catalog #Supplied with EL0013**
- Klenow reaction buffer 10X **Life Technologies Catalog #Supplied with EP0051**
- Tween 20 **Sigma Aldrich Catalog #P2287-100ml**
- PEG-8000 50% w/v **New England Biolabs Catalog #Supplied w/ B0216L (T4 RNA ligas**
- Adenosine Triphosphate (ATP) 100 mM **Thermo Fisher Scientific Catalog #R0441**
- 20X SSC **Sigma Aldrich Catalog #S6639-1L**
- Bovine Serum Albumin (BSA) **Contributed by users Catalog #BSA-50**
- dNTPs 100 mM ea. **Bioline Catalog #BIO-39025**
- PEG-4000 50% w/v **Thermo Fisher Scientific Catalog #Supplied with EL0013 (T4 DNA lig**

Note

Note that "N" in the splinter oligo represents any base such that the oligo is a mixture of various random sequences in this place.

In contrast, the "NNNNNN" in the indexing primers represents a specific string of bases (e.g., order "CGCTCAGT" in this place). Each sample will be indexed with a unique combination of indexing primers. Ideally, these combinations should not be reused in the lab. Be sure to follow Illumina's recommendations when chosing primer combinations (e.g., ensure adequate diversity in the bases, ensure each is at least 3 bp different from each other, don't use indexes that will begin with two dark cycles, etc.).

If you can afford it, you can oder all oligos HPLC purified, but I have found it not to have any detectable impact.

Gansauge and Meyer (2013) "strongly recommend checking the synthesis quality [of oligos] using acrylamide gel electrophoresis. Synthesis artifacts and impurities will lower the yield of library preparation. Moreover, incomplete single-stranded adapter oligonucleotides may serve as template for library preparation causing artifact formation". I have not done this before and have observed some artifacts that can be seen after running the CL104 positive oligo library on a gel. If artifacts are observed, re-order oligos.

	Oligos	Concentration	Working concentration	Synthesis scale	Purification	Supplier	Resuspension buffer	Sequence 5'-3'
	CL78	200 uM	na	250 nmol	Dual HPLC	IDT	TE	/5Phos/AGATCGGAAG/iSp9//iSp9//iSp9//3BioTEG/
	Splinter	100 uM	na	200 nmol	desalted	Eurogentec	TE	[SpC12][A][A][A]CTTCCGATCTNNNNNNNN[AmC6]
	CL53	500 uM	na	250 nmol	HPLC	IDT	TE	ACACGACGCTCTTC/3ddC/
	CL73	500 uM	na	250 nmol	HPLC	IDT	TE	/5Phos/GGAAGAGCGTCGTGTAGGGAAAGAG*T*G*T*A
	CL130	100 uM	na	100 nmol	HPLC	IDT	TE	GTGACTGGAGTTCAGACGTGTGCTCTTCC*GA*TC*T

	CL104	100 uM	0.1 uM	100 nmol	HPLC	IDT	TET buffer	/5Phos/TCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGA/3Phos/
	CL107	100 uM	10 uM	25 nmol	desalted	IDT	Water	TCATGTAACTCGCCTTGATCGT
	CL108	100 uM	10 uM	25 nmol	desalted	IDT	Water	TCGTCGTTTGGTATGGCTTC
	IS7	100 uM	10 uM	25 nmol	desalted	IDT	Water	ACACTCTTTCCCTACACGAC
	IS8	100 uM	10 uM	25 nmol	desalted	IDT	Water	GTGACTGGAGTTCAGACGTGT
	CL72_Custom_Seq_Primer	100 uM	na	25 nmol	desalted	IDT	Water	ACACTCTTTCCCTACACGACGCTCTTCC
	CL72_i5_index	100 uM	na	25 nmol	desalted	IDT	Water	GGA AGA GCG TCG TGT AGG GAA AGA GTG T
	CL105_CL106_Std	100 uM	Dilute to 10^11 - 10^2 copies/ul	4 nmol Ultramer	desalted	IDT	TET buffer	ACACTCTTTCCCTACACGACGCTCTTCCTCGTCGTTTGGTATGGCTTCTATCGUATCGATCGATCGACGATCAAGGCGAGTTACATGAAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
	P5-indexing primer	100 uM	10 uM	25 nmol	desalted	IDT	Water	AATGATACGGCGACCACCGAGATCTACACNNNNNNNNACACTCTTTCCCTACACGACGCTCTT
	P7-indexing primer	100 uM	10 uM	25 nmol	desalted	IDT	Water	CAAGCAGAAGACGGCATACGAGATNNNNNNNNNGTGACTGGAGTTCAGACGTGT

Table 1. Oligos needed. Store all oligos at -20 deg C.

Equipment needed

Thermalcycler
QuantStudio 3 qPCR machine
Thermalshaker
Heat block
Hybridisation oven
Magnetic rack (1.5 ml tubes)
P1000, P200, P100, P20, P10, P2 pipettes and extra-long filter tips
10% Household bleach solution
70% Ethanol

Kimwipes and paper towels
15 ml Falcon tubes
50 ml Falcon tubes
1.5 ml Safelock tubes
1.5 ml Lo-bind Safelock tubes
0.5 ml Lo-bind Safelock tubes
0.2 ml Lo-bind PCR tubes
8-well strip optical qPCR tubes with attached lid (0.1 ml profile)
Sharps container
UV glove box
96-well PCR plate rack
1.5 ml-2.0ml tube racks
15 ml tube racks
50 ml tube racks
Minispin
Vortex

BEFORE START INSTRUCTIONS

Note that you will need to adjust steps depending on the equipment you have (e.g., a Thermal shakers and hybridisation ovens can be used interchangably). If you only have heat blocks, you will need to keep beads moving by regular vortexing. For high-throughput, it may be advisable to use a 96-well plate magent coupled with 0.2 ml tubes rather than 1.5 ml tubes.

Always include the positive control oligo CL104 in the library preparation, as well as a no-template control and any extraction controls.

Note that CL78 should have a 5' phosphate, but this is missing in Gansauge and Meyer (2013).

Note that 0.1X BWT is NOT just a diluted version of 1X BWT. It only contains a tenth of the NaCl but is the same for most of the other reagents. Here, I call it WASH A as per Gansauge and Meyer (2013) to avoid confusion.

I have adjusted the volumes of buffers to make only 10 ml of the wash buffers, which is typically enough for a batch of 12 libraries. If you are doing more reactions, scale up the recipe.

Note that some oligos need to be resuspended in a specific buffer.

Note that the splinter oligo used here is not published in Gansauge et al. (2017), but was personally recommended to me by Matthias Meyer.

Store PEG and ATP at -20 deg C and avoid repeated rounds of freeze thawing.

Store MyOne C1 Streptavidin beads at 4 deg C in a fridge.

Preparation

30m

1

Note

Perform all reaction set-up steps in a reagent-only pre-PCR space inside a dedicated ultraclean environment. Add DNA and subsequent master-mixes to the reaction, and perform wash steps, in a separate pre-PCR space.

"Suit up" in this order: hair net, nitrile gloves, facemask, coveralls, gumboots, booties, second pair of gloves.

2 Prepare the space by decontaminating surfaces with 10% household bleach followed by 70% ethanol. UV irradiate pipettes and racks. Racks should be bleached between subsequent uses and UV irradiated.

3 Ensure ice is available. Thaw reagents on ice as needed. Keep enzymes on ice at all times. Do not vortex enzymes to mix but mix by flicking the tube gently. Pulse centrifuge all reagents before opening.

4 Label tubes.

	Tube	Qty	For ...
	1.5 ml Safelock Tube	10	5X SYBR
	0.5 ml Safelock Tube	8	25 mM dNTPs
	1.5 ml Safelock Tube	1	0.1 uM CL104
	1.5 ml Safelock Tube	1	0.1 uM CL105 1/500
	1.5 ml Safelock Tube	1	10 uM IS7
	1.5 ml Safelock Tube	1	10 uM IS8
	1.5 ml Safelock Tube	1	10 uM CL107
	1.5 ml Safelock Tube	1	10 uM CL108

	1.5 ml Safelock Tube	10	CL105_106 STD dilution series 10^11 - 10^2
	15 ml Falcon Tube	1	TE Buffer
	15 ml Falcon Tube	1	Bead binding buffer
	15 ml Falcon Tube	1	Wash A
	15 ml Falcon Tube	1	Wash B
	15 ml Falcon Tube	1	EBT buffer
	1.5 ml Safelock Tube	1	1% Tween 20
	1.5 ml Safelock Tube	1	2% Tween 20
	50 ml Falcon Tube	1	TET buffer
	0.2 ml Lo-bind PCR Tube	1	Purify CL78
	0.2 ml Lo-bind PCR Tube	1	Purify Splinter
	0.2 ml Lo-bind PCR Tube	1	CL78/ Splinter (DS1)
	0.2 ml Lo-bind PCR Tube	1	CL53/ CL73 (DS2)

	0.2 ml Lo-bind PCR Tube	# of samples + 2	Reaction tubes
	1.5 ml Lo-bind Tube	4	Step 16, Step 20, Step 38, Step 48 master mixes
	1.5 ml Lo-bind Tube	1	Beads wash
	15 ml Falcon Tube	1	Beads resuspension
	1.5 ml Lo-bind Tube	# of samples + 2	Reaction tubes 2
	0.5 ml Lo-bind Tube	# of samples + 2	Final library
	0.5 ml Lo-bind Tube	# of samples + 2	1/20 dilution of library
	1.5 ml Safelock Tube	2	Assay A and B master mixes
	8-strip optical qPCR Tubes	(((# of samples + 2)*2)+26)/8	Assay A and B

5 Prepare all necessary buffers and UV decontaminate where appropriate.

Note

Only add the SDS to the Bead Binding Buffer right before us. Discard Bead Binding Buffer after use.

Aliquot 5X SYBR into 500-ul batches and store at -20 deg C in foil.

Aliquot dNTPs into 50-ul batches and store at -20 deg C.

	A	B	C	D
	(Discard after use)	1 M Tris-HCl	100 ul	0.01 M
	(Exp. 1 month)	1 M Tris-HCl	100 ul	0.01 M
	(Exp. 1 month)	20% SDS	50 ul	0.1%
	(Exp. 1 year)	1 M Tris-HCl	100 ul	0.01 M
	(Exp. 1 year)	1 M Tris-HCl	100 ul	0.01 M
	(Exp. 1 year)	0.5 M EDTA	100 ul	0.001 M
	1% Tween 20	100% Tween 20	10 ul	1%
	2% Tween 20	100% Tween 20	20 ul	2%
	25 mM dNTPs	100 mM dATP	100 ul	25 mM
	5X SYBR	10,000X SYBR	2.5 ul	5X
	Bead Binding Buffer	5 M NaCl	2 ml	1 M
	Buffer	Reagent	Volume to add	Final concentration in solution
	EBT	1 M Tris-HCl	100 ul	0.01 M
	Stringency wash	20X SSC	50 ul	0.1X
	TE Buffer	Ultrapure water	9.88 ml	na
	TET buffer	1 M Tris-HCl	500 ul	0.01 M
	Wash A	5 M NaCl	200 ul	0.1 M
	Wash B	5 M NaCl	200 ul	0.1 M
		0.5 M EDTA	20 ul	0.001 M
		0.5 M EDTA	10 ul	0.0005 M
		100% Tween 20	5 ul	0.05%

	A	B	C	D
		20% SDS	250 ul	0.5%
		Ultrapure water	7.635 ml	na
		0.5 M EDTA	20 ul	0.001 M
		100% Tween 20	5 ul	0.05%
		20% SDS	250 ul	0.5%
		Ultrapure water	9.425 ml	na
		Ultrapure water	9.9 ml	na
		0.5 M EDTA	20 ul	0.001 M
		100% Tween-20	5 ul	0.05%
		Ultrapure water	9.675 ml	na
		100% Tween 20	5 ul	0.05%
		Ultrapure water	9.895 ml	na
		Ultrapure water	990 ul	na
		Ultrapure water	980 ul	na
		100% Tween 20	25 ul	0.05%
		Ultrapure water	49.375 ml	na
		DMSO	997.5 ul	na
		DMSO	4 ml	na
		100 mM dTTP	100 ul	25 mM
		100 mM dCTP	100 ul	25 mM
		100 mM dGTP	100 ul	25 mM

6

Before resuspending oligos, pulse centrifuge to collect the pellet at the bottom of the tube. Add the appropriate buffer (see Materials) and vortex thoroughly. Store at -20 deg C. Dilute out the working concentrations (below) and store at -20 deg C when not in use. Thaw on ice. Vortex and pulse centrifuge after each thaw. Before beginning library preparation, make sure you have enough of each working stock prepared!

Note

Note: Do not store oligos and adapters in the same box as enzymes or reagents!

The standards should be diluted in a totally different space, such as a teaching lab to ensure it does not contaminate the lab at extremely high concentration.

Also take extreme care with the positive control oligo as it will become a template for library preparation!

	Working stock	Reagent	Volume to add
	10 uM CL104	100 uM CL104	50 ul
		TET buffer	450 ul
	0.1 uM CL104	10 uM CL104	5 ul
		TET buffer	495 ul
	0.1 uM CL104 1/500	0.1 uM CL104	1 ul
	(i.e., 0.0002 uM)	TET buffer	499 ul
	10 uM IS7	100 uM IS7	50 ul
		Ultrapure water	450 ul
	10 uM IS8	100 uM IS8	50 ul
		Ultrapure water	450 ul
	10 uM CL107	100 uM CL107	50 ul

		Ultrapure water	450 ul
	10 uM CL108	100 uM CL108	50 ul
		Ultrapure water	450 ul
	10 uM CL105_106_STD	100 uM CL105_106_STD	50 ul
		TET buffer	450 ul
	10^11 copies CL105_106_STD	10 uM CL105_106_STD	10 ul
		TET buffer	592.25 ul
	10^10 copies CL105_106_STD	10^11 copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^9 copies CL105_106_STD	10^10 copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^8 copies CL105_106_STD	10^9 copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^7copies CL105_106_STD	10^8 copies CL105_106_STD	50 ul
		TET buffer	450 ul

	10^6 copies CL105_106_STD	10^7copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^5 copies CL105_106_STD	10^6 copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^4 copies CL105_106_STD	10^5 copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^3 copies CL105_106_STD	10^4 copies CL105_106_STD	50 ul
		TET buffer	450 ul
	10^2 copies CL105_106_STD	10^3 copies CL105_106_STD	50 ul
		TET buffer	450 ul

7 Pre-program the thermal cycler and thermalshaker.

Purify and ligate adapters

1h

8 Combine the following in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	CL78	20 ul	200 uM	20 uM	2 ul
	T4 RNA ligase buffer	20 ul	10 X	1 X	2 ul
	Klenow fragment	20 ul	10 U/ul	0.5 U/ul	1 ul
	T4 PNK	20 ul	10 U/ul	0.5 U/ul	1 ul
	Ultrapure water	20 ul	na	na	14 ul

9 Combine the following in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge.

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	Splinter	20 ul	100 uM	40 uM	8 ul
	T4 RNA ligase buffer	20 ul	10 X	1 X	2 ul
	Klenow fragment	20 ul	10 U/ul	0.5 U/ul	1 ul
	T4 PNK	20 ul	10 U/ul	0.5 U/ul	1 ul
	Ultrapure water	20 ul	na	na	8 ul

10 Incubate both reactions for 20 minutes at 37 deg C, followed by 1 min at 95 deg C in a thermal cycler with heated lid.

11 Combine the following in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge. This makes 10/20 uM of CL78/Splinter (DS1).

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	CL78 (purified)	40 ul	20 uM	10 uM	20 ul
	Splinter (purified)	40 ul	40 uM	20 uM	20 ul

12 Combine the following in a 0.2 ml Lo-bind PCR tube. Vortex and pulse centrifuge. This makes 200 uM of CL53/CL73 (DS2).

	Reagent	V2 (reaction volume)	C1 (stock concentration)	C2 (concentration in reaction)	V1 (volume to add)
	CL53	50 ul	500 uM	200 uM	20 ul
	CL73	50 ul	500 uM	200 uM	20 ul
	TE buffer	50 ul	na	na	9.5 ul
	NaCl	50 ul	5 M	0.05 M	0.5 ul

13 Incubate for 10 sec at 95 deg C, followed by a ramp down to 10 deg C at 0.1 deg C/sec in a thermal cycler with a heated lid.

14 Add 50 ul of TE buffer to DS2 to make 100 uM of DS2 (CL53/CL73).

Dephosphorylation, heat denaturation, and ligation of first ad...

1h 15m

15

Note

Note: on personal recommendation, I do not do the cleavage at abasic sites step using Endonuclease VIII as in Gansauge and Meyer (2013).

I also do not perform UDG treament as I find it beneficial to see the damage patterns in ancient DNA as a gauge of authenticity. However, some people prepare two libraries, one UDG treated and other other not.

Set a heat block or thermal shaker to 45 deg C and set another thermal shaker to 35 deg C.

16 Make up the following master mix in a 1.5 ml Lo-bind tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x _____ _ rxn
	T4 RNA ligation buffer	46 ul	10 X	1.74 X	8 ul	
	Tween 20	46 ul	2 %	0.087 %	2 ul	
	FastAP	46 ul	1 U/ul	1 U	1 ul	
	Ultrapure water	46 ul	na	na	23 ul	

17 Aliquot 34 ul per reaction into a 0.2 ml Lo-bind PCR tube.

18 To make the total reaction volume up to 46 ul, add:

Up to 12 ul DNA to each sample reaction. Make up the remainder with Ultrapure water. Typically input 3x10^8 - 3x10^11 double-stranded molecules; 1 fmol-1pmol single-stranded DNA; 13 pg-14 ng of ca. 40 bp DNA--this is typically 20% of the extract.

1 ul of 0.1 uM CL104 positive control oligo to the positive conrol reaction + 11 ul Ultrapure water. We are inputing 3.01x10^10 molecules of single-stranded CL104 into the library preparation.

12 ul of Ultrapure water to the no-template control reaction.

19 Incubate for 10 min at 37 deg C in a thermal cycler with a heated lid followed by 2 min at 95 deg C. Place immediately into an ice water bath.

20 Make up the following master mix in a 1.5 ml Lo-bind tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x _____ _ rxn
	PEG-8000	80 ul	50%	20%	32 ul	
	ATP	80 ul	100 mM	0.5 mM	0.4 ul	
	DS1 (CL78/Splinter)	80 ul	10/20 uM	0.125 /0.25 uM	1 ul	
	T4 DNA ligase	80 ul	30 U/ul	30 U	1 ul	

21 Aliquot 34.4 ul to the reactions from Step 19. Vortex and pulse centrifuge.

22 Incubate 1 hr at 37 deg C in a thermal cycler, followed by 1 min at 95 deg C. Transfer immediately to ice.

Note

While incubating, you can make up the wash buffers if they have not been prepared earlier.

23 **Pause point: Reactions can be frozen at -20 deg C for several days before proceeding. If you proceed immediately, skip Step 30 below.**

Immobilisation of ligation products on bead

30m

24 Allow MyOne C1 Streptavidin beads to come to room temperature. Vortex.

25 For every sample (including controls) aliquot 20 ul of MyOne C1 Streptavidin beads to a 1.5 ml Lo-bind tube. Include 20 ul extra for pipetting error. (e.g., if you have 5 samples + 2 controls, aliquot 160 ul of beads).

Note

Note that I have heard some find that beads do not stick well to Lo-bind tubes; I have not personally had an issue with this using a Dynamag, but if there is an issue, use non-Lo-bind tubes.

26 Allow the beads to separate from solution on a magnetic rack for 1-2 minutes. Discard the supernatant. Add 500 ul of Bead Binding Buffer. Vortex.

Note

Do not forget to add the SDS to the Bead Binding Buffer directly before use.

27 Repeat Step 26.

28 Discard the supernatant. Resuspend beads in 250 ul Bead Binding Buffer per sample including controls and pipetting error (e.g., if you have 5 samples + 2 controls, resuspend the beads in 2 ml of Bead Binding Buffer). Pipette up and down gently to resuspend to avoid generating excessive bubbles.

29 Aliquot 250 ul of bead suspension to new 1.5 ml Lo-bind Safelock tubes (1 per sample + controls).

30 Incubate ligation reactions from Step 22 for 1 min at 95 deg C and transfer to an ice water bath for 2-5 min.

Note

Remember to skip this step if you did not pause at Step 23.

31 Add the ligation reactions from Step 22 to the bead suspension. Vortex.

32 Incubate for 20 min at room temperature with inversion. Pulse centrifuge.

Note

Note that this step can be performed in a rotating hybridisation oven at room temperature or using a nutator / rocker.

33 Pellet the beads with the magnetic rack and discard the supernatant.

34 Add 200 ul of Wash A to each sample. Vortex. Pulse centrifuge. Pellet beads with a magnetic rack and discard the supernatant.

35 Add 100 ul Stringency Wash Buffer. Vortex. Incubate 3 min at 45 deg C in a heat block (or thermal shaker), vortexing every 30 sec. Pulse centrifuge. Pellet beads with a magenetic rack and discard the supernatant.

Note

While the samples are incubating, you can begin to make up the master mix at Step 38.

36 Set the heat block to 65 deg C so it has time to heat up before needed.

Note

Note that if you have several thermal shakers and heat blocks, these can all be pre-set to avoid having to wait for temperatures to change. Heat blocks take a very long time to cool down, so thermal shakers are preferred.

37 Add 200 ul of Wash B to each sample. Vortex. Pulse centrifuge. Pellet beads with a magnetic rack and discard the supernatant.

Primer annealing and extension

45m

38 Make up the following master mix in a 1.5 ml Lo-bind tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x _____ _ rxn
	Ultrapure water	48 ul	na	na	39.1 ul	
	Klenow reaction buffer	48 ul	10 X	1.04 X	5 ul	
	dNTPs	48 ul	25 mM	0.21 mM	0.4 ul	
	Tween 20	48 ul	1%	0.052 %	2.5 ul	
	CL130	48 ul	100 uM	2.083 uM	1 ul	

39 Add 48 ul of the master mix to each reaction from Step 37. Vortex and pulse centrifuge.

40 Incubate 2 min at 65 deg C in a heat block (or thermal shaker), and place immediately in an ice water bath for 2-5 min. Transfer the rack to room temperature.

41 Set the heat block back to 45 deg C so that it has time to cool down before needed.

42 Add 2 ul of Klenow fragment (10 U/ul) to the reactions from Step 40. Vortex and pulse centrifuge.

43 Incubate 5 min at 25 deg C (or room temperature), followed by 25 min at 35 deg C in a thermo shaker, with shaking at 1000 rpm. Do not allow beads to settle.

44 Remove the reactions and pellet the beads on a magnetic rack. Set the thermo shaker to 22 deg C.

Post-extension washes

10m

45 Add 200 ul of Wash A to each sample. Vortex. Pulse centrifuge. Pellet beads with a magnetic rack and discard the supernatant.

46 Add 100 ul Stringency Wash Buffer. Vortex. Incubate 3 min at 45 deg C in a heat block (or thermal shaker), vortexing every 30 sec. Pulse centrifuge. Pellet beads with a magenetic rack and discard the supernatant.

47 Add 200 ul of Wash B to each sample. Vortex. Pulse centrifuge. Pellet beads with a magnetic rack and discard the supernatant.

Ligation of second adapter

1h 5m

48 Make up the following master mix in a 1.5 ml Lo-bind tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x _____ _ rxn
	Ultrapure water	100 ul	na	na	73.5 ul	
	T4 DNA ligase buffer	100 ul	10 X	1 X	10 ul	
	PEG-4000	100 ul	50%	5%	10 ul	
	Tween 20	100 ul	1%	0.025 %	2.5 ul	
	DS2 (CL53/CL73)	100 ul	100 uM	2 uM	2 ul	
	T4 DNA ligase	100 ul	5 U/ul	0.1 U/ul	2 ul	

49 Add 100 ul of master mix to each tube. Vortex and pulse centrifuge.

50 Incubate for 1 hr at 22 deg C in the thermo shaker, shaking at 1000 rpm.

Note

Note: if the shaker has not cooled to 22 deg C yet, perform this step by hand vortexing at room temperature until the shaker is cool.

51 Remove samples and set shaker to 95 deg C. Pellet the beads in a magnetic rack and discard the supernatant.

Post-ligation washes

10m

52 Add 200 ul of Wash A to each sample. Vortex. Pulse centrifuge. Pellet beads with a magnetic rack and discard the supernatant.

53 Add 100 ul Stringency Wash Buffer. Vortex. Incubate 3 min at 45 deg C in a heat block (or thermal shaker), vortexing every 30 sec. Pulse centrifuge. Pellet beads with a magenetic rack and discard the supernatant.

54 Add 200 ul of Wash B to each sample. Vortex. Pulse centrifuge. Pellet beads with a magnetic rack and discard the supernatant.

Elution of the final library

5m

55 Add 25 ul EBT buffer to each sample. Vortex and pulse centrifuge.

56 Incubate for 2 min at 95 deg C in a thermo shaker (without shaking).

57 Transfer tubes immediately to the magnetic rack and pellet the beads. Transfer the supernatant to a clean 0.5 ml Lo-bind tube.

58 Create a 1in20 dilution of each library in Ultrapure water (1 ul library + 19 ul Ultrapure water). Vortex and pulse centrifuge.

59 Libraries can be stored at -20 deg C until amplification. For long-term storage, store at -80 deg C.

Quant the library

2h 30m

60 Make up the following master mix in a 1.5 ml Lo-bind tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x _____ rxn
	Ultrapure water	25 ul	na	na	15.9 ul	
	BSA	25 ul	10 mg /ml	0.4 mg/ml	1 ul	
	ABI Gold PCR Buffer	25 ul	10 X	1 X	2.5 ul	
	MgCl2	25 ul	25 mM	2.5 mM	2.5 ul	
	dNTPs	25 ul	25 mM	0.25 mM	0.25 ul	
	ABI Taq Gold DNA polymerase	25 ul	5 U/ul	0.05 U/ul	0.25 ul	
	SYBR Green	25 ul	5 X	0.12 X	0.6 ul	
	IS7	25 ul	10 uM	0.2 uM	0.5 ul	
	IS8	25 ul	10 uM	0.2 uM	0.5 ul	

Assay A master mix

61 Make up the following master mix in a 1.5 ml Lo-bind tube. Vortex and pulse centrifuge.

	Reagent	V2	C1	C2	V1	x __16__ rxn
	Ultrapure water	25 ul	na	na	15.9 ul	254.4 ul
	BSA	25 ul	10 mg /ml	0.4 mg/ml	1 ul	16 ul
	ABI Gold PCR Buffer	25 ul	10 X	1 X	2.5 ul	40 ul
	MgCl 2	25 ul	25 mM	2.5 mM	2.5 ul	40 ul
	dNTPs	25 ul	25 mM	0.25 mM	0.25 ul	4 ul
	ABI Taq Gold DNA polymerase	25 ul	5 U/ul	0.05 U/ul	0.25 ul	4 ul

	SYBR Green	25 ul	5 X	0.12 X	0.6 ul	9.6 ul
	CL107	25 ul	10 uM	0.2 uM	0.5 ul	8 ul
	CL108	25 ul	10 uM	0.2 uM	0.5 ul	8 ul

Assay B master mix

62 Add 24 ul of master mix to the corresponding PCR tubes. Pulse centrifuge the tubes.

63 Add 1 ul of DNA sample to the corresponding PCR tubes according to the scheme below. Pulse centrifuge the tubes.

	PCR NTC	CL105_106_STD 10^3	ssLib001 Neat	ssLib005 Neat	
	PCR NTC	CL105_106_STD 10^3	ssLib001 1in20	ssLib005 1in20	
	CL105_106_STD 10^6	CL105_106_STD 10^2	ssLib002 Neat	...etc.	
	CL105_106_STD 10^6	CL105_106_STD 10^2	ssLib002 1in20		
	CL105_106_STD 10^5	ssCL104 +VE Neat	ssLib003 Neat		
	CL105_106_STD 10^5	ssCL104 +VE 1in20	ssLib003 1in20		
	CL105_106_STD 10^4	ssNTC -VE Neat	ssLib004 Neat		
	CL105_106_STD 10^4	ssNTC -VE 1in20	ssLib004 1in20		

Assay A Plate set-up

	0.1 uM CL104 1/500	CL105_106_STD 10^4
	0.1 uM CL104 1/500	CL105_106_STD 10^4
	PCR NTC	CL105_106_STD 10^3

	PCR NTC	CL105_106_STD 10^3
	CL105_106_STD 10^6	CL105_106_STD 10^2
	CL105_106_STD 10^6	CL105_106_STD 10^2
	CL105_106_STD 10^5	
	CL105_106_STD 10^5	

Assay B Plate set-up

64 Take the strip tubes to a post-PCR space. Place in thermal cycler and run the following program:

95 deg C for 10 min

Followed by 50 cycles of:

95 deg C for 30 sec
60 deg C for 30 sec
72 deg C for 30 sec

65 Electrophorese 10 ul of the PCR product from the libraries (not standards) and controls on a 2% agarose gel.

Protocol

NAME

2% Agarose Gel Electrophoresis

CREATED BY

Alicia Grealy

PREVIEW

65.1 Make up 2 L of 1X TAE buffer:

50 ml of 40X TAE buffer
1950 ml of MilliQ water

Mix by inversion.

65.2 Using an electronic balance, weigh out 2.2 g of agarose powder on to a weigh boat using a spatula.

Note

Note that gel concentration can be adjusted. The more concentrated the gel, the greater the resolution of small fragment sizes.

65.3 Transfer the powder to a 200 ml conical flask.

65.4 Using the graduated measurng cylinder, measure out 110 ml of 1xTAE buffer. Add to the conical flask containing the agarose powder. Swirl the flask gently to mix.

65.5 Microwave the flask uncovered for 1 minute.

Safety information

A lid can loosly be placed over the flask but DO NOT tighten--allow steam to escape. Do not microwave for more than 1 minute at a time.

Do not fill flasks or beakers/Schott bottles more than half full with liquid!

65.6 Remove the flask from the microwave using oven mitts and swirl gently.

Safety information

The liquid is boiling! Use oven mitts to handle flask. Do not put your face over the opening to the flask as liquid can splash out!

65.7 Microwave the flask for a further minute but remove from the microwave if the agarose appears to boil excessively. Swirl to mix and examine near a light source to ensure the agarose has melted. Allow to cool for 5 minutes.

65.8 Place the gel casting tray into a rubber vice that will seal the ends tightly, or tape the ends with masking tape.

65.9 Place the assembly on a flat bench and use the spirit level to check it is level--adjust if needed.

65.10 Place a 20-well comb into the casting tray.

65.11 When the flask is cool to the touch, add 5 ul of SYBR Safe and swirl gently to mix. Avoid generating bubbles.

Safety information

Wait until the liquid is warm (not boiling) to cast the gel, or the tray may crack!

65.12 Pour the liquid gel slowly into the casting tray. Pop any bubbles that have formed using a clean pipette tip.

65.13 Let the gel set for 20-30 minutes at room temperature.

65.14 Allow residual gel to set in the flask, then scrape into the bin. Fill the flask half full with water and microwave until the water boils. Pour the water down the sink and clean the flask using a bottle brush.

65.15 When the gel is set, remove the combs gently.

65.16 Place the casting try and gel in the electrophoresis tank.

65.17 Fill the electrophoresis tank with 1 X TAE buffer to the fill line indicated on the tank.

65.18 Pipette 3 ul of 50 bp DNA ladder into the first well of the gel.

Note

The recommended volume will depend on the concentration of the ladder. Check the manufacterer's recommendations. If the ladder is not pre-mixed with loading dye, be sure to add 1-2 ul of loading dye before loading into the gel.

65.19 Place some Parafilm across a 96-well PCR plate rack and press down firmly to create small wells.

65.20 For each sample, pipette 1-2 ul of loading dye onto the Parafilm, taking care not to pierce the Parafilm.

65.21 Mix 10 ul of PCR product with the loading dye by pipetting gently up and down.

65.22 Transfer the 12 ul of PCR product/loading dye to the wells of the gel, taking care not to pierce the bottom of the well with the pipette tip.

Note

The volume each well can take will depend on the size of the comb used. Be sure not to overload the wells or product will float out the top of the well.

65.23 Place the lid on the gel tank and plug the electrodes into the appropriate power slots. Ensure the positive electrode is at the base of the gel.

Safety information

Take care when working with electricity and water!

Check electrical cords of all equipment and ensure none are damaged and that cords are not a tripping hazard. Do not use if the electrical cord is damaged in any way. Tag the instrument with warnings, make the area safe, and notify your line manager and anyone else in the immediate area that may be affected.

Use electrical equipment indoors only in an area free of explosive material, corrosive gas, powerful vibrations, direct exposure to sunlight, and temperature fluctuations. Use in a space where cables will not come into contact with liquids, be manually damaged, or interfere with other workplace operations.

Do not use electrical equipment with any other power adapter or cord than the one supplied.

65.24 Switch the power pack on a set the voltage to 80 V and the time to 1 hr and 10 min.

Note

Note that the voltage and time can be adjusted to suit what you are running on the gel. For amplicons (one small product), I will run the gel at 96 V for 30-40 min. For shotgun libraries, I will run the gel as above. The lower the voltage and longer it is run, the greater the separation of fragments will be.

65.25 Press 'Run' or 'Start' on the power pack and check to see that bubbles are rising from electrodes.

65.26 When the run is over, switch of the power pack, remove the lid, and remove the gel from the tank, taking care not to let it slide off the tray.

Safety information

Do not remove the lid to the electrophoresis tank until the power pack is switched off.

65.27 Place the gel on the UV transilluminator and photograph using the attached camera. Follow the manufactuerer's instructions to use the equipment.

Safety information

Take care working with UV. You should have UV safety training. Do not open the transilluminator while the UV is on! Use signage to warn others when the UV is on.

65.28 Discard the gel into a designated biohazard bin, and clean the UV dock with 70% ethanol.

65.29 Dispose of used tips into a designated sharps container.

65.30 Dispose of gel waste into a biohazard bag.

65.31 Used combs, beakers, flasks, and tray should be washed with warm water and placed on a rack to dry.

65.32 Gloves and chemical waste should be sealed in a biohazard bag for incineration.

66 Use the CT values from the qPCR to generate a standard curve for the standards in order to calculate how many template copies are present in each library. The positive control is used to calculate the efficiency of the library prep:

(# Copies of CL104 from Assay A / # copies CL104 from Assay B) * 100

Expected result

Library preparation efficiency should typically be between 30-70% according to Gansauge and Meyer (2013).

Molecule counts from the library preparation blank control should be less than 1x10^9, usually 1x10^8.

The relationship between input volume of DNA extract and out of library molecules should be linear. If it is not, either too much DNA was used for library preparation or the DNA extract is inhibited. Gansauge et al. (2017) recommend to create a few preps with various input amounts to determine this; however, most of the time, this is not feasible because it is expensive to prepare multiple libraries for one sample.

The Neat and 1in20 dilution of libraries should show be approximately 4.33 cycles apart. If they are not, there might be too much input DNA in the qPCR. Dilute futher and run the qPCR to get a more accurate estimate of library molecules.

Insert sizes typically range from 20-120 bp.

Indexing PCR will typically require 10-15 cycles of amplification.

This assay is also used to determine the number of cycles to give the indexing PCR, which needs to be stopped during the linear phase. See my library amplification protocol to proceed with the next step.

Index/amplify the library

2h 30m

67 Make up the following master mix in a 1.5 ml Lo-bind tube. Ensure to prepare enough master mix for 4 reactions per library plus pipetting error. Vortex and pulse centrifuge.

Note

Remember that each library will have it's own unique combination of forward and reverse indexing primers. **Do not add these to the master mix**, but add each to each reaction individually! Take great care not to cross-contaminate primers: only have one tube open at a time. Use qPCR tubes with individual capped lids (not strip lids!).

Note

Ideally, indexing combinations should never be reused in the lab. Be sure to follow Illumina's recommendations when chosing primer combinations (e.g., ensure adequate diversity in the bases, ensure each is at least 3 bp different from each other, don't use indexes that will begin with two dark cycles, etc.). For instance, the NextSeq cannot read "GG" a the start of an index (so indexes should not end in "CC" as they are sequenced in the reverse complement).

	Reagent	V2	C1	C2	V1	x ____ rxn
	Ultrapure water	25 ul	na	na	10.9	
	BSA	25 ul	10 mg/ml	0.4 mg/ml	1 ul	
	ABI Gold PCR Buffer	25 ul	10 X	1 X	2.5 ul	
	MgCl2	25 ul	25 mM	2.5 mM	2.5 ul	
	dNTPs	25 ul	25 mM	0.25 mM	0.25 ul	
	ABI Taq Gold DNA polymerase	25 ul	5 U/ul	0.05 U/ul	0.25 ul	
	SYBR Green	25 ul	5 X	0.12 X	0.6 ul	
	P5_indexing_primer	25 ul	10 uM	0.2 uM	0.5 ul	Don't add to master mix

	P7_indexing_primer	25 ul	10 uM	0.2 uM	0.5 ul	Don't add to master mix

68 Add 19 ul of master mix to the corresponding PCR tubes. Pulse centrifuge the tubes.

69 Add 0.5 ul of the corresponding forward indexing primer to the appropriate reaction tube. Pulse centrifuge the tubes.

70 Add 0.5 ul of the corresponding reverse indexing primer to the appropriate reaction tube. Pulse centrifuge the tubes.

71 Add 5 ul of DNA sample to the corresponding reaction tubes according to the scheme below. Pulse centrifuge the tubes.

e.g.,

	ssLib 001	ssLib 003	ssLib 005			
	ssLib 001	ssLib 003	ssLib 005			
	ssLib 001	ssLib 003	ssLib 005			
	ssLib 001	ssLib 003	ssLib 005			
	ssLib 002	ssLib 004	...etc.			
	ssLib 002	ssLib 004				
	ssLib 002	ssLib 004				
	ssLib 002	ssLib 004				

Note

These indexing reactions can be performed in larger reaction volumes using more of the library or indeed the entire library (as in Gansauge and Meyer 2013), and giving the reaction fewer PCR cycles. I am not sure which way would introduce less bias to the final results, but I feel that "putting all your eggs into one basket" may not be the best idea in case the reaction fails for an unforseen reason.

72 Take the strip tubes to a post-PCR space. Place in qPCR machine and run the following program:

95 deg C for 10 min

Followed by _____ cycles of:

95 deg C for 30 sec
60 deg C for 30 sec
72 deg C for 30 sec

Note

Note that the number of cycles to give should be determined based on the Assay A qPCR: stop while amplification is in the linear phase (before plateau). This PCR can be performed on a standard thermal cycler (and with your reagents of choice) but I prefer to run it on a qPCR as Assay A and B so I can monitor the amplification in real time. Ensure that you use a high-fidelity polymerase. You can use a proof-reading polymerase if doing standard PCR but do not use one with qPCR.

Purify the libraries

73 Pulse centrifuge the PCR tubes. Combine replicate PCR reactions into a 1.5 ml Lo-bine Safelock tube. Vortex and pulse centrifuge.

74 Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 1.6X beads : reaction volume (i.e., 160 ul). Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 35 ul of Ultrapure water.

Quantitate the libraries

75 Dilute the libraries 1 in 10 in Ultrapure water (i.e., 1 ul library in 9 ul Ultrapure water).

76 Use a LabChip GXII or equivalent fragment analyser (HiSense kit) to measure the molarity of the libraries between 160-500 bp.

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

Expected result

Libraries will be insert size + 136 bp, so the smallest fragments of interest will be ca. 166 bp (30 bp insert).

Pool libraries

77 Pool libraries in equimolar concentrations such that the total amount of DNA per library does not exceed 500-1000 ng.

Note

If you are proceeding directly with hybridisation capture, **STOP HERE and move to (e.g.) the protocol below**. Try to pool libraries such that the total amount of DNA per library does not exceel 500 ng (the recommended input amount per capture).

78 Use a Vivaspin 500 (MWCO 30,000 Da) centrifugal column to concentrate each library to 20-40 ul. Centrifuge at 15,000 rcf with the membrane facing outwards for 30 sec at a time.

https://www.sartorius.com/shop/ww/en/usd/applications-laboratory-filtration-ultrafiltration/vivaspin-500%2C-30%2C000-mwco-pes%2C-25pc/p/VS0121

Alternatively, concentrate the libraries using a SpeedyVac system, following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LED/manuals/DNA130%20User%20Manual%20final%20versionpdf.pdf

Size select and purify

79 Run each pool in duplicate across two lanes (20 ul each) of a PippinHT electrophoresis system (2% gel, Marker 20B), selecting fragments between 160-500 bp and following the manufacturer's instructions:

http://www.sagescience.com/wp-content/uploads/2015/10/PippinHT-Operations-Manual-Rev-B_460005.pdf

80 Combine replicates. Purify the libraries using SeraMag Speed Beads or SeraMag Select using a 2X beads : reaction volume (i.e., 160 ul). Follow the guidelines below:

https://www.gelifesciences.co.jp/catalog/pdf/SeraMagSelect_UserGuide.pdf

Elute in 25 ul of Ultrapure water.

Quantitate the final library

81 Dilute the libraries 1/2, 1/5, 1/10 in Ultrapure water (i.e., create a serial dilution in 10 ul volume).

82 Measure the concentration of the neat library and these dilutions in duplicate on the Qubit following the manufacturer's instructions.

https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0017209_Qubit_4_Fluorometer_UG.pdf

83 Measure the molarity of the neat library and dilutions on a LabChip GXII Hisense kit (or equivalent fragment analyser) following the manufacturer's instructions:

https://www.perkinelmer.com/Content/LST_Software_Downloads/LabChip_GX_User_Manual.pdf

84 Based on the average fragment length and Qubit measurement, calculate the molarity of the library dilutions. Create a standard curve to check that the concentrations are linear. If they can be "trusted", extrapolate the neat concentration based on the dilutions. Average all the measurements of the neat concentration to get the best estimate of the library molarity.

Sequencing

85 Dilute the library to between 2-4 nM in Ultrapure water.

Note

Note you will need CL72_custom_sequencing_primer to sequence. This can be spiked into well 12 (but select 'no custom primer' in the run set up) or into well 18 (select 'custom primer' in the run set up). Spiking the custom primers into the run is preferable so that the remaining Illumina primers are present and can sequence PhiX.

You do not need custom i5_indexing_primer to sequence off a MiSeq or NovaSeq because these instruments prime off P5. You do not need a custom i7 indexing primer because it uses primers already included in the kit. Note that for the NextSeq you **will** need custom i5_indexing_primer in addition to the CL72_custom_sequencing_primer.

86 Follow the manufacturer's instructions to perform the sequencnig run on your platform of choice.