Order list: Distributor information for reagents used in ISSR PCR and Library preparation

Protocol	Reagent	Supplier	Catalogue	Storage
ISSR PCR	2x Apex PCR Master Mix	Genesee Scientific	42-134	-25°C to -15°C
	5M Betaine	Fisher Scientific	AAJ77507AB	-25°C to -15°C
	UBC Primer Set #9	Integrated DNA Technologies	--	-25°C to -15°C
SparQ Library Preparation	SparQ DNA Frag & Library Prep Kit	QuantaBio	95194-024 95194-096	-25°C to -15°C
	1M Tris-HCl, pH 8.0	Invitrogen	15568025	Room temperature
	Y-yoke adapter	Glenn et al. 2016	--	-25°C
	Illumina iTru primers	Glenn et al. 2016	--	-25°C
Optional library reamplification (Kapa)	KAPA HiFi HotStart Library Amplification Kit	Roche	07958951001	-25°C
Bead reaction clean-ups	MyMag™ 96 Magnetic Plate	MAGBIO	MYMAG-96	--
	Axygen® AxyPrep FragmentSelect-I Kit	Corning	MAG-FRAG-I-50	20°C (Never freeze!)
Quality control	GeneRuler 1 kb Plus DNA Ladder	ThermoFisher Scientific	SM1331	-25°C to -15°C
	Qubit™ dsDNA BR Assay Kit	Invitrogen	Q32850	Room temperature

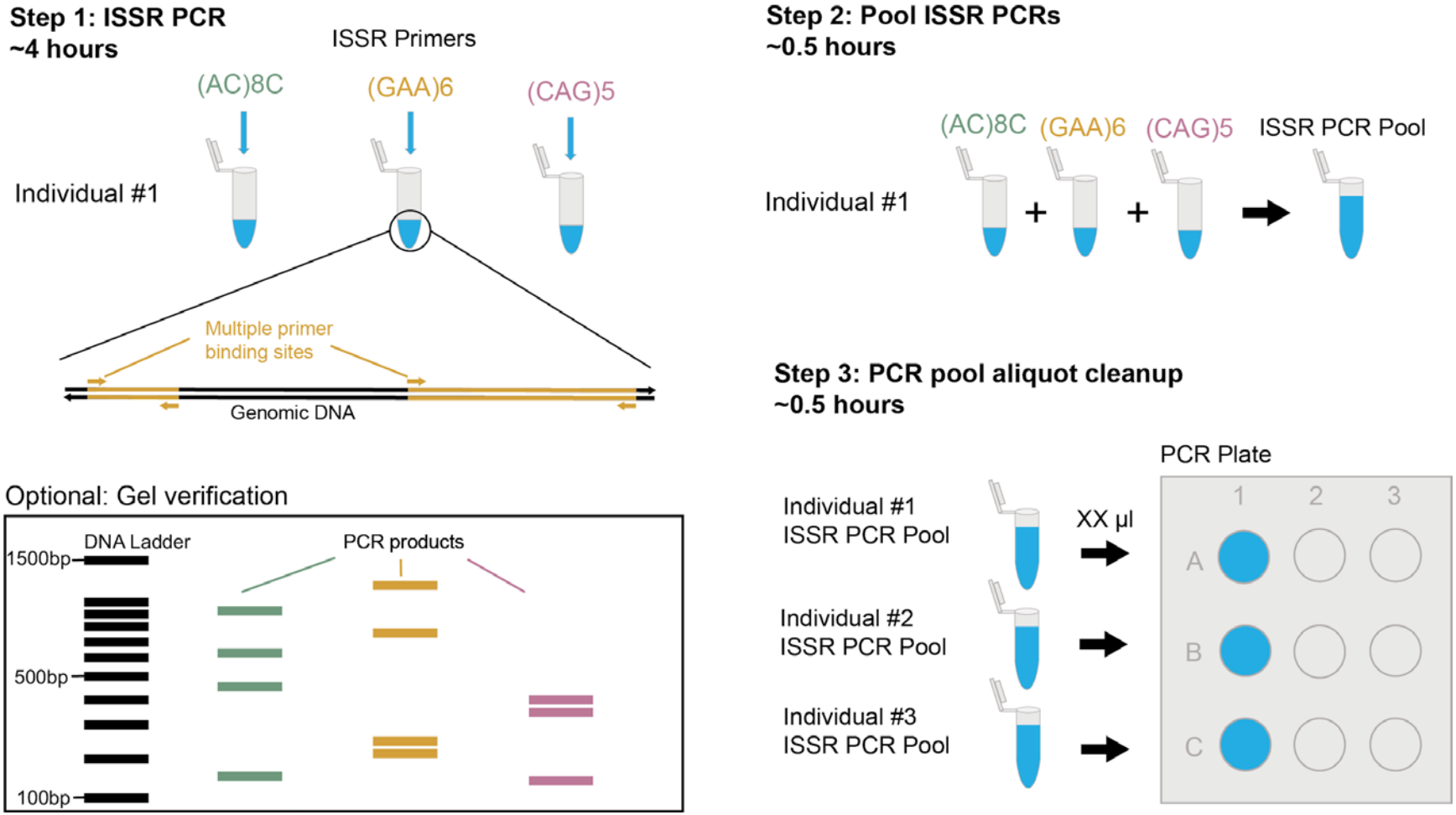
Glenn, T. C., Nilsen, R. A., Kieran, T. J., Sanders, J. G., Bayona-Vásquez, N. J., Finger, J. W., Pierson, T. W., Bentley, K. E., Hoffberg, S. L., Louha, S. and Garcia-De Leon, F. J., 2019. Adapterama I: universal stubs and primers for 384 unique dual-indexed or 147,456 combinatorially-indexed Illumina libraries (iTru & iNext). PeerJ, 7, p.e7755.

UBC Primer Set #9: University of British Columbia, Canada; paper available on GitHub (www.github.com/btsinn/ISSRseq)

ISSR primer ID	Primer motif
813	(CT)8T
814	(CT)8A
815	(CT)8G
817	(CA)8A
820	(GT)8T
824	(TC)8G
826	(AC)8C
834	(AG)8YT
836	(AG)8YA
840	(GA)8YT
843	(CT)8RA
845	(CT)8RG
848	(CA)8RG
855	(AC)8YT
856	(AC)8YA
857	(AC)8YG
858	(AC)8RT
859	(TG)8RC
860	(TG)8RA
868	(GAA)6
873	(GACA)4
caa5	(CAA)5
cag5	(CAG)5
gtt5	(GTT)5
gat5	(GAT)5
cat5	(CAT)5

Y-yoke adapter sequences ordered via Integrated DNA Technologies (IDT)

iTru_R2_stub_RCp	iTru_R1_stub
/5Phos/GATCGGAAGAGCACACGTCTGAAC TCCAGTCAC	ACACTCTTTCCCTACACGACGCTCT TCCGATCT



Critical steps of ISSRseq polymerase chain reaction (PCR), including the approximate time projected for preparation of 48 samples with 4 primer sets. PCR pool aliquot in Step 3 dependent on number of primer sets chosen to create library.

Step 1: ISSR PCR

Video protocol (begins at 14m): https://www.youtube.com/watch?v=MTclCwkQ7SA

Note before beginning: we recommend testing all 26 ISSR primers, plus any others you may choose to include, on a panel of selected samples to determine which should be used for amplification. We recommend 2-4 samples from different populations for the panel. After completion, all products should be run on a 1% agarose gel to visualize banding. Successful ISSR amplification will have multiple bands with sizes varying from a few thousand to as low as 200 base pairs. Once gel(s) have been run, determine which primers worked across all 2-4 samples used for testing, as these should be used for the rest of the experiment. Our experiment used 8 primers for *C. striata* and 26 for *C. bentleyi*, however, varying numbers can be used as it will only affect the number of PCR reactions.

Using the ISSR primers that were chosen, a PCR will need to be run for each sample with each primer (1 sample x 8 primers = 8 PCRs). After PCR, products can be run on an agarose gel to show amplification. The ISSR bands on the gel can optionally be scored and used as data (dominant markers), however, that is not necessary for this protocol.

ISSR PCR Master Mix

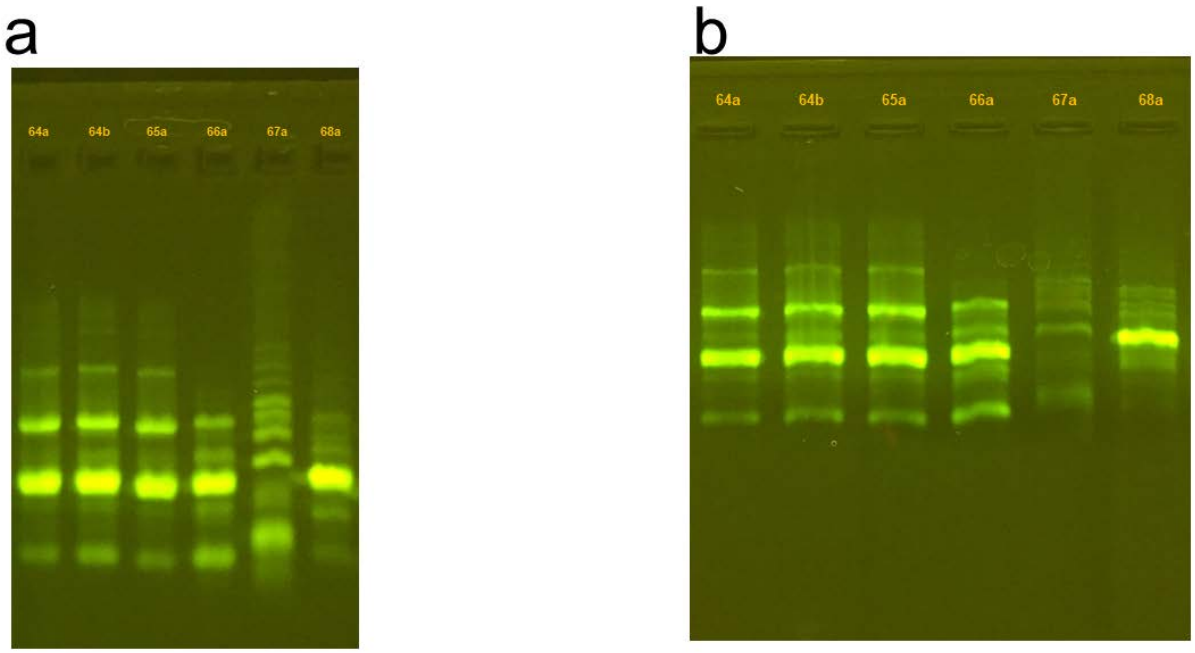
Reagent	µl per reaction
2x Apex PCR Master Mix	5.0 µl
5 M Betaine	0.5 µl
Nuclease-free ultrapure water	2.5 µl
10 µM ISSR primer	1.0 µl
Template DNA diluted to 20 ng/µl	1.0 µl
Total	10 µl

1) Prepare all reactions on ice. Create master mix from 2x Apex PCR Master Mix, 5 M Betaine, and Nuclease-free, ultrapure water by multiplying volumes based on total number of reactions + 2 in a single tube and mix.
2) Aliquot 8.5 master mix to each PCR well.
3) Separately add 0.5 of ISSR primer and 1.0 of Template DNA to each PCR well – pipette mix.
4) Proceed to thermocycler amplification (below).
5) Optional: after completion, all products should be run on a 1% agarose gel to visualize banding.

ISSR amplification thermocycler program

Step	Temperature	Time	Cycles
1	95°C	5 minutes	1
2	94°C	30 seconds	
3	50°C	45 seconds	30
4	72°C	2 minutes	
5	72°C	10 minutes	1
6	4°C	Hold	

Optional: Gel verification of single-primer amplicons



Example of gel verification of two ISSR primer PCR reactions (a, b) run on 6 individuals of *C. striata*.

Step 2: Pool ISSR PCR products for each accession

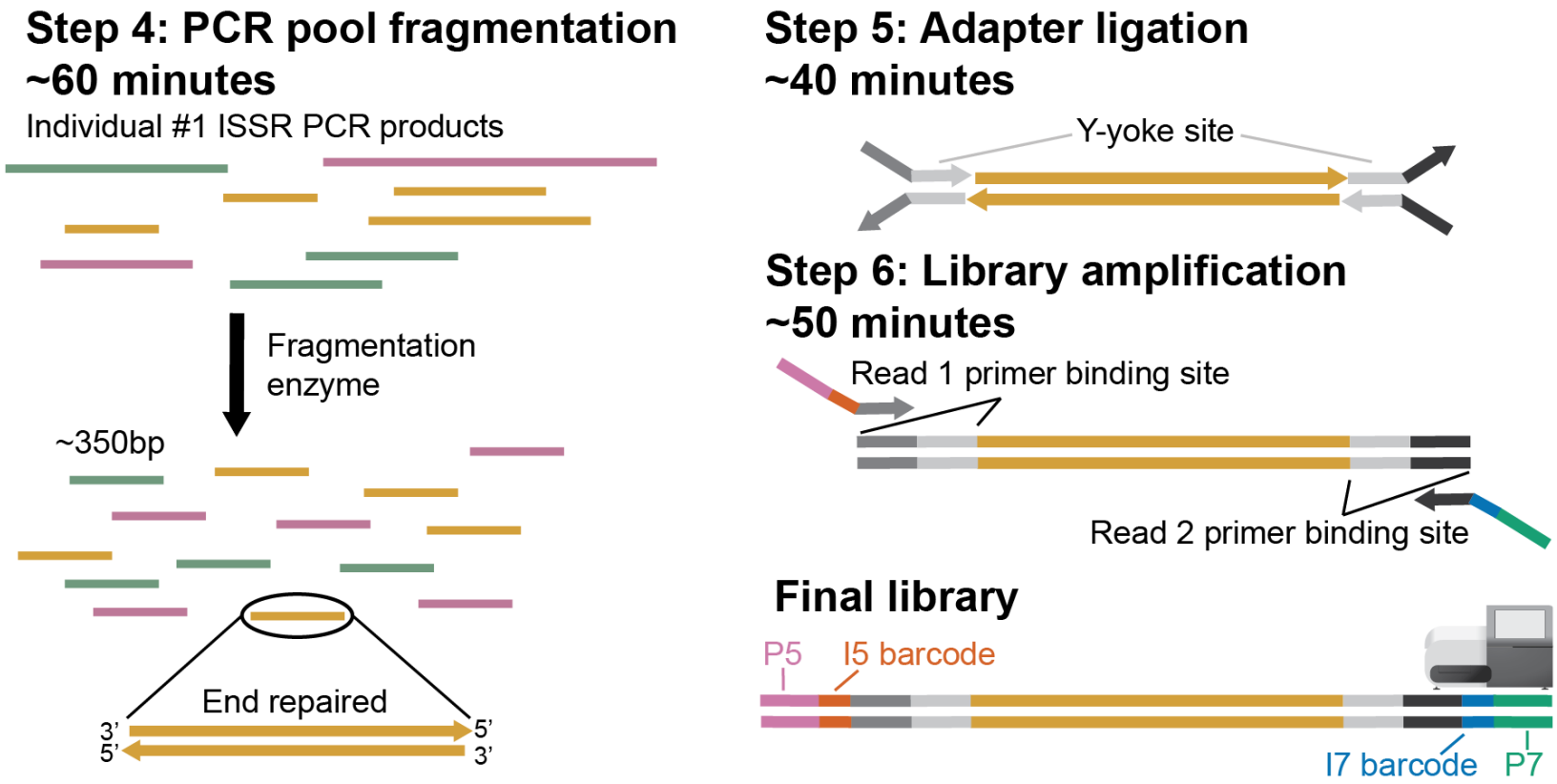
The technique used to pool ISSR PCRs is dependent on the number of primers utilized, in order to ensure sufficient volume for library construction. Two examples have been included below.

8 primer set example: 3.0 µl of each PCR reaction for each ISSR primer for each biological sample are pooled in a PCR plate (total pool 24 µl) for cleanup. The remaining PCR reactions should be stored in a -80°C freezer as backup. The 24 µl pool should then undergo a 1:1 bead cleanup to remove impurities (see Step 3 below).

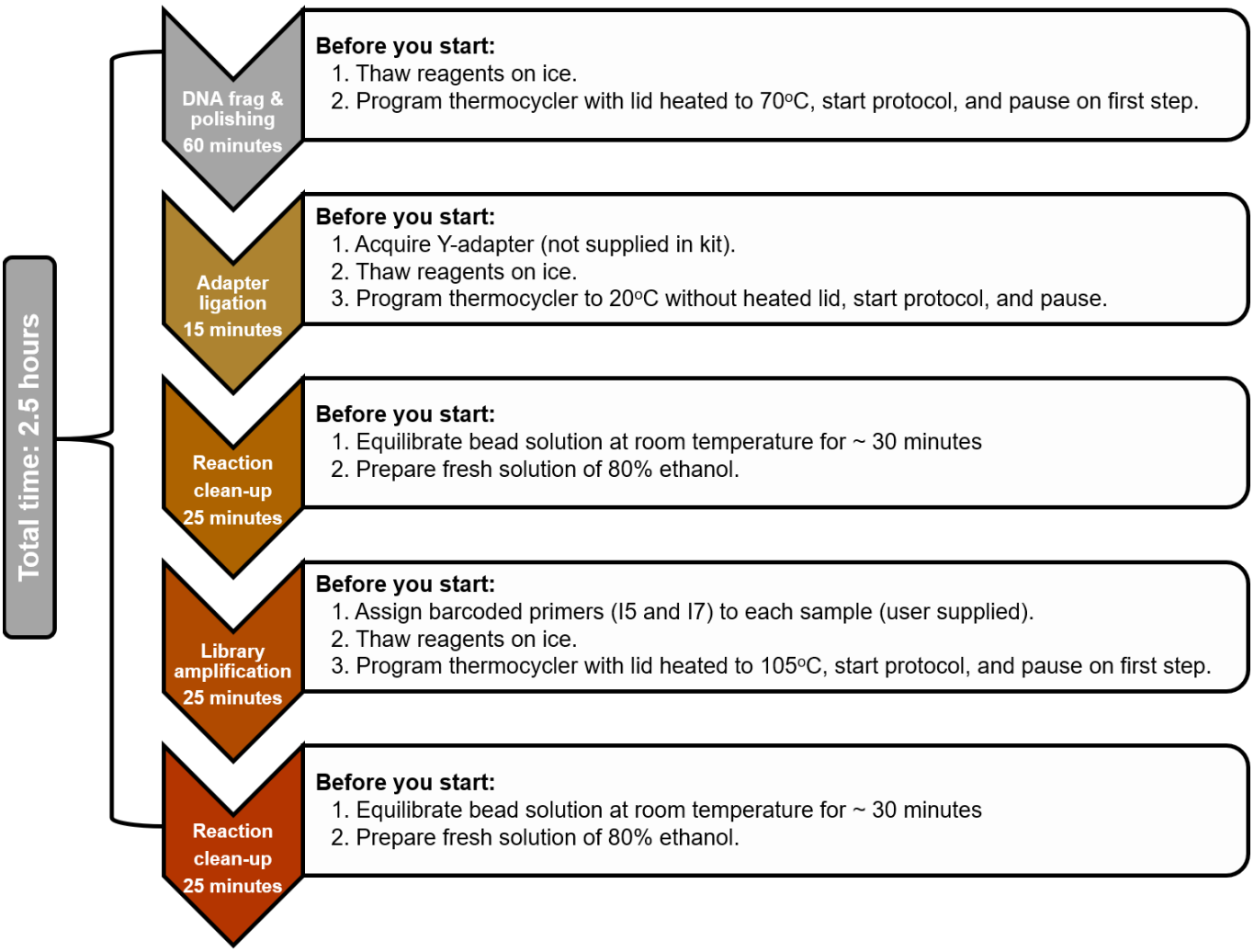
26 primer set example: The total PCR volume for each ISSR primer reaction for each biological sample are pooled and mixed (total pool volume 260 µl). A 25 µl aliquot of the PCR pool should be transferred to a new PCR plate for reaction cleanup and the remaining PCR pool should be stored in a -80°C freezer as backup. The 25 µl aliquot should then undergo a 1:1 bead cleanup to remove impurities (see Step 3 below).

Step 3: PCR pool aliquot cleanup protocol

1) Equilibrate AxyPrep MAG Fragment Select-I Kit beads to room temperature -- ~ 20 minutes.
2) Vortex beads and add 1:1 volume to each PCR pool aliquot – pipette to mix.
3) Incubate at room temperature for 5 minutes.
4) Pellet beads using the magnetic plate, and discard supernatant.
5) With the samples still on the magnetic plate, wash beads with 200 µl of *fresh* 80% ethanol.
6) Wait ~ 30 seconds and then remove and discard the ethanol – repeat steps 5 – 6, once.
7) Allow beads to air-dry on magnetic plate -- ~ 5-10 minutes.
8) Remove samples from magnetic plate and resuspend beads in desired volume + 2 µl of 10 mM Tris-HCL, pH 8.0.
9) Place on magnetic plate and transfer desired volume – 2 µl of supernatant to a thin-walled PCR tube.
10) Evaluate concentration of cleaned PCR pool aliquot using Qubit™ dsDNA BR Assay Kit.
11) Dilute cleaned PCR pool aliquot to 5 ng/µl to prepare for library preparation.
12) Samples can now be stored at -20°C.



Critical steps of ISSRseq library preparation and final library design for Illumina MiSeq/HiSeq sequencing. For further reference see Glenn et al. 2019.



Approximate time projected for each estimated for preparation of 48 samples.

Modified sparQ Fragmentase Protocol

Notes: All reactions in protocol have been calculated to be ½ the recommended volume of original sparQ kit protocol. NEVER vortex tubes of enzyme! Always mix by flicking with finger.

Prior to starting the protocol:

1) Prepare a *fresh* 80% ethanol solution of at least 200 ul per sample.
2) Prepare a solution of 10 mM Tris-HCL @ pH 8.0.
3) Remove DNase and RNase from work area (use 70% ethanol and RNAzap).
4) Thaw reagents on ice – finger flick DNA Frag & Polishing Buffer, DNA Ligase, and HiFi PCR Master Mix – others can be vortexed.
5) Program thermocyclers with the programs listed below.
6) Make a spreadsheet in excel with all samples, fragmentase time, and iTru barcodes.

Step 4: PCR Pool fragmentation

Start the DNA fragmentation and polishing thermocycler program prior to starting the protocol.

Note: The optimal time for fragmentase was **3.5 minutes** for our samples to have product between the target range of 300-500bps. However, that time may vary depending on average size of PCR amplicons. A test can optionally be run using a couple of samples and different fragmentation times to determine if 3.5 minutes results in the desired library size and, if not, fragmentase time should be adjusted accordingly. **The target fragment size should be 300-500 bp**.

DNA fragmentation and polishing thermocycler program – lid at 70°C

Step	Temperature	Time
1	4°C	Initially paused, then 1 minute
2	32°C	Varies (**3.5 minutes for ISSR**)
3	65°C	30 minutes
4	4°C	Hold

DNA fragmentation and polishing master mix

Reagent	µl per reaction
DNA Frag & Polishing Buffer (10X)	2.5 µl
Nuclease-free H20	5.0 µl
Total	7.5 µl

1) Set thermocycler block to hold at 4°C.
2) Set up master mixes on ice, *sans* the DNA Frag and Polishing Enzyme.
3) Label PCR tubes and add 5.0 µl of the DNA Frag and Polishing Enzyme to each.
4) Add 7.5 ul of the master mix and 12.5 µl DNA (cleaned PCR pool aliquot 5 ng/µl) to each tube – pipette mix (10-12x). Keep plate on ice.
5) Place each tube into the thermocycler.
6) Resume the thermocycler program once all samples are loaded.
7) Transfer tubes to ice at run completion.
8) Proceed immediately to adapter ligation.

Step 5: Adapter ligation

Thermocycler incubation program – lid NOT heated

Step	Temperature	Time
1	20°C	15 minutes

Adapter ligation reaction master mix

Reagent	µl per reaction
DNA Rapid Ligation Buffer (5X)	10 µl
DNA ligase	5.0 µl
Nuclease-free H2O	7.5 µl
Total	22.5 µl

1) Transfer 2.5 ul of 15 µM Y-yoke adapter (not supplied in kit) into each tube – pipette mix, and store on ice.
2) Prepare ligation reaction master mix – pipette mix and store on ice.
3) Add 22.5 µl of ligation reaction mix to the fragmented DNA and adapter tube – pipette mix.
4) Incubate at 20°C for 15 minutes – use a thermocycler *with heated lid disabled*.
5) Proceed immediately to adapter ligation cleanup.

Adapter ligation bead cleanup

1) Equilibrate AxyPrep MAG Fragment Select-I Kit beads to room temperature -- ~ 20 minutes.
2) Vortex beads and add 40 µl (0.8X) to each ligation sample – pipette to mix.
3) Incubate at room temperature for 5 minutes.
4) Pellet beads using the magnetic plate, and discard supernatant.
5) With the samples still on the magnetic plate, wash beads with 200 µl of *fresh* 80% ethanol.
6) Wait ~ 30 seconds and then remove and discard the ethanol – repeat steps 5 – 6, once.
7) Allow beads to air-dry on magnetic plate -- ~ 5-10 minutes.
8) Remove samples from magnetic plate and resuspend beads in 11.5 µl of 10 mM Tris-HCL, pH 8.0.
9) Place on magnetic plate and transfer 9.5 µl of supernatant to a thin-walled PCR tube.
10) Samples can now be stored at -20°C.

Step 6: Library amplification

Library amplification thermocycler program – set heated lid to 105°C.

Step	Temperature	Time	Cycles
1	98°C	2 minutes	1
2	98°C	20 seconds	8 (up to 10)
3	60°C	30 seconds	
4	72°C	30 seconds	
5	72°C	10 minutes	1
6	4°C	Hold	

Library amplification master mix

Note: User-supplied **Illumina iTru primers** that are compatible with the Y-yoke adapters should be utilized in this reaction in place of the sparQ kit supplied Primer Mix.

Reagent	µl per reaction
HiFi PCR Master Mix (2X)	12.5 µl
Illumina iTru primers	1.5 µl + 1.5 µl each I5 & I7 – added separately
Total	15.5 µl

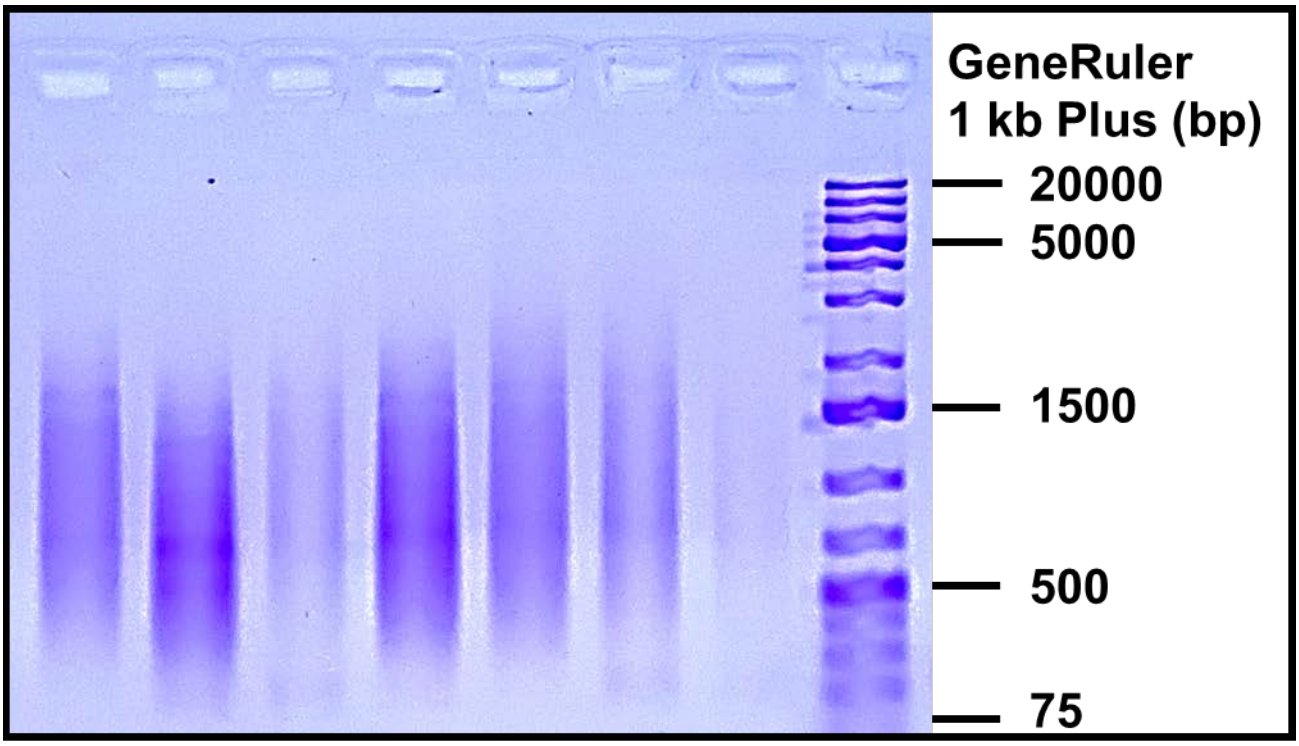
1) Add 12.5 µl of the library amplification master mix to each tube – pipette mix and store on ice.
2) Pulse spin tubes and proceed immediately to amplification.
3) After completion, proceed immediately to post-amplification cleanup.

Library amplification cleanup

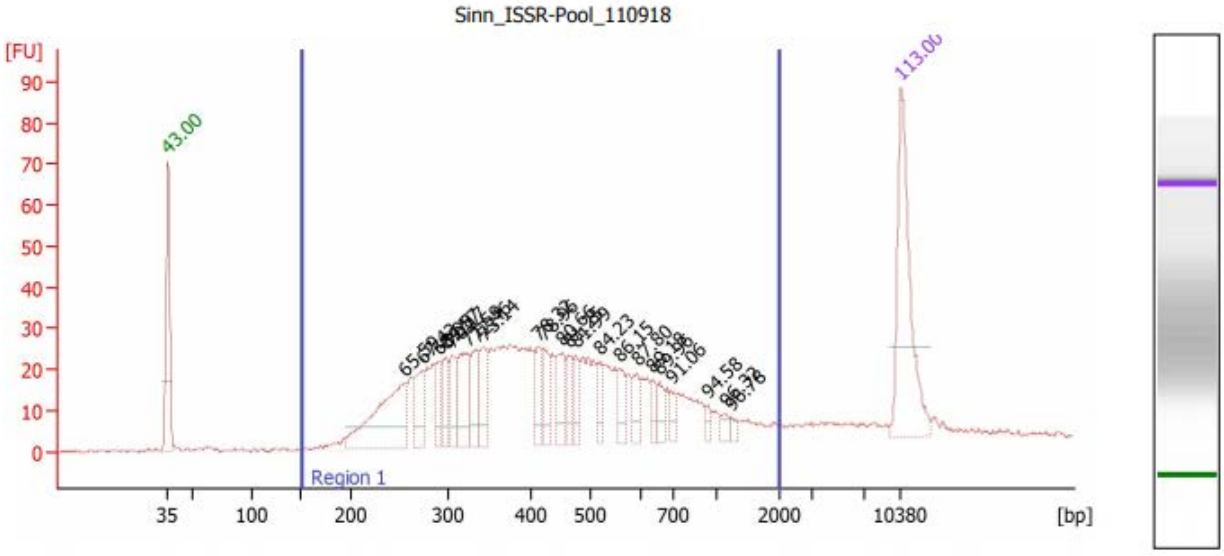
1) Equilibrate AxyPrep MAG Fragment Select-I Kit beads to room temperature -- ~ 20 minutes.
2) Vortex beads and add 25 µl (1X) to each ligation sample – pipette to mix.
3) Incubate at room temperature for 5 minutes.
4) Pellet beads using the magnetic plate, and discard supernatant.
5) With the samples still on the magnetic plate, wash beads with 200 µl of *fresh* 80% ethanol.
6) Wait ~ 30 seconds and then remove and discard the ethanol – repeat steps 5 – 6, once.
7) Allow beads to air-dry on magnetic plate -- ~ 5-10 minutes.
8) Remove samples from magnetic plate and resuspend beads in 24 µl of 10 mM Tris-HCL, pH 8.0.
9) Place on magnetic plate and transfer 22 µl of supernatant to tube for storage.
10) ISSRseq libraries can now be stored at -20°C.

Step 7a: Next Generation Sequencing Quality Control

1) After ISSRseq libraries are complete, run an agarose gel to determine success of amplification and that product is in the correct size range (300-500bp).
2) Assess ISSRseq library concentration with a Qubit™ dsDNA BR Assay Kit. (If concentration is too low (< 1ng/ul), re-attempt library amplification; see step 7b.)
3) Pool all sample ISSRseq libraries at equal molarity.
4) *Optional:* run ISSRseq library pool on an Agilent 2100 Bioanalyzer Instrument to assess final concentration and average fragment size.
5) *Optional:* if fragment size is out of desired range size selection can be completed either with AxyPrep MAG Fragment Select-I Kit beads or a Pippin Prep to select for DNA between 300 and 500bp.
6) Sequence library pool on an Illumina MiSeq or HiSeq platform.



Example of final ISSRseq library gel verification prior to library amplification cleanup and final pooling.



... Region table for sample 4 : Sinn_ISSR-Pool_110918

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
151	2,008	847.4	94	480	56.6	866.17	3,538.1	

Final ISSRseq library Agilent 2100 Bioanalyzer results depicting pool concentration and average sequence size.

Step 7b: Optional Library Re-amplification

Note: The sparQ kit used in this protocol does not contain extra HiFi PCR Master Mix (2X) reagent to repeat the library amplification step. As such, when resulting concentrations were low (< 1ng/ul), we chose to utilize a master mix from a separate PCR kit to boost library product for individual samples. Additionally, instead of using the Illumina iTru primers in this reaction, users can use the sparQ kit Primer Mix (not previously used) which contains both forward and reverse primers as we have done here. The sparQ kit Primer Mix is compatible with libraries flanked by the standard P5 and P7 adapter sequences allowing the user to boost library concentration for sequencing.

KAPA HiFi HotStart Library Amp Kit

Step	Temperature	Time	Cycles
1	98°C	45 seconds	1
2	98°C	15 seconds	10 (up to 18)
3	60°C	30 seconds	
4	72°C	30 seconds	
5	72°C	1 minute	1
6	4°C	Hold	

Library amplification master mix

Reagent	µl per reaction
KAPA HiFi HotStart ReadyMix (2X)	12.5 µl
Primer Mix (from sparQ kit)	1.00 µl
Total	13.5 µl

1) Add 12.5 ul of the library amplification master mix and 1.00 µl of Primer Mix to 11.5 µl of Adapter Ligated DNA (Library) to each tube – pipette mix and store on ice.
2) Pulse spin tubes and proceed immediately to amplification.
3) After completion, proceed immediately to post-amplification cleanup.

Library amplification cleanup

1) Equilibrate AxyPrep MAG Fragment Select-I Kit beads to room temperature -- ~ 20 minutes.
2) Vortex beads and add 25 µl to each ligation sample – pipette to mix.
3) Incubate at room temperature for 5 minutes.
4) Pellet beads using the magnetic plate, and discard supernatant.
5) With the samples still on the magnetic plate, wash beads with 200 µl of *fresh* 80% ethanol.
6) Wait ~ 30 seconds and then remove and discard the ethanol – repeat steps 5 – 6, once.
7) Allow beads to air-dry on magnetic plate -- ~ 5-10 minutes.
8) Remove samples from magnetic plate and resuspend beads in 24 µl of 10 mM Tris-HCL, pH 8.0.
9) Place on magnetic plate and transfer 22 µl of supernatant to tube for storage.
10) ISSRseq libraries can now be stored at -20°C.

11) Repeat Step 7a to assess library quality prior to sequencing.