Detailed ISSRseq Protocol

ISSR PCR Amplification

Isolated genomic DNA was diluted to 20ng/ul before proceeding. First, all 25 ISSR primers (from the University of British Columbia (Pharmawati et al. 2005)) must be tested on selected samples to determine which should be used for amplification. Pick 2-4 samples from different populations to test all primers on. The PCR reaction and conditions are below.

After amplification, all products should be run on an 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, 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 be scored and used as data, however, that is not necessary for this protocol.

Following PCR and gels, PCR product from each sample is pooled to have one pool per sample containing all products. Pooled samples should be cleaned with a 1:1 bead cleanup to remove impurities, followed by Qubit to determine DNA concentration. Pools should be diluted to 5ng/ul to prepare for library preparation.

Library Preparation of ISSR products

Library preparations can be made with preferred kits. We used the Quantabio sparQ DNA Library Prep Kit and reduced the reaction size from 50ul to 20ul. Our modified protocol is below. 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 conditions. A test should be run using a couple of samples to determine if 3.5 minutes results in the desired size and, if not, fragmentase time should be adjusted accordingly.

Sequencing preparation

After libraries are complete, an agarose gel should be run to show successful amplification and product in the correct size range (300-500bp). Libraries should be analyzed using a Qubit to determine DNA concentration. If concentration is too low (< 1ng/ul), the library must be redone or reamplified. We used the KAPA HiFi HotStart Library Amplification Kit to reamplify low concentration libraries (protocol below). Libraries should be pooled at equal molarity and run on a Bioanalyzer. Size selection should be run on a Pippin Prep to select for DNA between 300 and 500bp. Sequences were run on Illumina HiSeq and MiSeq.

ISSR PCR Amplification

PCR Reaction:

10ul reactions:

4.8ul Master Mix
3.07ul H20
.96ul primer
.38ul Betaine
.769ul DNA (diluted to 20ng/ul)

Thermocycler Conditions:

95°C – 4min
94°C – 30s
50°C – 45s **x30 cycles**
72°C – 2min
72°C – 10min
4°C - hold

Modified SparQ Fragmentase Protocol – ISSR Amplicons

Additional note: All reactions in protocol have been calculated to be ½ the recommended volume of original SparQ kit protocol

Prior to starting the protocol:

1) Perform bead cleanups on pooled PCR products, such that amplicons below XX bp are excluded.
2) Dilute samples to 5 ng/ul.
3) Prepare a ***fresh*** 80% ethanol solution. The Adapter Ligand clean-up will require 400uL/sample, and the Library Amplification clean-up requires 200uL/sample.
4) Prepare a solution of 10 mM Tris-HCL @ pH 8.0.
5) Remove DNase and RNase from work area (use 70% ethanol and RNAzap).
6) Thaw reagents on ice – finger flick DNA Frag & Polishing Mix, DNA Ligase, and HiFi PCR Master Mix – others can be vortexed.
7) Program thermocyclers with the programs listed below.
8) Make a spreadsheet in excel with all samples, fragmentase time, and iTru barcodes. You MUST know which barcodes code for which sample. Cannot use the same pair of primers for different samples, each sample must have a unique primer pair for later identification.

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

Reagent	ul per reaction
DNA Frag & Polishing Buffer (10X)	2
DNA (*pooled, cleaned PCR product @ 5ng/ul*)	8
Nuclease-free H20	5
DNA Frag Enhancer Solution	1
Total	16

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 **4 ul of the DNA Frag and Polishing Enzyme** to each.
4) Add 20 ul of the master mix and template DNA to each tube – pipette mix (5x). Keep plate on ice.
5) Place each tube into the thermocycler.
6) Resume the PCR program once all samples are loaded.
7) Transfer tubes to ice at run completion.
8) Proceed immediately to adapter ligation.

Adapter Ligation

Adapter ligation reaction mix

Reagent	ul per reaction
DNA Rapid Ligation Buffer (5X)	8
DNA ligase	4
Nuclease-free H2O	6
Total	18

Thermocycler Incubation Program – lid NOT heated

Step	Temperature	Time
1	20°C	25 minutes

1) Transfer **2 ul of 15 uM core-supplied Y-adapter** into each tube – pipette mix, and store on ice.
2) Prepare ligation reaction mix – pipette mix and store on ice.

3) Add 18 ul of ligation reaction mix to the fragmented DNA and adapter tube – pipette mix.
4) Incubate at 20°C for 25 minutes – use a thermocycler *with heated lid disabled*.
5) Proceed immediately to adapter ligation cleanup.

Adapter Ligation Cleanup

1) Equilibrate AMPure XP beads to room temperature -- ~ 20 minutes.
2) Vortex beads and add 32 ul to each ligation sample – pipette mix.
3) Incubate at room temperature for 5 minutes.
4) Pellet beads using the magnetic plate, and discard supernatant.
5) Remove from magnetic plate.
6) Wash beads with 200 ul of *fresh* 80% ethanol.
7) Place on magnetic plate, discard supernatant – repeat steps 5 – 7, once.
8) Place on magnetic plate and allow beads to air-dry -- ~ 5-10 minutes.
9) Resuspend beads in 9.2 ul of 10 mM Tris-HCL, pH 8.0. If pellets are cracking, add Tris-HCL to dry pellets until all are dry. Add Tris-HCL and let sit for 2 minutes to soften pellets.
10) Place on magnetic plate and transfer 7.6 ul of supernatant to a thin-walled PCR tube.
11) Samples can now be stored at -20°C. Can stop at this point and resume later.

Library Amplification

Library Amplification Master Mix

Reagent	ul per reaction
HiFi PCR Master Mix (2X)	10
Primer Mix	1.5 ul + (NOT for Illumina - skip) + 1.5 each i5 & I7 (sample-specific dual barcodes)
Total	13

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	
3	60°C	30 seconds	varies
4	72°C	30 seconds	
5	72°C	1 minute	1
6	4°C	Hold	

1) Add 10 ul 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 AMPure XP beads to room temperature -- ~ 20 minutes.
2) Vortex beads and add 20 ul (1X) to each ligation sample – pipette mix.
3) Incubate at room temperature for 5 minutes.

4) Pellet beads using the magnetic plate, and discard supernatant.
5) Remove from magnetic plate.
6) Wash beads with 200 ul of *fresh* 80% ethanol.
7) Place on magnetic plate and allow beads to air-dry -- ~ 5-10 minutes.
8) Resuspend beads in 19.2 ul of 10 mM Tris-HCL, pH 8.0. If pellets are cracking, add Tris-HCL to dry pellets until all are dry. Add Tris-HCL and let sit for 2 minutes to soften pellets.
9) Place on magnetic plate and transfer 17.6 ul of supernatant to tube for storage.
10) Samples can now be stored at -20°C.

KAPA HiFi HotStart Library Amplification Kit Protocol

This protocol is intended for library re-amplification if concentrations are too low, not for building libraries. Note the primer mix used in this protocol is the primer mix from the SparQ protocol that is not used for building libraries.

Reaction mix (25 ul rxns):

12.5 ul KAPA Master Mix

.75 ul primer mix (from SparQ kit)

11.75 ul Adapter Ligated DNA (Library)*

** can use 5.875 ul of DNA and 5.875 ul of molecular water if library sample is small.*

Thermocycler settings:

	98C	45 sec
10-18 cycles:	***98C***	***15 sec***
	60C	***30 sec***
	72C	***30 sec***
	72C	1 min
	4C	Hold

**10-18 cycles depending on your start concentration. For very low concentrations, increase the number of cycles.*

Qubit and gel to check for successful amplification