SCR Protocol (Full Volume)

This workflow uses the Santa Cruz Reaction (SCR) protocol by Kapp *et al*. 2021 for library building of museum specimens. The reaction volumes can be halved to reduce reagent costs, lower volumes have not been tested yet. Please cite the original publication if using this method: https://doi.org/10.1093/jhered/esab012

1. Oligonucleotides

All oligonucleotides are IDT Format (5' → 3') and ordered desalted from IDT. Order multiple batches and see oligonucleotide control recommendations detailed in original protocol by Kapp *et al* 2021.

scr_P5_adapter	/5AmMC12/ACACTCTTTCCCTACACGACGCTCTTCCGATCT
scr_P7_adapter	/5Phos/AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC/3AmMO/
scr_P5_splint	/5AmMC6/NNNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT/3AmMO/
scr_P7_splint	/5AmMC12/GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNN/3AmMO/

2. Reagents

Reagent	Provider	Cat #
T4 DNA Ligase (2,000,000 U/mL)	NEB	M0202M
T4 PNK (10,000 U/mL)	NEB	M0201L
ET SSB (500 ng/µL)	NEB	M2401S
ATP (100 mM)	Thermo Scientific	R0441
DTT (1 M)	Thermo Scientific	P2325
T4 RNA Ligase Buffer	NEB	B0216L
PEG 8000 (50%)	Sigma-Aldrich	89510
Tris-HCl pH 8.0 (1M)	Invitrogen	15568025
EDTA pH 8.0 (0.5M)	Invitrogen	15575020
Tween-20 (10%)	Teknova	T0710
NaCl (5M)	Sigma-Aldrich	S5150-1L
Glycerol (50%)	Invitrogen	15514011
MgCl2 (1M)	Invitrogen	AM9530G

3. SCR Reagent Preparation

TE Buffer (Store at RT)	10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)
EBT Buffer (Store at RT)	10 mM Tris-HCl (pH 8.0), 0.05% Tween-20
Adapter Dilution Buffer (Store at -20°C)	1X T4 RNA Ligase Buffer, 0.05% Tween-20
SSB Dilution Buffer (Store at -20°C)	20 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA (pH 8.0), 50% Glycerol
SCR Buffer (Store at -20°C)	666 mM Tris-HCl, 132 mM MgCl2

4. **Adapter-Splint Hybridization**

1. Resuspend all oligonucleotides to 100 µM using TE buffer.

2. Add the following components to two 0.2 mL tubes:

P5 tube	
Water	30.6 µL
100 µM scr_P5_**adapter**	6 µL
100 µM scr_P5_**splint**	8.4 µL
10X T4 RNA Ligase Buffer	5 µL

P7 tube	
Water	30.6 µL
100 µM scr_P7_**adapter**	6 µL
100 µM scr_P7_**splint**	8.4 µL
10X T4 RNA Ligase Buffer	5 µL

3. In a thermocycler with heated lid (105 °C**)**, hybridize the adapters and splints by incubating at 95 °C for 1 minute before ramping down to 10 °C at 0.1 °C per second.

4. Store hybridized P5 (12 µM) and P7 (12 µM) adapter stock solutions at -20 °C and freeze / thaw no more than 3 times.

5. Dilute adapter stocks with "adapter dilution buffer"
- P5 adapter/splint from 12 µM to 6 µM and 3 µM
- P7 adapter/splint from 12 µM to 6 µM, 3 µM, and 1.5 µM

Store hybridized P5 and P7 adapter/splints separately at -20°C and freeze / thaw dilutions no more than 3 times.

5. **ET SSB Dilutions**

Prepare the following ET SSB dilutions from 500 ng/µL stock solution using SSB Dilution Buffer:
- 328 ng/µL
- 164 ng/µL
- 82 ng/µL

Store ET SSB dilutions separately at -20°C.

6. **Reaction Mix Preparation**

Reagent	Stock Conc.	1 RXN (µL)
PEG 8000	50%	20
SCR Buffer	13.3X	3.75
DTT	1M	0.5
ATP	100mM	0.5
T4 PNK	10,000 U/mL	0.625
T4 DNA Ligase	2,000,000 U/mL	0.625

Notes:
- Warm PEG 8000 to 50 °C before pipetting into the Reaction Mix tube as the first component.
- Equilibrate PEG 8000 to RT before adding remaining reagents.
- Thoroughly mixing the Reaction Mix is essential, vortexing is recommended.
- Store at -20°C and freeze / thaw no more than five times.

7. **SPRI Solution Preparation**

1. Wash Beads:
   1.1 Mix SpeedBeads until all beads are in suspension.
   1.2 Transfer 1 mL of SpeedBeads to a 2.0 mL Eppendorf tube.
   1.3 Place tube on a magnetic rack and allow beads to fully concentrate (~1 min).
   1.4 While on the magnetic rack discard the supernatant.
   1.5 Add 1 mL of TE (10mM Tris, 1mM EDTA) to the tube.
   1.6 Cap tube and remove from the rack, then fully resuspend the beads by vortexing.
   1.7 Place tube on the magnetic rack and allow beads to fully concentrate (~1 min).
   1.8 While on the magnetic rack discard the supernatant.
   1.9 Repeat wash steps 1.5 – 1.8 for a second wash.
   1.10 Add 1 mL of TE to the tube, resuspend beads by vortexing.

2. Add 9 g of PEG-8000 powder to a 50 mL tube.
3. Add the following to the tube:
   - 10 mL of 5M NaCl,
   - 500 µL 1M Tris-HCl
   - 100 µL 0.5M EDTA
4. Add UltraPure water to the 50 mL tube up to ~49 mL.
5. Cap the 50 mL tube, then shake or rotate until PEG has completely dissolved.
6. Add 27.5 uL of Tween-20 to the 50 mL tube.
7. Add the 1 mL of washed SpeedBeads to the 50 mL tube.
8. Fill with UltraPure water to the 50 mL line of the 50 mL tube.
9. Gently shake until SpeedBeads are mixed.
10. Wrap 50mL tube in foil and store in the dark at 4°C.

Workflow:

Protocol choice by quantity of dsDNA

Note: This method uses a tiered adapter and SSB system based on the DNA input into the reaction. Use this table to select the correct dilution set for each DNA extract.

Protocol	dsDNA* (ng)	P5 (µM)	P7 (µM)	SSB (ng/µL)
SCR tier 1	29 - 75	12	6	328
SCR tier 2	15 - 29	6	3	164
SCR tier 3	7 - 15	3	1.5	82

(Original protocol by Kapp *et al*. 2021 has additional tiers)

Gather the following reagents before starting:

- Thaw and equilibrate the **Reaction Mix** and appropriate **ET SSB dilution** to room temperature before pipetting.
- Thaw the appropriate **P5 and P7 adapter-splint dilutions** and place them on ice.

1. **Combine the extracted DNA and SSB**
   1.1 Combine the DNA extract (if less than 20 µL, fill to 20 µL with EBT buffer) and necessary SBB dilution in a 96-well plate
   1.2 Vortex 30s, and spin down x2. [Kapp *et al*., pulse-vortexes the sample mix 5 times at maximum speed prior to briefly spinning down in a mini centrifuge].

SCR	
DNA extract	20 µL
ET SSB [328, 164, 82 ng/µL]	2 µL

2. **Denature the input DNA**

   2.1 Heat DNA/SSB's for 3 min at 95°C in a thermocycler with a 105°C heated lid.
   2.2 Immediately cold shock in a cooling block or on ice for 2 min.
   2.3 Spin down reactions for 5 seconds then place back on ice.

3. **Add Adapters**

3.1 While the reactions are on ice or in a cooling block, add the following:

SCR	
[12, 6, 3 µM] annealed P5 adapter/splint	1 µL
[6, 3, 1.5 µM] annealed P7 adapter/splint	1 µL

Notes:

- Add to strip tubes then use multichannel to add to plates
- Can combine P5 + P7 beforehand but must be quick to add to DNA
- Do not vortex the combined adapters very vigorously or will damage them
- **Do not store combined adapters**

4. **Add Reaction Mix**

4.1 While the reactions are on ice or in a cooling block, add 26 µL reaction mix
4.2 Foil seal plates and pulse vortex for 10 seconds, spin down x2
crucial to mix well after adding reaction mix

Notes:

- Add reaction mix to strip tubes then add with multichannel.
- Solution quite viscous so pipette slowly and pipette mix to get everything out of the tips
- Total reaction volume = 50 µL

5. **Incubate Reactions**

5.1 Incubate reactions for 1 hour in a pre-heated thermocycler at 37°C, with the lid set to 50°C [Kapp *et al*. 2021, incubated for 45 minutes].

6. **Clean SCR Reactions**

[Kapp *et al*., uses a a MinElute PCR Purification Kit (Qiagen Catalog No.28004) following the manufacturer's instructions and elutes in 50 µL buffer EBT].

6.1 Spin down reactions for 5s
6.2 Add the following to the reactions:

SCR 0.7x bead clean	
EBT buffer	55 µL
18% PEG SPRI	75 µL

6.3 Mix and incubate for 10 – 30 minutes at RT
6.4 Continue SPRI clean (See section 10. Bead Clean with Stamp Magnet Protocol),
6.5 Elute in 25 µL of **HEATED** (~37°C) EBT buffer.

**** Safe Stop Point - can stop here and store reactions in fridge until ready for qPCR****

7. **qPCR**

Quantitative PCR is recommended to inform the optimal cycle number for each sample during index PCR.

7.1 Prepare the following reaction for each library:

	1 reaction (per well)
LUNA SYBR qPCR Master Mix (K0221)	10 µL
H2O	8 µL
10 µM Primer IS7	0.5 µL
10 µM Primer IS8	0.5 µL
Library	1 µL
	20 µL

*NOTE: IS7/8 primers are at 100 µM, can dilute with water to make up 10 µM

7.2 qPCR cycle conditions:

- 95°C for 10 minutes
- 40 cycles of the following:
    - 95°C for 30 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
- Measure the fluorescence at the end of each extension step.

- Optimal cycle number for index PCR = CT value +1
- Safe range for batching libraries = CT value -1 to CT value +1 (but can also batch between -1 to +3 if necessary)

8. **Index PCR**

Optimal cycle number can be determined by qPCR (see step 7). Batch reactions together based on CT values.

8.1 Prepare the following reaction for each library:

Amplitaq Gold 360 Master Mix	25 µL
20 µM i5 index	2.5 µL
20 µM i7 index	2.5 µL
Library	20 µL
	50 µL

8.2 Cycle the reactions in a thermocycler using the following conditions:

- 95°C for 10 minutes
- Library specific number of the following cycle conditions:
    - 95°C for 30 seconds
    - 60°C for 30 seconds
    - 72°C for 60 seconds
- Final extension of 72°C for 7 minutes

**** Safe Stop Point ****

9. **Clean Index PCR Reaction**

Follow a 1.2x ratio bead clean ie. if 50 µL product then 60 µL beads if using Cytiva SPRI beads.

9.1 Add 60 µL of SPRI (no EBT)
9.2 Continue bead clean (See section 10. Bead Clean with Stamp Magnet Protocol)
9.3 Elute in **25 µL** of heated EBT buffer

Samples now ready for QC, pooling and sequencing

- Use Tapestation for post-indexing QC and to determine sub-pools
- Use **Ampure XP** beads only for **final bead** cleans (ie. post pooling), do not add any EBT for these cleaning steps.

10. **Bead Clean with Stamp Magnet Protocol:**

(Steps 6 and 9 of Library Prep protocol)

Two possible stamps from https://ariumlab.com/product-category/extractors/

1. 0.2ml 96 Well Extractor (aka “wide magnet”)
2. HV 0.2mL 96 Well Extractor with Stand (aka “thin magnet”)

If using wide magnet use un-skirted PCR plate (comb) and wide bottomed 96-well plates.
If using thin magnet use provided comb and PCR plates
Ensure volume of sample and beads is appropriate for plates
Ensure EBT and beads are at room temperature before starting

1. Prepare two wash plates with fresh 80% ethanol (100-200 uL) per well.
2. Prepare one elution plate with 22 - 25 µL of EBT per well.
3. If using wide magnet transfer samples (~50 uL) into wide well Kingfisher plates first.
4. Add EBT and / or 18% PEG SPRI beads to sample based on protocol stage:

	0.7x Post adapter ligation	1.2x Post indexing
Sample	50 µL	50 µL
EBT buffer	55 µL	NA
18% PEG SPRI	75 µL	60 µL

5. Vortex and incubate for 10 - 30 minutes at RT.
6. Add the tip comb to the magnet, add the magnet to the plate and let the beads concentrate until the solution is clear (~3 mins).
   NB. Keep the remaining supernatant in case the bead clean fails (labelled “safety 1”).
7. Transfer the magnet with the beads into the first ethanol wash plate
   a. Move the magnet up/down/side-to-side for about 30 seconds
   b. Move the magnet into the second wash plate and repeat the above steps.
8. Remove the magnet from the ethanol washes and let the excess ethanol dry off (~1 min).
   NB. Ensure the beads do not over-dry.
9. Place the magnet into the heated (~37°C) EBT solution, quickly release the comb from the magnet & mix the beads with the EBT using the comb.
10. Incubate beads in EBT for 15 minutes at RT.
11. Place the magnet back into the plate and let the beads concentrate (~1-2 minutes).
12. KEEP the ELUANT and use further.
13. Resuspend the beads in 20 uL EBT and store in the fridge as a backup (labelled “safety 2”).

Safety 1 and 2 can be discarded after confirmation that the bead clean worked as anticipated.