

Nov 20, 2025

Version 2

Santa Cruz Reaction (SCR): Single-Stranded Ancient DNA Library Protocol V.2

DOI

<https://dx.doi.org/10.17504/protocols.io.5jyl8d4p7g2w/v2>

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Protocol Citation: Alba Rey-Iglesia, Deon de Jager, Vanssy Li, Eline Lorenzen 2025. Santa Cruz Reaction (SCR): Single-Stranded Ancient DNA Library Protocol. protocols.io <https://dx.doi.org/10.17504/protocols.io.5jyl8d4p7g2w/v2> Version created by **Deon de Jager**



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Protocol status: Working

We use this protocol and it's working

Created: November 20, 2025

Last Modified: November 20, 2025

Protocol Integer ID: 233049

Keywords: stranded ancient dna library protocol this protocol, stranded ancient dna library protocol, ssdna library, stranded ancient dna library, ancient dna library, santa cruz reaction method, scr, protocol, dna, santa cruz reaction, end of the protocol, sequencing

Abstract

This protocol describes the construction of single-stranded ancient DNA libraries using the Santa Cruz Reaction method (Kapp et al. 2021: <https://doi.org/10.1093/jhered/esab012>).

At the end of the protocol, you will have ssDNA libraries, which will need to be indexed before sequencing.

The protocol was originally written up by Alba Rey-Iglesia and subsequently converted to a protocols.io version by Deon de Jager and Vanssy Li.



Protocol materials

☒ 50% PEG 8000 **New England Biolabs Catalog #B0216L**

☒ DTT, 1M **Thermo Fisher Catalog #P2325**

☒ 100mM ATP **Thermo Scientific Catalog #R0441**

☒ T4 Polynucleotide Kinase - 2,500 units **New England Biolabs Catalog #M0201L**

☒ T4 DNA Ligase (2,000,000 units/ml) - 100,000 units **New England Biolabs Catalog #M0202M**

☒ UltraPure™ 1M Tris-HCl, pH 8.0 **Invitrogen - Thermo Fisher Catalog #15568025**

☒ Magnesium chloride (MgCl₂) solution (1 M) **Invitrogen - Thermo Fisher Catalog #AM9530G**

☒ T4 RNA Ligase Reaction Buffer - 3.0 ml **New England Biolabs Catalog #B0216L**

☒ 10% Tween-20 Solution **Teknova Catalog #T0710**

☒ UltraPure 0.5M EDTA pH 8.0 **Invitrogen - Thermo Fisher Catalog #15575020**

☒ UltraPure™ Glycerol, 50% **Invitrogen - Thermo Fisher Catalog #15514011**

☒ 5M Sodium chloride solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5150-1L**

☒ ET SSB 500 µg/ml **New England Biolabs Catalog #M2401S**

☒ MinElute PCR Purification Kit **Qiagen Catalog #28004**

☒ Buffer EB **Qiagen Catalog #19086**

☒ Buffer PB **Qiagen Catalog #19066**

☒ Buffer PE **Qiagen Catalog #19065**



Before start

- This protocol should be done in an ancient DNA clean lab facility.
- Clean all surfaces with 5% bleach solution followed by 70% ethanol before and after use. Clean all equipment with 70% ethanol before and after use.
- UV-treat buffers PE, PB, and elution buffer for 10 min in a UV cross-linker before use.
- Make sure the Adapter-Splint Hybridization has been done (this only needs to be done once whenever adapters and splints are ordered):

Protocol

NAME

Santa Cruz Reaction (SCR): Adapter-Splint Hybridization

CREATED BY

Vanssy Li

Preview

- **When referring to "adapters" in the protocol below, we are referring to the adapter-splint hybrid oligos.**
- Remember to include **negative controls**! A library build negative control, and your DNA extraction negative control.
- Flick/vortex and spin down everything before using.
- Mix by pipetting when using a reagent, and mix by pipetting when adding it to the sample.
- **Vortex heavily** - it might seem like too much, but it is needed!
- Please follow advice and guidelines from the original protocol ([Kapp et al. 2021](#)) as these are not necessarily repeated here!

Samples & Tiers

- 1 Using Tables 1 and 2, decide which tier (1-5) is appropriate for each of your DNA extractions (samples).
 - Use any tier that is convenient for the library negative control (does not have to be tier 5).

Note

It makes life easier if you use as few tiers as possible across the samples, within reason. This can be achieved by adjusting the volume of DNA extract added (the rest of the 20 uL is made up with EB buffer) as the tiers are based on the absolute amount of DNA (in ng) used.

- 1.1 **Table 1** Samples and their designated tiers based on the amount (ng) of input DNA (also see Table 2).

A	B	C	D	E	F	G	H
Sample ID	Extraction ID	Library ID	DNA extract conc. from Qubit (ng/uL)	DNA extract volume to add (uL) (max 20 uL)	EB volume to add (uL)	DNA amount (ng)	Tier
Sample-001	EXT-001	SCR-001					
Sample-002	EXT-002	SCR-002					
Sample-003	EXT-003	SCR-003					
Sample-004	EXT-004	SCR-004					
Sample-005	EXT-005	SCR-005					
Sample-006	EXT-006	SCR-006					
Sample-007	EXT-007	SCR-007					
Sample-008	EXT-008	SCR-008					

	A	B	C	D	E	F	G	H
	Sample-009	EXT-009	SCR-009					
	Sample-010	EXT-010	SCR-010					
	Sample-011	EXT-011	SCR-011					
	Sample-012	EXT-012	SCR-012					
	Sample-013	EXT-013	SCR-013					
	Sample-014	EXT-014	SCR-014					
	Extraction Negative Control	EXT-015	SCR-015		20	0		
	Library Negative Control	NA	SCR-016	NA	NA	20		

2 Selecting the appropriate tiers based on your input DNA (ng).

Once you have determined which tiers are required, make the necessary dilutions of P5 and P7 adapters, and SSB proteins (see the next two sections below for how to do that).

2.1 **Table 2** Concentration tiers for single-stranded binding proteins (SSB) and P7 and P5 adapters.

	A	B	C	D	E
	DNA input (ng)	SSB conc. (ng/uL)	P5 conc. (uM)	P7 conc. (uM)	Tier
	30+	328	12	6	1
	15-29	164	6	3	2
	7-14	82	3	1.5	3
	4-7	41	1.5	0.75	4
	4	20.5	0.75	0.375	5



P7 & P5 Adapter Dilutions for Tiers

- 3 Once you have determined the appropriate tiers to use for your samples, make up the required dilutions in adapter dilution buffer (see next step).
 - Note: The SCR protocol implements an asymmetric P5:P7 adapter molar ratio that reduces adapter-dimer formation.

3.1 Adapter dilution buffer (store at -20°C):

Required reagents:

1.  T4 RNA Ligase Reaction Buffer - 3.0 ml **New England Biolabs Catalog #B0216L**
2.  10% Tween-20 Solution **Teknova Catalog #T0710**
3. ddH₂O
 - Final composition of buffer: 1X T4 RNA Ligase Buffer, 0.05% Tween-20.
 - Table 3 gives the recipe to make 500 µL:

Table 3 Adapter dilution buffer components. Makes 550 µL.

A	B	C
Reagent	Volume (µL)	Final conc.
10X T4 RNA Ligase Buffer	50	1X
10% Tween-20 solution	2.5	0.05%
ddH ₂ O	447.50	-
Total	500	-

3.2 Dilution of adapters into appropriate tiers:

Store at -20°C, do not freeze-thaw more than 4 times!

Required reagents:

1. Adapter dilution buffer (made in the previous step).
2. P5 adapter-splint hybrid (12 µM) - see Adapter-Splint Hybridization protocol.
3. P7 adapter-splint hybrid (12 µM) - see Adapter-Splint Hybridization protocol.

Protocol

NAME

Santa Cruz Reaction (SCR): Adapter-Splint Hybridization

CREATED BY

Vanssy Li

Preview

Table 4 P5 adapter dilutions.

	A	B	C	D	E
	P5 adapter dilutions				
	<i>Tier</i>	<i>Final adapter conc. (uM)</i>	<i>P5 adapter vol. (uL)</i>	<i>Adapter dilution buffer vol. (uL)</i>	<i>Total volume (uL)</i>
	1	12	24	0	24
	2	6	12	12	24
	3	3	6	18	24
	4	1.5	4	28	32
	5	0.75	4	60	64

Table 5 P7 adapter dilutions.

	A	B	C	D	E
	P7 adapter dilutions				
	<i>Tier</i>	<i>Final adapter conc. (uM)</i>	<i>P7 adapter vol. (uL)</i>	<i>Adapter dilution buffer vol. (uL)</i>	<i>Total volume (uL)</i>
	1	6	20	20	40
	2	3	14	42	56
	3	1.5	8	56	64
	4	0.75	4	60	64
	5	0.375	4	124	128

**Note**

Store dilutions at -20°C, **freeze/thaw maximum of 4 times** – make appropriate size aliquots! Only need 1 µL of each adapter PER SAMPLE, so, for example, highest tier concentration for P5 will make at least 20 reactions, lowest tier P7 will make at least 120!!

- Have a working subpacket containing the currently active P5 and P7 dilutions, and include a note or write on the packet how many times each dilution has been thawed & frozen.

SSB Proteins Dilutions for Tiers

- 4 As with the adapters, make appropriate dilutions of the SSB proteins based on the required tiers.

4.1 SSB dilution buffer (store at -20°C for up to 1 month):

Required reagents:

1. UltraPure™ 1M Tris-HCl, pH 8.0 Invitrogen - Thermo Fisher Catalog #15568025
2. 5M Sodium chloride solution Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5150-1L
3. DTT, 1M Thermo Fisher Catalog #P2325
4. UltraPure 0.5M EDTA pH 8.0 Invitrogen - Thermo Fisher Catalog #15575020
5. UltraPure™ Glycerol, 50% Invitrogen - Thermo Fisher Catalog #15514011
6. ddH₂O
 - Final composition of buffer: 20 mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA (pH 8.0), 25% glycerol. (Note: the original publication lists 50% glycerol as the final concentration, but this is impossible, as the glycerol stock is already at 50%, thus it is interpreted as meaning the stock (50%) should be at half its original concentration in the final reaction, thus 25%.)
 - Table 6 gives the recipe to make 500 µL:

Table 6 SSB dilution buffer components. Makes 500 µL.

A	B	C
Reagent	Volume (µL)	Final conc.
1M Tris-HCl, pH8.0	10	20 mM
5M NaCl	2	20 mM



	A	B	C
	1M DTT	0.25	0.5 mM
	0.5M EDTA, pH8.0	0.1	0.1 mM
	50% glycerol	250	25%
	ddH ₂ O	237.65	-
	Total	500	-

4.2 Dilution of SSB proteins into appropriate tiers:

Store at -20°C for up to 1 month only!

Reagents required:

1. SSB dilution buffer (made in previous step).
2.  ET SSB 500 µg/ml New England Biolabs Catalog #M2401S

Table 7 SSB protein dilutions.

	A	B	C	D	E
	SSB dilutions				
	Tier	SSB final conc. (ng/uL)	SSB stock vol. (uL)	SSB dilution buffer vol. (uL)	Total volume (uL)
	1	328	20	10.5	30.5
	2	164	10	20.5	30.5
	3	82	5	25.5	30.5
	4	41	5	56	61
	5	20.5	5	117	123

Note

Since these do not last, it's worth making smaller batches and only of the tier/s you will use.
NOTE 2 uL is needed per sample, so the lowest tier (20.5ng/uL) will make 60 reactions if made as above!!



Santa Cruz Reaction (SCR) Buffer preparation

- 5 **The SCR Buffer is a component of the SCR Master Mix (next step) and is prepared beforehand.**

Store at -20°C, do not freeze-thaw more than 4 times!

Required reagents:

1.  UltraPure™ 1M Tris-HCl, pH 8.0 Invitrogen - Thermo Fisher Catalog #15568025
2.  Magnesium chloride (MgCl₂) solution (1 M) Invitrogen - Thermo Fisher Catalog #AM9530G
3. ddH₂O

Table 7 SCR buffer components. Recipe makes enough for 40 reactions, store in appropriate size aliquots.

A	B	C
Reagent	Volume (uL)	Final conc.
1M Tris-HCl, pH8.0	100	666 mM
1M MgCl ₂	19.8	132 mM
ddH ₂ O	30.2	-
Total	150	-

SCR Master Mix preparation

- 6 **SCR Master Mix recipe - prepare on the same day that you need it.**

However, can be stored at -20°C, in which case do not freeze-thaw more than 4 times.

Note

MIXING OF MASTER MIX IS SUPER IMPORTANT – It has to be mixed for at least 5 min by flicking and vortexing continually. This happens at Step 6.4 below which provides more specific instructions.

ALSO, once adapters have been added to the DNA in the library build (below), pulse vortex the smaller tubes 5 times and spin down and repeat x3 – make sure you see the whirlpool inside the tube so you know it's mixing!! Mix as much as possible.

Required reagents:

1.  50% PEG 8000 New England Biolabs Catalog #B0216L
2. SCR buffer (prepared in previous step)

3.  DTT, 1M Thermo Fisher Catalog #P2325
4.  100mM ATP Thermo Scientific Catalog #R0441
5.  T4 Polynucleotide Kinase - 2,500 units New England Biolabs Catalog #M0201L (10,000 U/mL)
6.  T4 DNA Ligase (2,000,000 units/ml) - 100,000 units New England Biolabs Catalog #M0202M

Table 8 SCR Master Mix components for 16 samples. Volume in final column is for 16 samples + 10% extra for pipetting error. **Note:** In the next steps, 26uL of SCR Master Mix gets added to 20uL DNA + 2uL SSB dilution + 1uL P5 dilution + 1uL P7 dilution, to give a final volume of 50uL - hence the "Final conc. in 50uL" column.

	A	B	B	C	D
	Reagent	Stock conc.	Vol 1x sample (uL)	Final conc. in 50 uL	Vol x18 for 16 samples (uL)
	50% PEG 8000	50%	20	20%	360
	SCR buffer	13.3X	3.75	1X	67.5
	1M DTT	1M	0.5	10 mM	9
	100mM ATP	100mM	0.5	1 mM	9
	T4 PNK 10,000 U/mL	10,000 U/mL	0.625	0.125 U/mL	11.25
	T4 DNA Ligase 2,000,000 U/mL	2,000,000 U/mL	0.625	25 U/mL	11.25
	Final volume (uL)		26	-	

- 6.1 Heat an aliquot of 50% PEG 8000 to 45°C for 3 min in a heating block. 
- 6.2 Add the heated PEG 8000 as the first component in the master mix, then allow to cool (normal rack is fine).
- 6.3 Add all other master mix reagents.
- 6.4 **Here comes the 5 min bit:** Start a timer for 5 min. Flick the master mix tube while inverting for 30s, then pulse-vortex 3x and spin down very briefly in centrifuge (or )



micro-centrifuge, if available). Repeat these steps until the 5 min is over.

- 6.5 If you don't use the SCR master mix straight away, try to keep it cold without freezing it (i.e. an ice rack that has warmed up for a couple of minutes outside the freezer).

Library Build - On ice!!

7 SCR library build:

Take ice rack out of freezer now or after step 7.1 (depending on how fast you work). It is super important that the reaction in 7.3 does not freeze when it comes out of the thermocycler, but is still kept ice-cold. Thus, taking an ice rack out now allows it to warm up slightly so it doesn't freeze the reaction. An ice bath (if you have one) is preferential to ice rack as it is much less likely to freeze the reaction.

- 7.1 In a PCR strip tube, combine 20µL of DNA extract with 2µL of tier-specific SSB dilution; mix by pipetting. If sample is less than 20µL then top up with buffer EB/EBT.
- 7.2 Pulse vortex 5x and briefly spin down.
- 7.3 Denature DNA by heating in a thermocycler at 95°C for 3 min. Ensure thermocycler is already set to 95°C when samples are placed inside.
- 7.4 Remove from thermocycler and place immediately on ice rack/bath for 2 min (speed here is super important - we don't want the DNA to re-anneal), then spin down and transfer back to ice block to continue.
- 7.5 Add 1 µL of input specific splinted P5 adapter (hybrid) to each tube (mix by pipetting or flicking - spin down briefly after flicking).
- 7.6 Add 1 µL of input specific splinted P7 adapter (hybrid) to each tube (mix by pipetting or flicking - spin down briefly after flicking).
- 7.7 Add 26 µL of SCR mastermix to each tube. **Mix mastermix thoroughly before adding to tubes** by flicking and vortexing a couple of times (as in step 6.4).
- 7.8 Pulse vortex 5x at max speed, ensuring mixing, spin down (repeat 3 times total). Make sure you see the whirlpool inside every tube so you know it is mixing!
- 7.9 Incubate reactions (50 µL total volume) at 37°C for 45 min. Ensure thermocycler already sits at 37°C when samples are put in, hold at 4°C or 10°C at the end of incubation, but



proceed to clean up immediately after incubation.

Clean up of libraries

- 8 **Clean up with *MinElute* PCR Purification Kit columns** - immediately after library build! (not Monarch columns as with aDNA extractions).

Note

REMINDER: UV-treat buffers for 10 min in a UV cross-linker before use, if not already done.

Required reagents:

-  MinElute PCR Purification Kit **Qiagen Catalog #28004** - store at 4°C. Remove just before use.
- EBT buffer: 5 mL Buffer EB + 2.5 µL Tween-20 (
 -  Buffer EB **Qiagen Catalog #19086**
 -  10% Tween-20 Solution **Teknova Catalog #T0710**)
- Or TET buffer instead of EBT: 10 mM Tris-HCl, 1 mM EDTA, 0.05% Tween-20.
-  Buffer PB **Qiagen Catalog #19066**
-  Buffer PE **Qiagen Catalog #19065**

8.1 Add 10x volume of buffer PB: 1x volume of library (e.g. 500 µL PB and 50 µL library) to the MinElute spin column. Mix by pipetting or by inverting a few times to mix.

8.2 Centrifuge at 6,000g for 1 min, discard the flow-through that has been collected in the collection tube into a 50 mL falcon tube labelled as "Qiagen waste".

Either change the collection tube (can use a 2 mL eppie with the lid removed as a substitute) or tap the collection tube upside-down onto a 4x folded tissue paper and reuse the same collection tube. The latter reduces plastic use & waste and is thus more environmentally friendly without having an adverse effect on your libraries.

8.3 Add 700 µL of Buffer PE (wash buffer) to each column.

8.4 Centrifuge at 8,000g for 1 min, discard the flow-through as before and change collection tubes again or reuse the same collection tube as before (see step 8.2).



- 8.5 Centrifuge the spin columns at 12,000g for 2 min to dry the membrane from any residual buffers.
- 8.6 Discard the collection tube and place the column in a new **LoBind 1.5ml Eppendorf tube** (with lid attached).

Note

If LoBind tubes are not available, it is essential to use EBT or TET buffer in the next step, as this helps to prevent the binding of DNA to the plastic of the tube.

It is obviously crucial to retain as much DNA as possible when working with aDNA libraries due to the low concentration and complexity of the libraries and the fact the source of the DNA is usually finite, so every step should limit DNA loss as much as possible.

- 8.7 Apply 15 µL of EB buffer directly to membrane and incubate for 10 min at 37°C.



Note

When using LoBind tubes, standard Buffer EB can be used (without Tween-20), as the LoBind tubes are designed to reduce the binding of DNA. Although note that a recent study (Gilardet et al. 2025) showed that using TET (which includes Tween-20) for elution of aDNA extractions increases complexity of the final library - granted, that was for extractions, but it can't hurt for libraries either.

Citation

Gilardet A, Lord E, García GO, Xenikoudakis G, Douka K, Wooller MJ, Rowe T, Martin MD, Le Moullec M, Anisimov M, Heintzman PD, Dalén L (1970) . A High-Throughput Ancient DNA Extraction Method for Large-Scale Sample Screening.

<https://doi.org/10.1111/1755-0998.14077>

LINK

- 8.8 Centrifuge at 12,000g for 1 min.



8.9 Repeat steps 8.7 and 8.8 for a final library volume of 30 uL.

8.10 Store libraries at -20°C.

Protocol references

Kapp, J. D., Green, R. E., & Shapiro, B. (2021). A Fast and Efficient Single-stranded Genomic Library Preparation Method Optimized for Ancient DNA. *Journal of Heredity*, 112(3), 241-249. <https://doi.org/10.1093/jhered/esab012>