Lucigen®
Simplifying Genomics

NxSeq® 20 kb Mate Pair Library Kit Protocol

Generating Libraries using 10-20 kb inserts

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SP001 Rev. C

Product Description

The NxSeq® Long Mate Pair Library Kit is designed to generate mate pair libraries for sequencing on Illumina platforms. This protocol describes the use of the NxSeq Long Mate Pair Library Kit reagents to generate mate pair libraries using 10-20 kb insert sizes.

When combined with fragment library sequencing data, mate pair library sequences enable superior genome assembly, closure, and finishing. Applications include de novo genome assembly, chromosomal rearrangement detection, haplotyping, and BAC sequencing.

In order to generate 10, 15, and 20kb libraries, the following reagents are required:

Catalog #	Description	Kit Size
13000-1	NxSeq® Long Mate Pair Library Kit	5 libraries
13100-1	NxSeq® Long Mate Pair Library and Index Kit	5 libraries + 12 indices (5 libraries each)
13200-1	NxSeq Long Mate Pair Library Index Kit	12 indices, 5 libraries each
13300-1	NxSeq® Long Mate Pair Library Kit, Box 1	5 libraries
13400-1	NxSeq® Long Mate Pair Library Kit, Box 2	5 libraries

10-20 kb Protocol Workflow

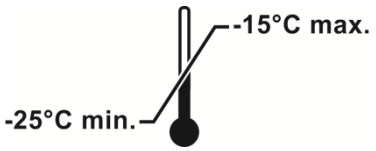
This protocol replaces steps 1-10 in the User's Manual (MA160). After the completion of the protocol outlined in this document, users will return to the protocol in the User's Manual (MA160). The restriction enzyme testing described in the User's Manual is required prior to starting the 10-20 kb protocol outlined in this document.

This protocol describes the use of two methods for step 5 (Size Selection); these methods include BluePippin Isolation and Gel Isolation using SeaKem Gold Agarose gels and Elutraps. Step 1 (Shearing) through step 4 (Ligation of Adaptor) and Step 6 (Ligation of Insert to Coupler) through Step 8 (Clean up) are the same regardless of the size selection method.

Refer to this section in the standard protocol (MA160: NxSeq® Long Mate Pair Library Kit User's Manual) for additional details on the overall mate pair library workflow.

Components and Storage

Store all kits and components at -20°C



NxSeq® Long Mate Pair Kit – Box 1

Reagent Name	# tubes in kit (A943016)	Cap Identifier	Map Identifier
Elution Buffer	1	EB	EB: Elution Buffer
End Repair Tailing Buffer[1]	2	ERB	ERB: E.R. Buffer
End Repair Enzyme Mix[1]	1	ERE	ERE: E.R. Enzyme
Klenow Fragment	1	KF	KF: Klenow
Adaptor[1]	1	ADT	ADT: Adaptor
Ligase	1	LIG	Lig: Ligase

[1] Reagents only in Box 1

NxSeq® Long Mate Pair Kit – Box 2

Reagent Name	# tubes in kit (A943018)	Cap Identifier	Map Identifier
Elution Buffer (EB)	5	EB	EB: Elution Buffer
Klenow Fragment	1	KF	KF: Klenow
Ligase	1	LIG	LIG: Ligase
Coupler Mix	1	CM	CM: Coupler
10X Ligase Buffer	1	10X	10X: Ligase Buffer
Nuclease 1	1	N1	N1: Nuclease 1
Nuclease 2	1	N2	N2: Nuclease 2
Biotin Wash Buffer	4	BWB	BWB: Biotin Wash
Biotin Capture Buffer	1	BCB	BCB: Biotin Buffer
Biotin Capture Reagent	1	BCR	BCR: Biotin Reagent
Tailing Buffer	1	TB	TB: Tailing Buffer
Junction Code™ Reagent	1	JC	JC: Junction Code
T4 Polynucleotide Kinase	1	PNK	PNK
Accura HotStart 2X Master Mix	1	AMM	AMM: Accura 2X MM
Primer Mix, Index 12	1	12	12: Index 12

Customer-Supplied Reagents and Equipment

Note: The customer-supplied reagents and equipment vary depending on the size selection method.

Reagent	Recommended Vendor	Catalog #	Used for 2-8 kb Protocol	Blue Pippin (Step 5A)	SeaKem / Elutrap (Step 5B)
SeaKem Gold Agarose	Lonza	50152	No	Yes	Yes
50X TAE agarose gel running buffer	Thermo Scientific	B49	No	Yes	Yes
Lambda DNA- HindIII digest	NEB	N3012S	No	Yes	Yes
Lambda DNA Mono Cut Mix	NEB	N3019S	No	Yes	Yes
3 M NaOAc pH7.0	Ambion	AM9740	No	Yes	Yes
100% Isopropanol	Various	Various	No	Yes	Yes
GlycoBlue	Ambion	AM9516	No	Yes	Yes
10% SDS Solution	Ambion	AM9822	No	Yes	Yes
Proteinase K	NEB	P8107S	No	Yes	Yes
Ethidium Bromide Solution at 10 mg/mL	Bio-Rad	161-0433	No	Yes	Yes
10X Loading Dye	Various	Various	No	Yes	Yes
0.75% Agarose cassettes, Dye Free, Low Range	Sage Science	BLF7510	No	Yes	No
S1 marker	Sage Science	*	No	Yes	No
Loading solution	Sage Science	*	No	Yes	No
0.1% Tween 20	Sage Science	*	No	Yes	No
3 M Sodium Acetate	Life Technologies	AM9740	No	Yes	No
HpyCH4V Restriction Enzyme	NEB	R0620S	Yes	Yes	Yes
*Rsa*I Restriction Enzyme (10 U/µL)		R0167S	Yes	Yes	Yes
*Alu*I Restriction Enzyme (10 U/µL)		R0137S	Yes	Yes	Yes
HaeIII Restriction Enzyme (10 U/µL)		R0108S	Yes	Yes	Yes
CutSmart™ Buffer		B7204S (comes with Restriction Enzymes)	Yes	Yes	Yes
*Acc*II Restriction Enzyme (10 U/µL)	Takara	1002A	Yes	Yes	Yes
RsaI, AluI, HaeIII, AccII and RE Buffer	Life Technologies	A25653	Yes	Yes	Yes
Dynabeads MyOne Streptavidin C1	Life Technologies	65001	Yes	Yes	Yes
Agencourt AMPure XP Magnetic Beads	Beckman Coulter	A63881 or A63882	Yes	Yes	Yes

Reagent	Recommended Vendor	Catalog #	Used for 2-8 kb Protocol	Blue Pippin (Step 5A)	SeaKem / Elutrap (Step 5B)
100% Ethanol	Various	Various	Yes	Yes	Yes
Nuclease Free Water (not DEPC-treated)	Ambion	AM993	Yes	Yes	Yes
1.5 mL Eppendorf DNA LoBind Microcentrifuge tubes	Eppendorf	22431021	Yes	Yes	Yes
0.2 mL thin wall PCR tubes	Various	Various	Yes	Yes	Yes
Qubit® dsDNA HS Assay Kit	Invitrogen	Q32854	Yes	Yes	Yes
Bioanalyzer DNA Kits. Options include • Agilent High Sensitivity DNA Kit • Agilent DNA 12000 Kit (optional)	Agilent Technologies	5067-4626 5067-1508	Yes	Yes	Yes

*Included with 0.75% Agarose cassettes, Dye Free, Low Range.

Equipment	Recommended Vendor	Catalog #	Used for 2-8 kb Protocol	Blue Pippin (5A)	SeaKem /Elutrap (5B)
Wide Bore Pipet Tips 200 µL	Axygen	TF-205-WB-L-R-S	No	Yes	Yes
Wide Bore Pipet Tips 1000 µL	Axygen	TF-1005-WB-R-S	No	Yes	Yes
Eppendorf Centrifuge	Eppendorf	5424	No	Yes	Yes
Electrophoresis supplies:	Various	Various	No	Yes	Yes
• SeaKem Gold Agarose • Markers (1K plus and 100 bp) • Marker (Lambda DNA HindIII digest) • Marker (Lambda DNA Mono Cut Mix)	Lonza Lucigen NEB NEB	50152 50020-1 or 50010-1 N3012S N3019S	No	Yes	Yes
Covaris g-TUBE™	Covaris	520079 or 520104	No	Yes	Yes
Refrigerator 4C° (calibrated to 4 – 5°C)	Various	Various	No	Yes	Yes
BluePippin	Sage Science	n/a	No	Yes	No
Elutrap	Whatman	10 447 700	No	No	Yes
Long wave UV 365 nM (Blak-Ray Lamp)	UVP, Inc	Model UVL-56	No	No	Yes

Equipment	Recommended Vendor	Catalog #	Used for 2-8 kb Protocol	Blue Pippin (5A)	SeaKem /Elutrap (5B)
2100 Bioanalyzer	Agilent	Various	Yes	Yes	Yes
Electrophoresis supplies: • Agarose • Markers (1K plus and 100 bp)	Various	Various	Yes	Yes	Yes
	Lucigen	50020-1 or 50010-1	Yes	Yes	Yes

Prior to starting: Restriction Enzyme Selection

Before proceeding with library construction, you must identify the restriction enzyme(s) needed to digest the gDNA to 400–900 bp (desired final library after PCR amplification). This step is critical to ensure the kit performs as designed and the sequencing coverage is uniform.

Refer to this section in the standard protocol (MA160: NxSeq® Long Mate Pair Library Kit User's Manual) for detailed instructions.

General Recommendations

- Use Eppendorf Lo-Bind 1.5 mL tubes throughout the protocol.
- Thaw all kit reagents on ice prior to use.
- Use wide bore tips to handle High Molecular Weight DNA
- Use a Qubit Fluorometer or equivalent to perform all sample quantification throughout the protocol.
    - The ratios of material used in each ligation step throughout the protocol have been optimized for the best performance.
    - The materials provided in the kit are quantified using Qubit® 2.0 Fluorometer (Life Technologies).
    - The use of other quantification methods (e.g. gel image, A260/A280), may lower the efficiency of the kit and result in insufficient material to sequence.

Detailed Protocol

1. Shear DNA to Appropriate Size

During this step, the genomic DNA (gDNA) is sheared to an average size range that is larger than the desired insert size. See *Appendix B: The effect of the size range of sheared DNA on insert size* for additional information on shearing and size selection.

Notes:

- gDNA used must be free of contaminating RNA.
- gDNA used must be of a high molecular weight (>20 kb).
- gDNA must be resuspended in Low TE (0.1 mM EDTA; 10 mM Tris pH 8) or in 10 mM Tris pH 8.5.
- Tagmentation from Illumina should not be used for shearing., Tagmentation will add additional nucleotides, and the use of Tagmentation has not been tested with the mate pair kit.

A sample loss of 20-60% is expected during shearing and clean up. The percentage of sample loss will vary depending on the shearing method used. This expected loss should be taken into account when determining the amount of gDNA to shear.

Use the table below to determine the recommended amount of starting gDNA and shearing method for your final desired insert size.

Final Desired insert size	Recommended amount of starting material	Recommended shearing conditions
10kb	15 µg in 150 µL Elution Buffer (EB)	g-TUBE™; 6200 RPM for 2 minutes each orientation using an Eppendorf model 5424 Centrifuge
15kb	15 µg in 150 µL Elution Buffer (EB)	g-TUBE™; 5500 RPM for 2 minutes each orientation using an Eppendorf model 5424 Centrifuge
20 kb	15 µg in 150 µL Elution Buffer (EB)	g-TUBE™; 4500 RPM for 3 minutes each orientation using an Eppendorf model 5424 Centrifuge

1.1 Options for shearing

Covaris g-TUBE™ (Covaris, Woburn, MA)

1.2 Size confirmation of sheared gDNA

- Confirm the correct size of the sheared gDNA:
  - Visualize on a 0.3 % SeaKem Gold agarose gel in 1X TAE buffer; 70 V, 75 minutes. Use the λ-HindIII and λ–Monocut ladders (See Figure 2 for example gel image).

1.3 Quantification of Sheared gDNA

- Quantify the sample from step 1.3 Purified, Sheared gDNA using Qubit® dsDNA HS Assay Kit with the Qubit® 2.0 Fluorometer according to manufacturer's instructions.

- Minimum amount and concentration of DNA required to proceed: DNA should be in either Low TE (0.1 mM EDTA, 10 mM Tris pH 8) or 10 mM Tris pH 8.5.

Insert Size	Minimum Amount DNA Required	Minimum Concentration DNA Required
10 kb	15 µg for g-TUBE™ shearing*	≥ 100 ng/µL
15 kb		
20 kb		

* More DNA might be needed, depending on DNA shearing method.

Optional Safe Stopping Point: DNA can be stored at -20C.

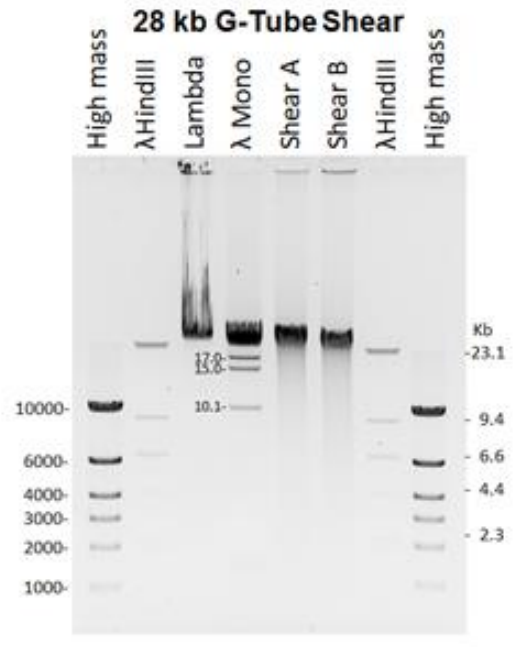


Figure 2. Sheared genomic DNA. Genomic DNA was sheared to approximately 28 kb with a g-TUBE™ and visualized on a 0.3% SeaKem Gold agarose gel. Markers include: High Mass Ladder, Lambda-HindIII, Lambda genomic DNA, and Lambda Mono Cut Mix.

2. End Repair

During this step, the sheared gDNA from step 1 is end-repaired. Each end-repair reaction is limited by the number of DNA molecules. Therefore, the number of reactions performed at this step is determined by the insert size:

Insert Size	Recommended # of reactions
10 kb	8 (938 ng each reaction)
15 kb	16 (938 ng each reaction)
20 kb	16 (938 ng each reaction)

2.1 NxSeq® Long Mate Pair Kit – Box 1 Reagents

Reagent	Cap Identifier
End Repair Tailing Buffer	ERB
End Repair Enzyme Mix	ERE

2.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Purified, Sheared gDNA	From step 1.4
Nuclease Free Water	Ambion
0.2 mL thin wall PCR tubes	Eppendorf
Thermocycler	User

2.3. Protocol

- Add the following reagents to 0.2 mL thin wall PCR tubes (number of reactions determined in table in step 2: End Repair).

Reagent	Amount (for each reaction)
Purified, sheared gDNA	938 ng
Nuclease-Free Water	Up to 23 µL
End Repair Tailing Buffer (ERB)	25 µL
End Repair Enzyme Mix (ERE)	2 µL
Total	**50 µL**

- Mix by pipetting up and down 10 times using a wide bore tip.
- Place tube(s) in a thermocycler and incubate according to the following parameters:

Step	Temperature	Time
1	25°C	20 minutes
2	72°C	25 minutes
3	4°C	Hold

- Proceed directly to step 3: A-Tailing.

3. A-Tailing

During this step, the End-Repaired gDNA from step 2.3 is A-tailed. The number of reactions performed during this step is the same as the number of reactions performed in step 2. End Repair.

3.1 NxSeq® Long Mate Pair Kit – Box 1 Reagents

Reagent	Cap Identifier
Klenow Fragment	KF

3.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
End-repaired gDNA	From step **2.3**
Thermocycler	User

3.3 Protocol

- Using the tubes containing the End-repaired, sheared DNA, set up the A-tailing reaction; add each reagent in the following order.

Reagent	Volume (µL) (for each reaction)
End-repaired gDNA	50
Klenow Fragment (KF)	2
Total	**52**

- Mix by pipetting up and down 10 times with a wide bore tip.
- Place the tube in a thermocycler and incubate according to the following parameters:

Step	Temperature	Time
1	37°C	20 minutes
2	70°C	15 minutes
3	4°C	Hold

- Proceed directly to step 4. Ligation of Adaptor.

4. Ligation of Adaptor

During this step, the A-tailed gDNA from step 3.3 is ligated to the adaptor.

NOTE: Do not vortex the adaptor. Mix by pipetting up and down and spin down briefly prior to use.

4.1 NxSeq® Long Mate Pair Kit – Box 1 Reagents

Reagent	Cap Identifier
Adaptor	ADT
Ligase	LIG

4.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
A-tailed gDNA	From step **3.3**
10% SDS Solution	Ambion
Proteinase K	NEB
3 M NaOAc pH7	Ambion
100% Isopropanol	Various
GlycoBlue	Ambion
Thermocycler	User

4.3 Ligation

- In the tube with the A-tailed gDNA, set up the ligation reactions; add each reagent in the following order.

Reagent	Volume (µL) (for each reaction)
A-tailed gDNA	52
Adaptor (ADT)	6
Ligase (LIG)	4
Total	**62**

- Mix by pipetting up and down 10 times with a wide bore tip.
- Place tube in the a thermocycler and incubate according to the following parameters:

Step	Temperature	Time
1	25°C	30 minutes
2	4°C	Hold

- Spin the tubes briefly to collect materials at the bottom of the tubes.
- Pool ligation reactions
    - For 10kb libraries, pool reactions 1-8 into clean 1.5 mL LoBind tubes.
    - For 15 and 20kb libraries, pool reaction 1-8 and 9-16 into two clean 1.5 mL LoBind tubes.

- Calculate the total volume of pooled ligation reactions and record the value.

Reagent	Volume (µL) (for each reaction)	Volume (µL) (reactions 1-8)	Volume (µL) (reactions 9-16)
Ligated Material	62	496	496
10% SDS Solution	4	32	32
Proteinase K	4	32	32
Total	**70**	**560**	**560**

- Mix by inverting tube 10 times. Spin briefly to collect material in the bottom of the tube.
- Place tube in the thermomixer or heat block and incubate according to the following parameters:

Step	Temperature	Time
1	37°C	30 minutes

4.4 Precipitate Ligated Material

- In the tube containing the material treated with Proteinase K, set up the precipitation reactions; add each reagent in the following order.

Reagent	Volume (µL) (reactions 1-8)	Volume (µL) (reactions 9-16)
Proteinase K treated DNA	560	560
3 M NaOAc	56	56
GlycoBlue	1	1
100% Isopropanol	900	900

- Mix by inverting tube 10 times.
- Incubate at Room Temperature for 10 minutes

- Centrifuge at **Room Temperature (25°C)** for 30 minutes at 15000 RPM. Note: SDS will precipitate at lower temperatures.

- Remove supernatant being careful not to disturb the blue pellet.
- Immediately add 600 µL of 70% Ethanol.

- Centrifuge at Room Temperature for 5 minutes at 15000 RPM.
- Remove supernatant with a pipette while being careful not to disturb the pellet.

- Air dry for 10minutes.
- For each tube, resuspend the pellet in:
    - **60 µL Elution Buffer (EB) with a wide bore tip for the BluePippin protocol in Section 5A.**
    - **50 µL Elution Buffer (EB) with a wide bore tip for the SeaKem / Elutrap protocol in Section 5B.**

- Incubate the eluted sample for 15 min at Room Temperature.
- Centrifuge at high speed (12,000-15,000 rpm) for 5 minutes to remove insoluble material.

- Transfer and pool the supernatants into a single, clean 1.5 mL LoBind tube.

5A. Size-select the Adaptor-Ligated DNA with BluePippin

During this step, the Precipitated Insert with Ligated Adaptor from step 4.4 is cleaned up using the BluePippin.

5A.1 NxSeq® Long Mate Pair Kit – Box 1 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

5A.2 Reagents / Equipment Needed

Reagent	Supplied By
Precipitated Insert with Ligated Adaptor	From step 4.4
BluePippin	Sage Science
0.75% Agarose cassettes, Dye Free, Low Range	Sage Science
S1 marker	Sage Science
Loading solution	Sage Science
0.1% Tween 20	Sage Science
3 M Sodium Acetate	Ambion
100% Isopropanol	User
70% Ethanol (Prepare fresh daily)	User
1.5 mL LoBind Microcentrifuge tubes	Eppendorf
Ethidium Bromide Solution 10 mg/mL	BioRad
10X Loading Dye	Various

5A.3 BluePippin Size Selection

- Prepare the 0.75% agarose dye free cassette according to the following instructions:
  - Gently tap any bubbles out from behind the elution modules.
  - Remove electrophoresis buffer from all five elution modules and replace it with fresh electrophoresis buffer.
  - Seal the elution modules shut with the provided tape.
  - Ensure that the buffer level is sufficient in all chambers.
  - Fill each sample well completely with electrophoresis buffer, and then remove 40 µL from each well.
  - Calibrate the instrument and test the cassette. Do not use any lanes that fail.

- Prepare the insert for loading onto the BluePippin
  - Add:
    - **10 kb**: 20 µL of BluePippin loading solution to the tube containing 60 µL of resuspended insert DNA. **Do not vortex to mix.**
    - **15 and 20 kb**: 40 µL of BluePippin loading solution to the tube containing 120 µL of resuspended insert DNA. **Do not vortex to mix.**
  - Mix by pipetting up and down (about 10 times) with a wide bore pipet tip until the sample is equilibrated with loading solution.
  - Spin the tube briefly to collect materials at the bottom of the tubes.

- Load the cassette with 40 µL of S1 marker in one lane, designated as the reference lane.

- Using a wide bore tip, load 40 µL of the resuspended insert DNA plus loading solution into each of the following lanes:
    - For 10kb insert: Two of the four remaining lanes.
    - For 15 and 20kb inserts: Four remaining lanes.



NOTE: This protocol is optimized for 3-4 µg of DNA loaded per lane. If your amount of input DNA is outside of this range, additional optimization may be required.

- Under the Protocol Editor tab, program the BluePippin run with the following parameters:
    - Range Mode Settings: see table below
    - BluePippin Cassette Definition: see table below.
    - Indicate the reference lane loaded with the S1 marker by choosing the appropriate flag and select "apply reference to all lanes."
    - For the four sample lanes, select the "range" box.
    - For the four sample lanes, enter the desired start value and a desired end value from the table below.

Minimum Insert Size	Range Mode Settings	BluePippin Cassette Definition
10 kb	Bpstart = 10000, Bpend = 50000	0.75%DF Marker S1 high-pass 6-10kb vs3
15 kb	Bpstart = 15000, Bpend = 50000	0.75%DF Marker S1 high-pass15-20 kb
20 kb	Bpstart = 18000, Bpend = 50000	0.75%DF Marker S1 high-pass15-20 kb

- Save the parameters as a named .pprot file.
- Under the Main tab, click on start. The required run time is dependent on the minimum insert size. For example, 10kb insert will take approximately 2 hours whereas a 20kb insert will take up to 4-5.
- After the run ends, allow the size selected samples to sit in the cassette for **a minimum of 45 minutes.**



NOTE: Keeping the samples in the cassette for longer than 45 minutes increases the recovery of high molecular weight DNA samples from the elution modules. Samples can remain in the cassette for as long as 14-16 hours (overnight).

- Slowly extract the 40 µL size selected DNA samples from each of the two or four sample lane elution modules.
    - Use a regular 20-200 µL tip; wide bore tips will not fit in the elution module,
    - **Do not pipet the samples up and down.**
- Combine the two or four lanes for each sample into a single 1.5 mL Lo-Bind tube.
- To each of the two or four-sample lane elution modules, add 40 µL of 0.1% Tween 20.

- Allow the solution to sit in the elution module for 1 minute. **Do not pipet the sample up and down.**

- Gently and slowly extract the 40 µL sample from each sample lane elution module and add it into the 1.5 mL Lo-Bind tube for a total of ~160 uL for 1 0Kb samples and ~320 µL for 15 Kb and 20 Kb samples.

5A.4 Protocol: Precipitation of Captured Insert DNA

- In the tube with the size selected sample, set up the precipitation reaction; add each reagent in the following order.

Reagent	Volume (µL) 10kb insert	Volume (µL) 15 and 20kb insert
Insert DNA	160	320
3 M NaOAc, pH 7.0 (0.1X)	16	32
GlycoBlue	1	1
100% Isopropanol (1.5X)	240	480

- Mix by inverting the tube 10 times.
- Incubate at -20°C for a minimum of 10 minutes up to overnight (14-16 hours).

- Centrifuge at 4°C for 30 minutes at 15,000 RPM.
- Remove supernatant being careful not to disturb the blue pellet.

- Immediately add 600 µL of 70% Ethanol.
- Centrifuge at 4°C for 5 minutes at 15,000 RPM.

- Remove supernatant with a pipette while being careful not to disturb the pellet.
- Air dry for 10 minutes.

- Carefully re-suspend the pellet in 50 µL Elution Buffer (EB).
- Incubate eluted sample for 15 min at RT.

5A.5 Concentration: Quantify using Qubit according to manufacturer's instructions.

- Record the concentration in ng/µL.
- Confirm the minimum amount and concentration of DNA required to proceed.

Insert Size	Minimum Amount DNA Required	Minimum Concentration DNA Required
10 kb	500 ng	10.0 ng/µL
15 kb	750 ng	15.0 ng/µL
20 kb	1000 ng	20.0 ng/µL

5A.6 Size: Confirm the correct size selection

- Visualize on 0.3% SeaKem Gold agarose gel. Run with Lambda DNA-HindIII digest (30 ng) and Lambda DNA Mono Cut Mix (200 ng). Bands at 10, 15, 17 and 23 kb should be visible (See Figure 3).

- Proceed directly to step 6: Ligation of Insert to Coupler.

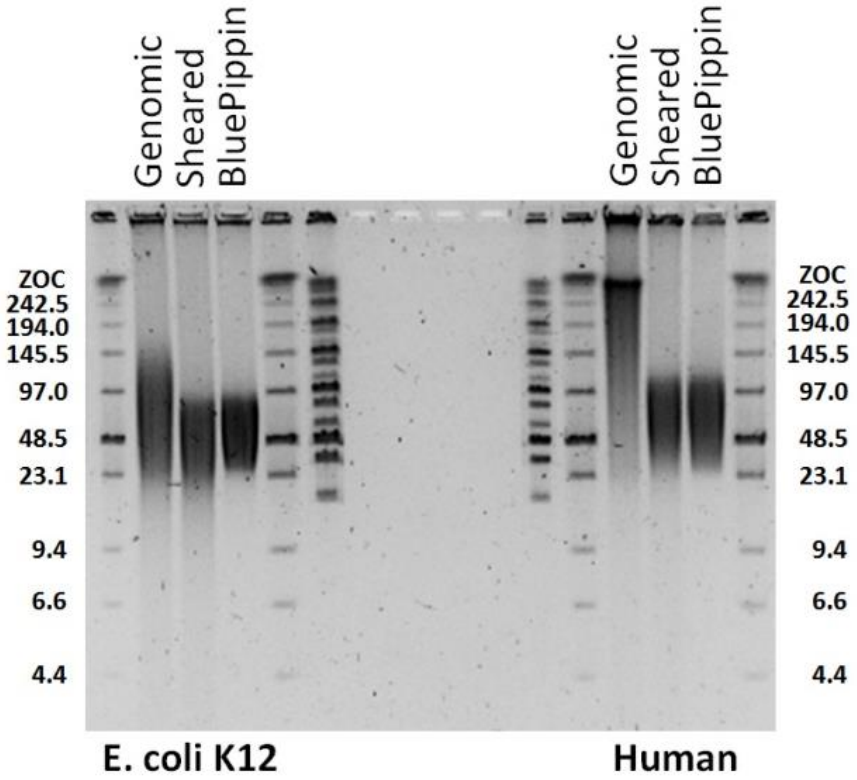


Figure 3. PFGE analysis of un-sheared genomic DNA, Sheared DNA and Size Selection with the BluePippin for inserts 20 kb and above.

5B. Insert Size Selection using SeaKem Gold Agarose and Elutrap™

During this step, the Precipitated Insert with Ligated Adaptor from step 4.4 is cleaned up using SeaKem Gold Agarose and Elutrap.

5B.1 NxSeq® Long Mate Pair Kit – Box 1 Reagents

Reagent	Cap Identifier
Elution Buffer	EB

5B.2 Reagents / Equipment Needed

Reagent	Supplied By
Precipitated Insert with Ligated Adaptor	From step 4.4
SeaKem Gold Agarose	Lonza
50X TAE agarose gel running buffer	Thermo Scientific
Ethidium Bromide Solution 10 mg/mL	BioRad
Electrophoresis supplies	Various
10X Loading Dye	Various
Elutrap	Whatman
3 M NaOAc pH7.0	Ambion
GlycoBlue	Ambion
100% Isopropanol	User
1.5 mL LoBind Microcentrifuge tubes	Eppendorf

5B.3 Protocol: Agarose Gel Size Selection

- Prepare a SeaKem Gold agarose gel.
    - Add SeaKem Gold Agarose to 1X TAE according to the table below.

Insert size	SeaKem Gold Agarose (g)	1X TAE (mL)
10 kb	1.0	100
15 kb	0.5	100
20 kb	0.3	100

    - Heat the TAE and agarose mixture to boiling to dissolve agarose.
    - Cool the solution for 10 sec in a cold-water bath and add 15 µL of Ethidium Bromide (10 mg/mL).
    - Pour into an electrophoresis casting tray (approximately 15 cm wide and 10 cm long) with a comb to form 1 cm X 2 mm wells.

- When polymerized, place agarose gel into an electrophoresis chamber containing 1X TAE buffer.
- Add:
    - **10 kb**: 50 µL 10X Loading Dye to the 50 µL pooled, precipitated insert with ligated adaptors from step 4.4 (8 µg / tube).
    - **15 and 20 kb**: 100 µL 10X Loading Dye to the 100 µL pooled, precipitated insert with ligated adaptors from step 4.4 (16 µg / tube).

- Load 50 µL sample into 2-4 internal wells (4 µg/well).
- Load 2.0 µL each Lambda DNA HindIII digest into two outer wells (approximately 1 µg).

- Run gel according to the table below:

Insert size	Conditions
10 kb	100 Volts; 60 minutes
15 kb	100 Volts; 60 minutes
20 kb	70 Volts; 75 minutes

Note: Reversing electrode polarity for 20 seconds at 1 min and 30 min into the electrophoresis can help decrease small insert contamination.

- Excise the insert DNA band from the gel using a long wave UV hand held lamp and a single edge razor blade.
    - Cut the agarose gel at the lower edge of the Lambda HindIII 23 kb bands and make a second cut approximately 5 mm above the first cut.
    - Remove the approximately 1 cm X 5 mm agarose plugs containing the insert DNA, and place in a 1.5 mL tube (See example in Figures 4 and 5).

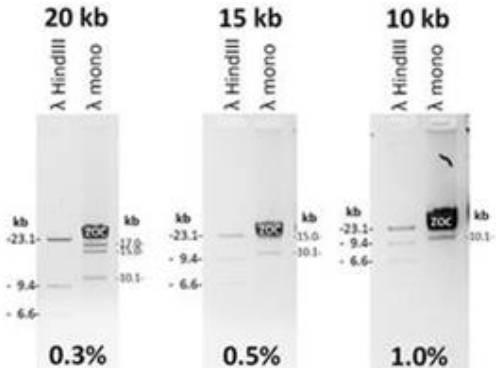


Figure 4: Zone of Compression (ZOC). Inserts located in the Zones of Compression are excised for elution using the Elutrap™.

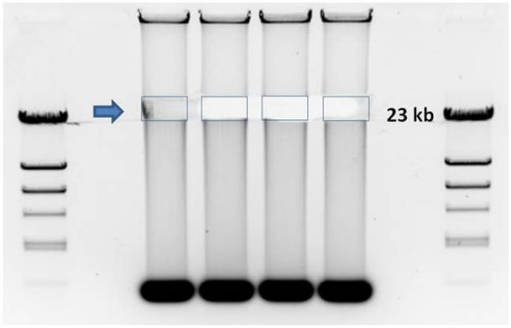


Figure 5. Gel-Isolated Insert. Sheared, adapted insert was gel isolated on a 0.3% SeaKem Gold agarose gel. The marker is a Lambda-HindIII digest. The lower bands are un-ligated adaptor.

5B.4 Protocol: Elutrap Capture of Insert DNA

- Set up the Elutrap according to manufacturer's instructions.
- Place two agarose plugs in the Elutrap next to the white BT2 filter (stacked horizontally or vertically side by side). 15 and 20 kb libraries require 2 Elutraps each.
- Apply 150 Volts for 5 hours.
- After electrophoresis, reverse the polarity of the electrodes and apply 200 Volts for 20 seconds to remove DNA from the BT1 membrane.
- Remove the buffer, containing the insert, from the sample chamber with a 1000 mL pipette tip and place into a fresh 1.5 mL LoBind tube (approximately 400 to 500 mL). Take care not to puncture the fragile white BT2 membrane.



Note: The amount of sample removed will be variable.

5B.5 Protocol: Precipitation of Captured Insert DNA.

- Measure the volume of the sample removed from the sample chamber (X in table below). Split into two tubes if the volume of the mixture exceeds 1.5 mL.
- Add the following to the tube(s) containing the insert DNA.

Reagent	Volume	Example #1 Volume (µL)	Example #2 Volume (µL)
Gel Excised Insert DNA (step 5B.3)	X	400	500
3 M NaOAc, pH 7.0 (0.1X)	0.1 X	40	50
GlycoBlue	1 µL	1	1
100% Isopropanol (1.5X)	1.5X	660	825

- Mix by inverting the tube 10 times.
- Incubate at -20°C for a minimum of 10 minutes up to overnight (14-16 hours).
- Centrifuge at 4°C for 30 minutes at 15,000 RPM.
- Remove supernatant being careful not to disturb the blue pellet.
- Immediately add 600 µL of 70% Ethanol.
- Centrifuge at 4°C for 5 minutes at 15,000 RPM.
- Remove supernatant with a pipette while being careful not to disturb the pellet.
- Air dry for 10 minutes.
- Carefully re-suspend the pellet(s) using the following amounts of Elution Buffer (EB):
  - Single Tube: 50 µL
  - Two Tubes: 25 µL in each tube
- Incubate eluted sample for 15 min at RT.
- If sample is split into two tubes and resuspended in 25 µL of Elution Buffer (EB), pool tubes into clean 1.5mL tube.

- Combine 15 and 20 kb insert Elutrap samples (2 Elutraps each).

5B.5 Concentration: Quantify using Qubit according to manufacturer's instructions

- Record the concentration in ng/µL.
- Confirm the minimum amount and concentration of DNA required to proceed, using the table below.

Insert Size	Minimum Amount DNA Required	Minimum Concentration DNA Required
10 kb	500 ng	10.0 ng/µL
15 kb	750 ng	15.0 ng/µL
20 kb	1000 ng	20.0 ng/µL

5B.6 Size: Confirm the correct size selection:

- Visualize on 0.3% SeaKem Gold agarose gel. Run with Lambda DNA-HindIII digest (30 ng) and Lambda DNA Mono Cut Mix (200 ng). The 10, 15, 17 and 23 kb bands should be visible (See Figures 6 and 7).

- Proceed directly to step 6: Ligation of Insert to Coupler.

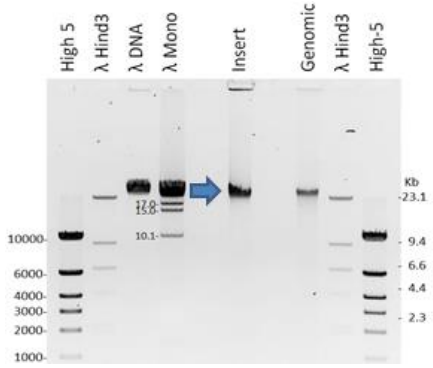


Figure 6. Gel-Isolated Insert DNA. 20 kb insert DNA was gel-isolated on a 0.3% SeaKem Gold agarose gel and eluted with an Elutrap electro-eluter. Purified insert DNA was visualized on a 0.3% SeaKem Gold agarose gel. Markers include High Mass Ladder, Lambda-HindIII, Lambda genomic DNA, and Lambda Mono Cut Mix.

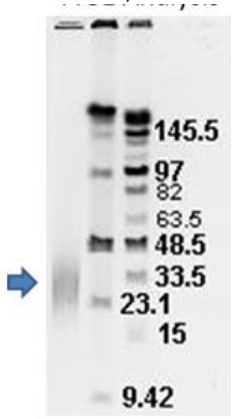


Figure 7. Optional Method for Visualization: FIGE analysis of purified insert DNA. Insert DNA (arrow) was separated on a 0.6% agarose gel by Field Inversion Gel Electrophoresis using a BioRad CHEF-DR III System.

6. Ligation of Insert to Coupler

During this step, the size-selected DNA with adaptor from step 5A.6 or 5B.6 is ligated to the coupler.

6.1 NxSeq® Long Mate Pair Kit – Box 2 Reagents

Reagent	Cap Identifier
Coupler Mix	CM
10X Ligase Buffer	10X
Ligase	LIG

6.2 User-Supplied Reagents / Equipment

Reagent	Supplied By
Size-selected DNA with Adaptor	From step 5A.6 or 5B.6
Nuclease Free Water	Ambion
Refrigerator (set at 4-5°C)	Various
Thermomixer or heat block (set at 70°C)	Eppendorf
Pipet designed for volumes under 2µL	Various

6.3 Determine Amount of Insert Required

- Use the following equation to determine the amount of size-selected DNA with adaptor material is required for step 6.4 Protocol.

NOTE: The optimal condition for this step is to use equal amounts of insert and coupler in the ligation reaction. The size of the coupler included in the kit is 2000 bp and the amount of coupler specified for each reaction is 100 ng.

$$\frac{\boldsymbol{insert\ size\ bp}}{\boldsymbol{2000\ bp}}\boldsymbol{x\ 100\ ng = ng\ insert = Y}$$

Example: $\frac{\boldsymbol{5000\ bp}}{\boldsymbol{2000\ bp}}\boldsymbol{x\ 100\ ng = \ 250\ ng}$

6.3.1 Calculate and record the amount of insert required.

- Use the following equation to determine the required volume of size-selected DNA with adaptor required (X), based on the concentration determined in step 5A.5 or 5B.5 (Z) and the amount of insert (Y) in ng as calculated above.

$$\frac{\boldsymbol{Y}\ (Amount\ in\ ng)}{\boldsymbol{Z}\ (\boldsymbol{Concentration\ in}\frac{ng}{uL})} = \boldsymbol{X\ volume\ in\ uL}$$

6.3.2 Calculate and record the volume of insert required (X volume in uL).

6.4 Protocol

IMPORTANT NOTE: For 10 and 15 kb insert ligations, the insert will be ligated in a single tube. For 20 kb insert ligations, the insert will be split into two tubes with 500ng in each tube.

- In a single fresh 1.5 mL LoBind tube (10 and 15 kb) or two fresh 1.5 mL LoBind tubes (20 kb), set up the following ligation reactions to generate the Ligated Insert/Coupler; add each reagent in the following order.

Reagents for 10 and 15 kb inserts

	Tube 1: Volume (µL)
Size-selected Insert with adaptor:	X (calculated in step 6.3.2)
Coupler mix (CM)	3.0
Nuclease-free water	Up to 356.5
10X Ligase Buffer (10X)	40
Ligase (LIG)	0.5
Total	**400**

Reagents for 20 kb inserts

	Tube 1: Volume (µL)	**Tube 2: Volume (µL)**
Size-selected Insert with adaptor: 500 ng each reaction	X/2 (calculated in step 6.3.2)	X/2 (calculated in step 6.3.2)
Coupler mix (CM)	1.5	1.5
Nuclease-free water	Up to 356.5	Up to 356.5
10X Ligase Buffer (10X)	40	40
Ligase (LIG)	0.5	0.5
Total	**400**	**400**

NOTE: Use a pipet designed for volumes under 2 µL to pipet the Ligase.

- Mix gently by inverting tube(s) 10 times.
- Incubate and heat kill the Ligated Insert/Coupler from step 6.4 according to the table below:

Step	**Temperature**	**Time**
1	4-5ºC	Overnight (14-16 hours)
2	70ºC	15 minutes

- Place the tube(s) on ice for 2 minutes.
- Spin the tube(s) briefly to collect materials at the bottom of the tube(s).

- Proceed directly to Step 7: Exonuclease Treatment.

7. Exonuclease Treatment

During this step the Heat Killed Ligated Insert/Coupler from step 6.4 is treated to remove any linear DNA.

7.1 NxSeq® Long Mate Pair Kit – Box 2 Reagents

Reagent	Cap Identifier
Nuclease 1	N1
Nuclease 2	N2

7.2 Reagents / Equipment Needed

Reagent	Supplied By
Heat Killed Ligated Insert/Coupler	From Step 6.4.
Thermomixer or heat block (set at 37°C)	Eppendorf
Thermomixer or heat block (set at 80°C)	Eppendorf

7.3 Protocol

- In the tube(s) with the Heat Killed Ligated Insert/Coupler, set up the exonuclease treatment; add each reagent in the following order.

Reagent	Volume (µL) per Tube
Heat Killed Ligated Insert/Coupler	400
Nuclease 1 (N1)	7
Nuclease 2 (N2)	5
Total	**412**

- Mix gently by pipetting up and down 10 times with a wide bore tip.
- Place tube(s) in a thermomixer or heat block and incubate according to the following parameters.

Step	Temperature	Time
1	37°C	30 minutes
2	80°C	30 minutes

- Place the tube(s) on ice for 2 minutes.
- Spin the tube(s) briefly to collect materials at the bottom of the tube(s).

- Proceed directly to Step 8: Clean-up of Exonuclease Treated Insert/Coupler.

8. Clean Up of Exonuclease Treated Insert / Coupler

In this step, the Exonuclease Treated Insert/Coupler from step 7.3 is cleaned.

8.1 Protocol: Precipitation of Exonuclease treated Insert DNA.

- Add the following to the sample of Exonuclease Treated Insert / Coupler.

Reagent	Volume (µL) per Tube
Exonuclease Treated Insert / Coupler	412
3 M NaOAc (0.1X)	41
GlycoBlue	1
100% Isopropanol	680

- Mix by inverting the tube(s) 10 times.
- Incubate at -20°C for a minimum of 10 minutes up to overnight (14-16 hours).

- Centrifuge at 4°C for 30 minutes at 15,000 RPM.
- Remove supernatant being careful not to disturb the blue pellet.

IMPORTANT NOTE: Frequently the DNA is spread up the side of the tube and is difficult to see. Always add the Elution Buffer to the side of the tube and elute the DNA by washing the side of the tube until the DNA is resuspended.

- Immediately add 600 µL of 70% Ethanol.
- Centrifuge at 4°C for 5 minutes at 15,000 RPM.

- Remove supernatant with a pipette while being careful not to disturb the pellet.
- Air dry for 10 minutes.

- Carefully re-suspend the pellet(s) in a total of 35 µL Elution Buffer (EB) using a wide bore tip. For two tubes, add 17.5 µL Elution Buffer (EB) to each tube.

- Incubate eluted sample for 15 min at RT.
- For 20kb inserts, combine the two tubes into a clean 1.5mL Lo-bind tube for a total of 35 µL.

Continue with Step 11: Restriction Enzyme Digest through Step 20 in the standard protocol (MA160: NxSeq® Long Mate Pair Library Kit User's Manual)
See Appendix A of this document for the expected size distribution of 20 kb inserts.

Optional Safe Stopping Point: DNA can be stored at -20C.

Appendix A. Size Distribution of 20kb Inserts

Size distributions of sequenced 10, 15, and 20 kb Long Mate Pair Libraries are shown below. Qualified long span mate pairs were mapped against reference genomes, and the pair distance distribution was plotted in CLC Genomics Workbench.

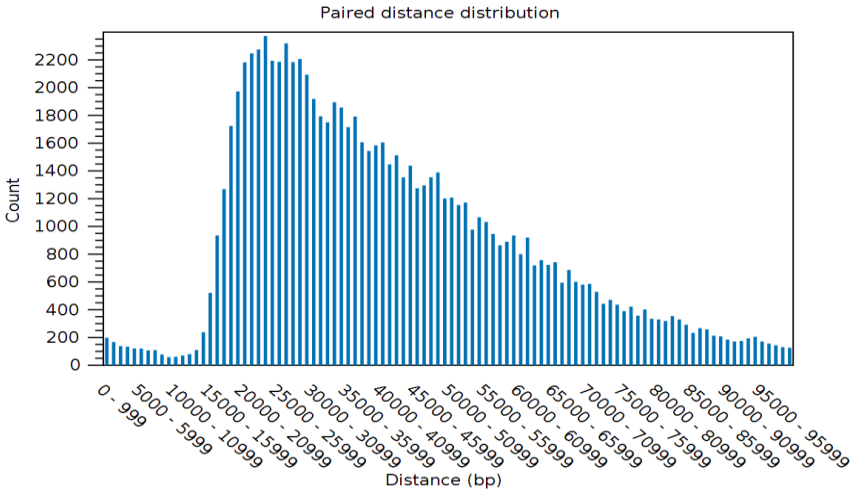


Figure 7: Size Distribution of 20 kb Insert Size Selected with BluePippin. Qualified long span mate pairs were mapped against the repeat masked reference genome GRCh38, and the pair distance distribution was plotted in CLC Genomics Workbench. High Pass; 20-50 kb range mode (35 kb selection); 15-20 kb definition, Marker S1.

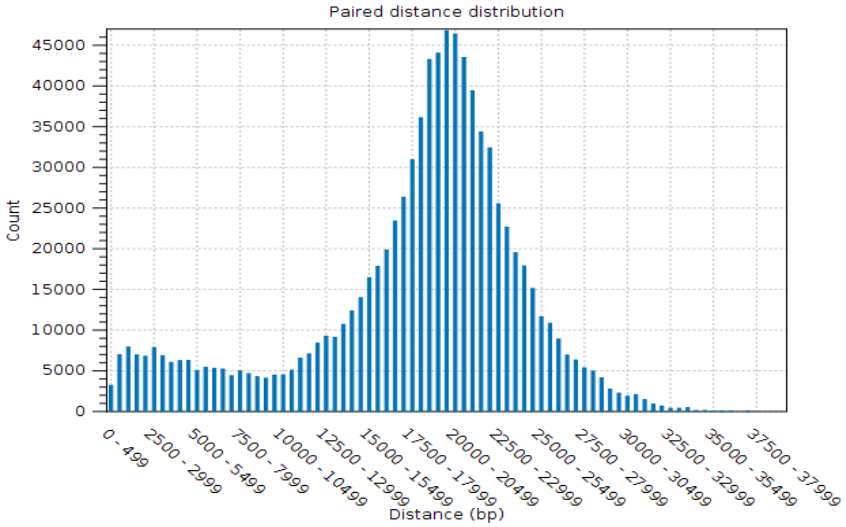


Figure 8: Size Distribution of 20kb Insert Size Selected with Elutrap. Sequencing data was mapped against the reference E. coli strain K12 and plotted as Paired Distance Distribution.

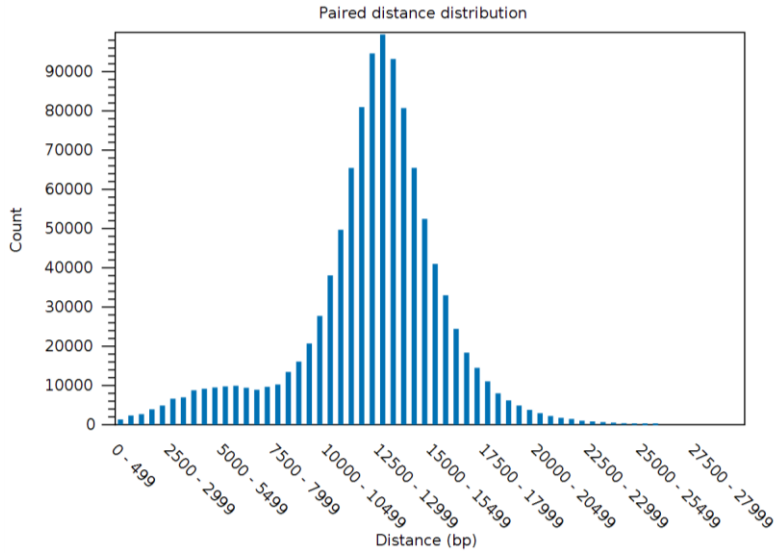


Figure 9. 15 kb Mate Pair: E. coli, Elutrap.

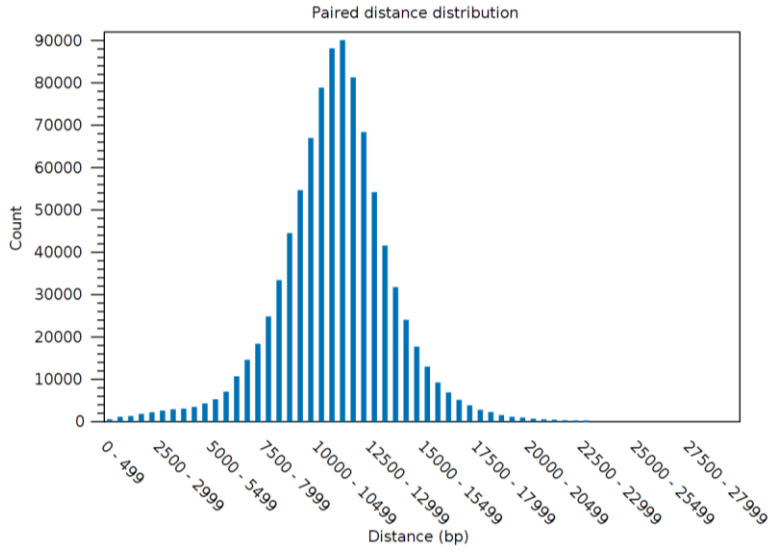


Figure 10. 10 kb Mate Pair: E. coli, Elutrap.

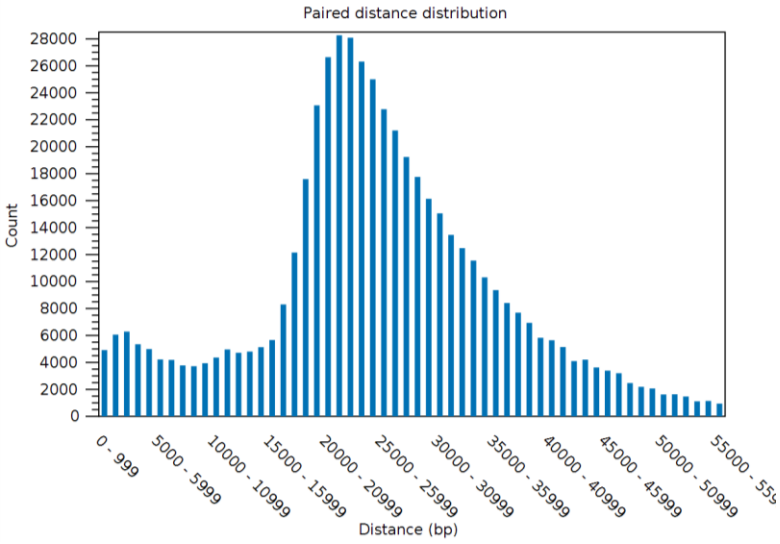


Figure 11. 20 kb Mate Pair: Thermus aquaticus; Elutrap.