FLI-Seq™ User Guide

This product is for research use only and is not intended for diagnostic or therapeutic uses.

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Chapter 1: Overview

Introduction into FLI-Seq™.

Fast-**L**ibrary of **I**nserts Sequencing or FLI-Seq™ is a method for generating Next-Generation Sequencing libraries from pooled CRISPR screens. Probes are used to enrich for genomic DNA inserts containing the guide RNA sequence, resulting in a purified pool of substrate DNA for subsequent PCR reactions.

The FLI-Seq kit offers:

Reduced number of PCR reactions and cycles
High throughput and robust workflow
High reproducibility with accurate data
Unbiased target identification with high specificity

Important Note

Before initiating this procedure thoroughly review the required equipment and materials list.

This procedure is to be followed by trained lab personnel.

Term	Temperature
Room Temperature	20 – 25°C
Ice	0 – 4°C
Freeze	-20°C
Volume units	
µL is microliter	One millionth (10^{-6}) of a liter
mL is milliliter	One thousandth (10^{-3}) of a liter

Precautions

1. This kit contains chemicals which may be hazardous. FLI-Seq Buffer contains SDS and other detergents, which may irritate upon contact. Personal protection equipment (PPE) should be worn during the entirety of this procedure and only trained personnel should conduct experimental steps.
2. We recommend using DNA low-retention tubes for all steps (e.g. Eppendorf DNA LoBind 1.5 mL [cat. #022431021] and VWR 0.2 mL strip tubes and caps [cat. #95000-094]).
3. We recommend using TipOne® RPT Ultra Low Retention Filter Tips from USA Scientific [USA Scientific RPT tips cat. #1180].
4. Ensure all tubes are fully closed during wash and incubation steps.
5. Store all reagents at listed storage temperatures when not in use.
6. All thermomixer incubation steps are done with interval mixing (15 seconds ON / 15 seconds OFF) at 1,250 rpm.
7. For bead drying steps, ensure that all liquid has evaporated before starting elution.
8. Do not over-dry beads.
9. Do not freeze beads.
10. Always ensure beads are completely resuspended before transferring.

IMPORTANT: *Materials listed below are for FLI-Seq experimental set up ONLY. See Appendix A for supplemental information*.

- *This guide has been optimized for samples defined as genomic DNA (gDNA) isolated from 4 × 10^6 cells. The included FLI-Seq reagents are sufficient for sequencing library preparation from up to 600 µg of isolated genomic DNA (gDNA) or 1 × 10^8 cells.*

- *This kit was not tested on samples below 750,000 cells (7.5×10^5) or less than 5 µg of gDNA.*

Reagents Included:

Reagent	Storage
3× FLI-Seq Buffer	–20°C
FLI-Seq Probes, 1 µg/µL	–80°C
Bead Elution Buffer	–20°C
sgDNA Primers	–20°C
Indexed Primers (505-508, 705-708)	–20°C
2× PCR Mix	–20°C
FLI-Seq Beads	4°C

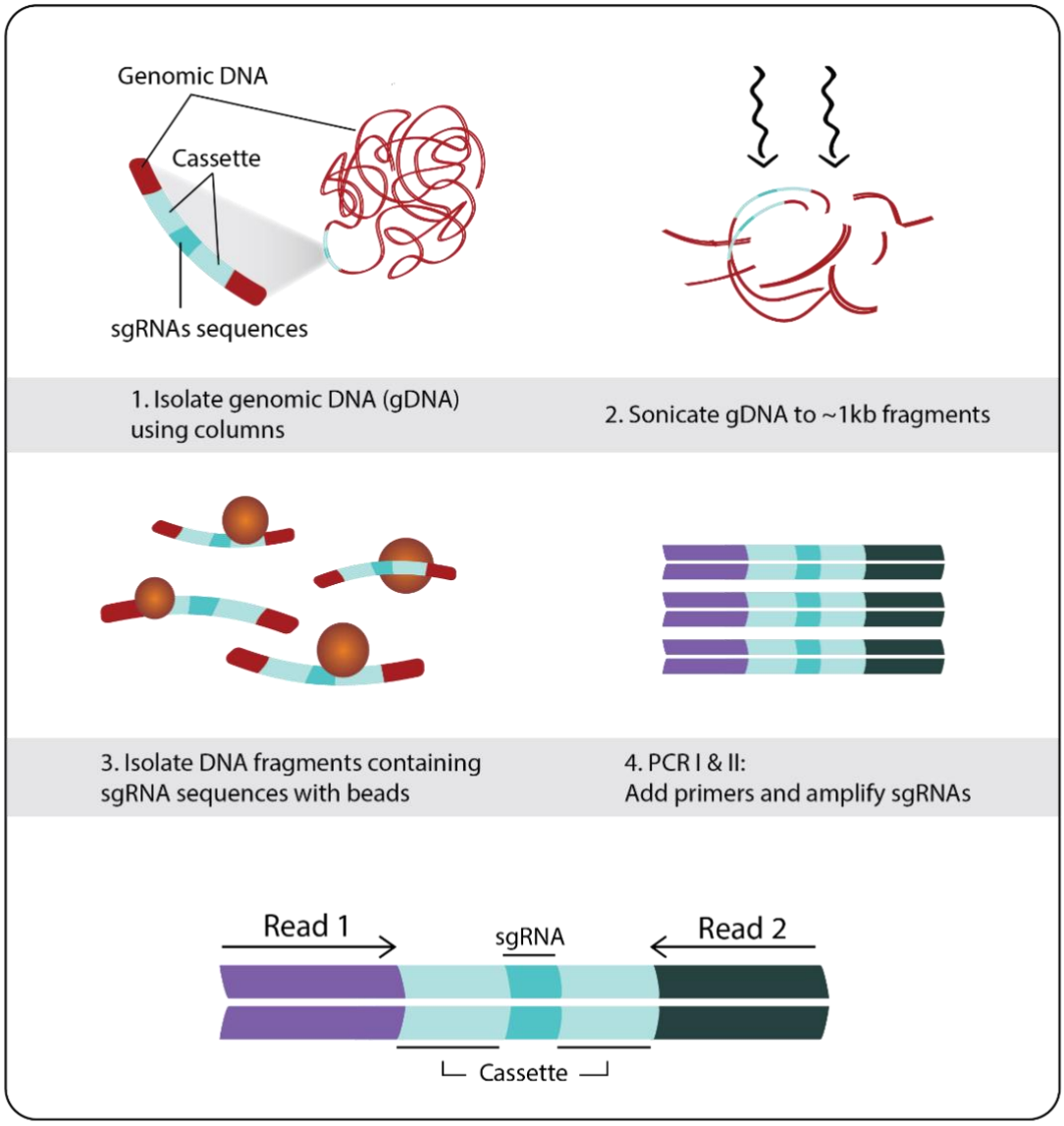
Required Equipment:

Equipment	Vendor and Catalog Number (tested at Eclipse)
Low or variable energy sonicator	QSonica cat. #Q800R2-110
T100 PCR Thermocycler	BioRad cat. #1861096
Microcentrifuge	Eppendorf cat. # 2231010057
Mini centrifuge	Sigma cat. # Z681717
Thermomixer with 1.5 mL heatblock	Eppendorf cat. #5382000015
Magnetic rack for 1.5 mL tubes	ThermoFisher Scientific cat. #12321D
Magnetic rack for 0.2 mL strip tubes	EdgeBio, cat. #57624
TapeStation (or Bioanalyzer)	Agilent cat. # G2991AA
Tube rotators	VWR cat. # 10136-084

Reagents Not Included:

Reagent	Vendor Catalog Number
Low-retention filter pipette tips	USA Scientific RPT tips cat. #1180
PCR 8-Well Tube Strips	VWR cat. #20170-004
1.5 mL DNA LoBind Micro-centrifuge tubes	Eppendorf cat. #022431021
50 mL conical centrifuge tubes	Fisher Scientific cat. #14-432-22
DNase-free RNase Cocktail Enzyme Mix	Thermofisher AM2286
Zymo DNA Clean and Concentrator™-5 kit	Zymo Research cat. #4014/4013
Ethanol, 200 proof, for molecular biology	Sigma-Aldrich cat. #E7023 or equivalent
1M DTT	Sigma 43816 or equivalent
2M and 100 mM NaOH	Sigma-Aldrich cat. #79724 or equivalent
3M HCl	VWR #BDH7375-1 or equivalent
Agencourt AMPure XP beads	Beckman Coulter cat. # A63881
DNA quantification method	Agilent cat. #5067
DNA isolation method	Qiagen cat. #69504
Nuclease-free water	VWR cat. #95000-094

FLI-SEQ WORKFLOW



Chapter 2: Genomic DNA Isolation and Fragmentation

Overview

In this section, genomic DNA (gDNA) will be isolated and sheared to ~ 1kb fragments using sonication.

Consumables

- Qiagen's DNeasy Blood and Tissue gDNA isolation kit or equivalent
- Agilent's Genomic DNA ScreenTape or equivalent

Preparation

1. Preparation Note: Cool the sonicator chiller to 4°C.

Procedure

Isolate genomic DNA

Isolate genomic DNA (gDNA) using Qiagen's DNeasy Blood and Tissue Kit or equivalent method. See Appendix A for modified protocol. Final concentration of eluted gDNA should be between 200-300 ng/µL. If the concentration is above 300 ng/µL, dilute it to 200-300 ng/µL with Qiagen elution buffer (AE).

Optional Stopping Point*: *If stopping here, freeze isolated gDNA at –20°C

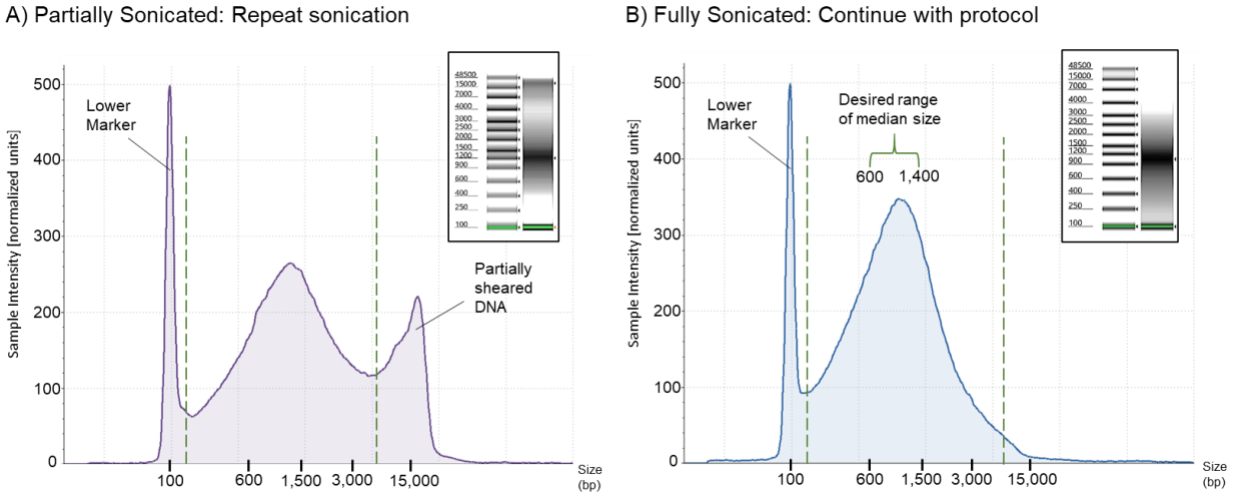
Next stopping point: ~1-2 hrs

Shear genomic DNA

Desired fragment size is ~**1 kb**. Median fragment sizes of 600-1400 bp are acceptable. Avoid fragmenting below 300 bp as this will compromise final library yield. If average fragment size is above 1500 bp (Figure 1A), repeat sonication in 1-2 cycle intervals until average fragment size is approximately 1 kb (Figure 1B).

1. Sonicate gDNA samples with an energy setting at 20% amplitude for 5 cycles of 10 seconds ON / 10 seconds OFF.
   - **Note:** If using a BioRuptor® sonicator, sonicate on the "low" setting for 4 cycles of 30 seconds ON / 30 seconds OFF.
2. Measure 1 µL of sheared gDNA sample on TapeStation or equivalent to determine fragment sizes.
   - **Note:** Protocol has been optimized using Agilent4200 TapeStation. See Agilent4200 TapeStation manual for operational instructions.
   - **Note:** We recommend using Agilent's Genomic DNA ScreenTape and following the manufacturers protocol.
   - **Note:** If concentration of the sample is above the recommended maximum of 100 ng/µL, dilute sample as necessary to fall within TapeStation quantification range.

Figure 1. TapeStation gDNA ScreenTape Sonication Optimization



3. Calculate total amount of gDNA in micrograms.
   - For TapeStation, set region area to 70-4000 bp, record concentration and multiply by total volume of sample in µL. If sample was diluted for TapeStation, multiply by dilution factor. The result is the total amount of DNA in µg.

4. Calculate total amount of probes needed. Probe quantity should be 14% of total DNA amount by mass.

   - Example: for 40 µg of total sheared gDNA: 40 µg * 0.14 = 5.6 µg of probe needed

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Optional Stopping Point: *If stopping here, freeze sheared gDNA at –80°C*

Next stopping point: ~5-6 hrs

Chapter 3: Capture guide-RNA sequence from gDNA

Overview

In this section, guide-RNA (sgRNA) sequences will be captured from sheared gDNA.

Consumables

- 3× FLI-Seq Buffer (thaw at room temperature then store at room temperature until use)
- FLI-Seq Probes (thaw at room temperature then store on ice until use)
- Bead Elution Buffer (thaw at room temperature then store on ice until use)
- FLI-Seq Beads (store at room temperature)
- 1M DTT
- 2M and 100mM NaOH
- 3M HCl
- Nuclease-free water
- DNase-free RNase Cocktail Enzyme Mix
- Zymo DNA Clean & Concentrator™-5 kit

Preparation

1. Preheat thermomixer with 1.5 mL tube holder to **95°C**.
2. Prepare 2M NaOH, 100mM NaOH and 3M HCl
3. Centrifugation steps are done at room temperature.

Procedure

Aliquot probes

1. Aliquot quantity of probes calculated from step 4 in Chapter 2 for each sample to a new 1.5 mL LoBind tube. (e.g. for 5.6 µg of probes needed, aliquot 5.6 µL of probe).
2. Dilute probes with nuclease-free water to a total volume of **200 µL**.
   - **Note:** For samples using >20 µg total probes, bring total volume up to 10 µL per 1 µg probe (e.g. for 25 ug total probes, bring volume up to 250 µL).
3. Store diluted probes on ice.

Wash FLI-Seq beads

1. Fully resuspend **FLI-Seq Beads** by pipetting.
2. In a new 1.5 mL tube, aliquot **17 µL** of **FLI-Seq Beads** per **1 µg** of probe used for each sample (e.g. for 40 µg of gDNA and 5.6 µL of probe, use 95.2 µL of FLI-Seq beads).

3. Place 1.5 mL tube with FLI-Seq beads on magnet until separation is complete and supernatant is transparent.
4. Carefully aspirate and discard supernatant without disturbing beads.
5. Resuspend beads in 1 volume of 3× FLI-Seq Buffer (e.g. if using 95.2 µL of FLI-Seq beads, add 95.2 µL 3× FLI-Seq buffer).
6. Place tube on magnet for 30 seconds until separation is complete and supernatant is transparent.
7. Carefully aspirate and discard supernatant without disturbing beads.
8. Repeat FLI-Seq bead wash steps for a total of two washes (repeat steps 5-7).
9. Spin capped samples in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
10. Place tube back on magnet for 10 seconds until separation is complete and supernatant is transparent.
11. While on magnet aspirate and discard all residual liquid without disturbing beads.
12. Resuspend beads in 0.5× volume of final probes (determined from "Aliquot probes" step 2) with 3× FLI-Seq Buffer (e.g. if probes were diluted in 200 µL, resuspend washed beads in 100 µL of 3× FLI-Seq buffer).

Couple probes to beads

1. Add resuspended **washed FLI-Seq Beads** to probes (from "Aliquot probes" step 3) and mix quickly by inverting tube several times.
2. Couple probes to beads at room temperature on a tube rotator for 15 min.
3. Place 1.5 mL tube with beads and probes on magnet.
4. Slowly invert closed tubes as beads start to separate to capture any beads from cap.
5. After separation is complete, discard supernatant without disturbing beads.
6. Spin all samples in mini-centrifuge for 3 seconds.
7. Place samples back on magnet and allow 30 seconds to separate.
8. Pipette and discard any excess liquid without disturbing beads.
9. Resuspend probe-coupled beads in **3× FLI-Seq Buffer using 0.5× volume of sheared gDNA** (see Appendix A: Genomic DNA Isolation) (e.g. if volume of sheared gDNA is 130 µL, resuspend probe-coupled beads in 65 µL of 3× FLI-Seq buffer).
10. Keep resuspended probe-coupled beads at room temperature while preparing sheared gDNA in next step.

Denature sheared gDNA

1. To sheared gDNA sample, add 2% of 1M DTT (e.g. if volume of sheared gDNA is 130 µL, add 2.6 µL DTT).
2. Incubate gDNA sample in preheated thermomixer at 95°C for 1 minute with interval mixing at 1,250rpm.
   - **Note:** for volumes >250 µL, increase time at 95°C to 2-3 min.
3. Decrease temperature to 75°C **without** removing tubes from thermomixer.

- **Note:** Do not allow cooling and renaturation of gDNA fragments

Couple denatured gDNA to beads/probes

1. When the thermomixer temperature reaches 75°C, resuspend probe-coupled beads by pipette mixing until homogenous ("Couple probes to beads", step 10).
2. Place probe-coupled beads in thermomixer.
3. Keeping both gDNA samples and probe-coupled beads in thermomixer, open tube caps and directly add entire volume of probe coupled-beads to sheared gDNA sample.
4. Reduce thermomixer temperature to 67°C.
5. Incubate with interval mixing at 1,250rpm according to **Table 1**:

Table 1. Coupling Thermomixer Temperatures

Temperature	Time
67°C	5 minutes
64°C	5 minutes
61°C	2 hours, 15 minutes

- **Preparative Note:** After 2hrs of incubation, thaw **Bead Elution Buffer** at room temperature.

6. Prepare 600 µL 1× FLI-Seq buffer according to **Table 2** in a fresh 1.5 mL LoBind tube.
7. Prewarm and keep 1x **FLI-Seq Buffer** at **37°C**
   - **Note:** For samples ≥100 µg gDNA, make 1.2mL volume of 1× FLI-Seq buffer for each sample.

Table 2. 1× FLI-Seq buffer (per sample):

Reagent	Volume (µL)	
	<100 µg gDNA	≥100 µg gDNA
3× FLI-Seq buffer	200	400
Nuclease-free water	400	800
Total:	600	1200

8. Prepare **gDNA elution mix** according to **Table 3** in a fresh 1.5 mL LoBind tube.
   - **Note:** For samples ≥100 µg total gDNA, make double the volume of elution buffer.

Table 3. gDNA elution mix (per sample):

Reagent	Volume (µL)	
	<100 µg gDNA	≥100 µg gDNA
Bead Elution Buffer	54	108
DNase-free RNase Cocktail Enzyme Mix	6	12
Total:	60	120

Wash gDNA-coupled beads

- **Note:** Samples are processed one at a time for steps 1-4 to maintain sample temperature at 37°C.
- **Preparative Note:** Preheat thermomixer with 1.5 mL tube holder to **37°C**

1. Remove sample from 61°C thermomixer ("Couple denatured gDNA to beads/probes", step 5) and immediately place on magnet.
2. When separation is complete and liquid is transparent (no longer than 20 seconds), carefully aspirate and discard supernatant.
3. Add 600 µL (or 1.2 mL for samples ≥100 µg total gDNA) of **prewarmed 1× FLI-Seq buffer** and quickly invert to mix until homogenous.
4. Incubate sample at **37°C** in thermomixer for at least 5 minutes with interval mixing at 1,250rpm.
   - **Note:** Samples remain at this step while processing additional samples through steps 1-4
5. Once all samples have been processed, place on magnet.
6. Slowly invert closed tubes as beads start to separate to capture any beads from cap.
7. After separation is complete, discard supernatant without disturbing beads.
8. Spin all samples in mini-centrifuge for 3 seconds.
9. Place samples back on magnet and allow 30 seconds to separate.
10. Pipette and discard any excess liquid without disturbing beads.
11. Do not dry beads – continue with next step.

Degrade FLI-Seq probes and elute sgRNA sequence-enriched gDNA fragments

1. Add 59 µL **gDNA elution mix** ("Couple denatured gDNA to beads/probes", step 8) to each sample.
2. Pipette mix thoroughly until combined.
   - **Note:** Add 120 µL for samples with initial total gDNA ≥100 µg
3. Incubate samples in thermomixer at **37°C** for 9 minutes with interval mixing at 1,250rpm.
4. Once incubation is complete, remove samples from thermomixer and place at room temperature.
   - **Preparative Note:** Set thermomixer temperature to **70°C**.
5. Add 4 µL of **2 M NaOH** to each sample. Pipette to mix.
   - **Note:** Add 8 µL for samples with initial total gDNA ≥100 µg
6. Incubate samples in thermomixer at **70°C** for 10 minutes with interval mixing at 1,250rpm.
7. Once incubation is complete, remove samples from 70°C.
8. Spin all samples in mini-centrifuge for 3 seconds.
9. Place samples on magnet and allow 30 seconds for bead separation.
10. **Transfer** supernatant to a new 1.5 mL LoBind tube.
    - **Note:** This is your first gDNA elution.
11. Resuspend beads in 30 µL **100 mM NaOH**.
    - **Note:** Resuspend in 60 µL for samples with initial total gDNA ≥100 µg

12. Incubate samples in thermomixer at 70°C for 2 minutes with interval mixing at 1,250rpm.
13. Once incubation is complete, remove samples from 70°C.
14. Spin all samples in mini-centrifuge for 3 seconds.
15. Place samples on magnet and allow 30 seconds for bead separation.
16. Transfer supernatant to first gDNA elution for each sample (step 10).
17. Neutralize NaOH by adding 3.67 µL of 3 M HCl to gDNA elution.
    - Note: Add 7.34 µL for samples with initial total gDNA ≥100 µg
18. Pipette to mix and proceed accordingly.
    - Note: Sample solution may become cloudy.
    - Note: Final sample volume will be around 95 µL for samples with total gDNA <100 µg or around 195 uL for samples with total gDNA ≥100 µg

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Optional Stopping Point: *If stopping here, freeze sgRNA sequence-enriched gDNA fragments at – 20°C*

Next stopping point: ~1 hr

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sgRNA sequence-enriched gDNA cleanup with Zymo DNA Clean and Concentrator kit

- Preparative Note: Ensure 100% EtOH is added to the DNA Wash Buffer concentrate upon first usage.
- Preparative Note: Centrifugation steps are done at room temperature.

1. Add 7× volume of DNA Binding Buffer to each sample and pipette mix until combined.
    - Note: For <100 µg initial gDNA samples with final elution volume of 95 µL, add 665 µL DNA binding buffer.
    - Note: For ≥100 µg initial gDNA samples with final elution volume of 190 µL, add 1330 µL DNA binding buffer.
2. Transfer all liquid (up to 780 µL) to corresponding labeled filter-columns in collection tubes.
3. Centrifuge at 7,000 × g for 30 seconds. If residual sample is left, centrifuge until all liquid has passed through filter-column.
4. Double bind the sample by transferring the flow-through from the collection tube back into the filter-column.
5. Centrifuge at 7,000 × g for 30 seconds. Discard flow-through.
    - Note: If sample volume exceeds 780 µL, repeat steps 2-5 until entire sample volume has been double bound to filter-column.
6. Add 250 µL DNA Wash Buffer to each column.
7. Centrifuge at 7,000 × g for 30 seconds. Discard flow-through.
8. Repeat steps 6-7 once for a total of 2 washes.
9. 'Dry' spin at 10,000 × g for 1 minute to remove any residual ethanol.
10. Transfer each filter-column to a new labeled 1.5 mL LoBind tube. Discard used collection tubes.

11. Open columns' caps and allow to air dry for 3 minutes.
12. Elute all samples by adding 12 µL of **Bead Elution Buffer** directly to each filter.
13. Incubate at room temperature for 1 minute.
14. Centrifuge at 10,000 × g for 30 seconds.
15. Perform a second elution by adding 12 µL of **Bead Elution Buffer** directly to each filter.
16. Incubate at room temperature for 1 minute.
17. Centrifuge at 12,000 × g for 1 minute. Discard filter-columns.
    - **Note:** Elution volume will be ~23 µL.
18. If proceeding to next step, store all samples at room temperature.

Optional Stopping Point: *Freeze purified DNA at –20°C*

Next stopping point: ~2-3 hrs

Chapter 4: Amplification of sgDNA sequences

Overview

In this section, sgRNA sequences will be amplified from enriched genomic DNA.
The PCR amplification will be split into two separate iterations: PCR1 with primers flanking sgRNAs, and PCR2 to introduce full-length sequencing adapters.

Consumables

- Agencourt AMPure XP or equivalent (not provided; equilibrate to room temperature before use)
- sgDNA Primers, 20 µM (thaw at room temperature then store at room temperature until use)
- Bead Elution Buffer (thaw at room temperature then store on ice until use)
- 2× PCR Mix (thaw at room temperature then store on ice until use)
- 50(5,6,7,8) Index Primer (thaw at room temperature then store at room temperature until use)
- 70(5,6,7,8) Index Primer (thaw at room temperature then store at room temperature until use)
- Nuclease-free water

Preparation

1. Centrifugation steps are done at room temperature.
2. Prepare fresh 80% ethanol in Molecular Biology Grade water in a fresh 50 mL tube. Store at room temperature for up to 1 week.

Procedure

Determine PCR amplification cycles

1. Determine the total number of PCR cycles required (X) to obtain **100 fmoles** of final library by referring to Table 4 "**PCR cycles estimation table"**, or by using the formula:

$$X = \log_2(120{,}000/N)$$

X = *total number of PCR cycles required between PCR1 and PCR2 for 100 fmoles of library*
N = *number of 10^6 cells used in library prep (for 7 million cells use the number 7 in this formula).*

Table 4. PCR cycles estimation table:

Cell number (estimated by gDNA yield)	gDNA (µg)	Total number of PCR cycles	PCR1 number of cycles	PCR2 number of cycles
1 Million (1×10^6)	6.7	17	9	8
2 Million (2×10^6)	13.5	16	8	8
4 Million (4×10^6)	27	15	8	7
8 Million (8×10^6)	54	14	7	7
> 25 Million (25×10^6)	> 150	12	6	6

- **Example:** For **7×10^6 cells** used in library prep:

$$X = \log_2(120000/7)$$
$$X = \log_2(17143)$$
$$X = 14 \text{ (e.g. the total number of PCR cycles (PCR1 + PCR2))}$$

- **Note:** For incompletely selected cells, polyploid cells, or if you need a greater yield of library; increase the total number of PCR cycles by 1-3.

2. Calculate the number of PCR cycles in PCR1 and PCR2 as follows:
   # of cycles in **PCR1** = **X/2** rounded up.
   # of cycles in **PCR2** = **X/2** rounded down.
   If # of cycles in **PCR2** > 9, then use only 9 cycles.
   If # of cycles in **PCR2** < 6, then use at least 6 cycles for **PCR2** and use X–6 cycles for **PCR1**.
3. Determine total number of PCR reactions needed for amplification: **use one 50 µL PCR reaction per 3×10^6 cells** or **20 µg of gDNA** used in library preparation.
   - **Example:** For 20×10^6 cells that yielded 120 µg of gDNA, split PCR into 6 separate 50 µL reactions by diluting DNA sample with 5× volume of Nuclease-free water and utilizing 23 µL per PCR1 reaction.

PCR1

1. Prepare PCR1 reaction mix according to **Table 5** in a fresh 1.5 mL LoBind tube.
2. Mix then store on ice until use.
   - **Note**: Dilute DNA with Nuclease-free water in order to run no more than the yield from 20 µg starting gDNA per PCR1 reaction.

Table 5. PCR1 reaction mix (per sample):

Reagent	Volume (µL)
Enriched gDNA	23
sgDNA Primers	2
2× PCR Mix	25
Total:	**50**

3. Amplify samples in a thermocycler according to **Table 6**.

Table 6. PCR1 Thermocycler Program

Step	Temperature	Time	Cycles
1	98°C	0:30	1
2	98°C	0:15	≤10
3	67°C	1:00	
4	72°C	1:00	
5	72°C	2:00	1
6	5°C	0:05	

4. Following PCR1 amplification, store samples at room temperature and immediately proceed to next step.

First PCR amplification cleanup

- **Preparative Note**: Allow AMPure XP beads (not provided) to equilibrate at room temperature for 5 minutes.

1. Manually shake or vortex AMPure XP beads to resuspend until homogenous.
2. Add 2× PCR volume of AMPure XP beads to each sample.
3. Pipette to mix until homogenous (e.g. For each 50 µL PCR reaction, add 100 µL AMPure XP beads).
4. Incubate sample at room temperature for 10 minutes with pipette mixing every 5 minutes.
5. Place samples on magnet and allow to incubate for 1 minute or until separation is complete and liquid is transparent.
6. Carefully aspirate and discard supernatant without disturbing beads.
7. Add 150µL of **80% EtOH** without disturbing beads.
8. Move samples to different positions on magnet to wash thoroughly.
9. Carefully add an additional 150µL of **80% EtOH**.
10. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
11. Carefully aspirate and discard supernatant.
12. Repeat steps 7-11 for a total of two washes.
13. Spin capped tubes in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
14. Place samples on magnet.
15. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
16. While on magnet, aspirate and discard all residual liquid without disturbing beads.
17. Allow beads to air-dry for 5 minutes or until beads no longer appear wet and shiny.
    - **Note:** Do not over-dry.
18. Once completely dry, carefully remove tubes from magnet.
19. Add 21 µL **Bead Elution Buffer** to each sample.

20. Pipette mix until sample is homogenous.
21. Incubate for 5 minutes at room temperature.
22. Place samples on magnet.
23. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
24. Carefully transfer 20 µL of eluted sample to a new 0.2 mL PCR 8-well strip tube.

Optional Stopping Point: *Freeze purified PCR1 product at –20°C*

Next stopping point: ~2-3 hr

PCR2

1. Prepare PCR2 reaction mix according to **Table 7** in a fresh 1.5 mL LoBind tube.
2. Mix then store on ice until use.
   - **Note:** PCR reactions derived from the same sample will be pooled after PCR clean up, use the **same primer pair** per sample.

Table 7. PCR2 reaction mix (per sample):

Reagent	Volume (µL)
PCR1 product	20
50(5,6,7,8) Index Primer	2.5
70(5,6,7,8) Index Primer	2.5
2× PCR Mix	25
Total:	**50**

   - **Note:** If samples are multiplexed during high-throughput sequencing, ensure that all samples pooled together have a unique combination of indexing primers.
   - **Note:** Final library pool concentration and conditions are set by high-throughput sequencing provider. See manufacturer manual or Illumina website for additional details.
3. Amplify samples in a thermocycler according to **Table 8**.

Table 8. PCR2 Thermocycler Program

Step	Temperature	Time	Cycles
1	98°C	0:30	1
2	98°C	0:15	≤8
3	70°C	1:00	
4	72°C	1:00	
5	72°C	2:00	1
6	5°C	0:05	

4. Following PCR2 amplification, store samples at room temperature and immediately proceed to next step.

Second PCR amplification cleanup

- **Preparative Note**: Allow AMPure XP beads (not provided) to equilibrate at room temperature for 5 minutes.

1. Manually shake or vortex AMPure XP beads to resuspend until homogenous.
2. Add 1.4× PCR volume of AMPure XP beads to each sample (e.g. For each 50 µL PCR reaction, use 70 µL AMPure beads).
3. Pipette to mix until homogenous.
4. Incubate sample at room temperature for 10 minutes with pipette mixing every 5 minutes.
5. Place samples on magnet and allow to incubate for 1 minute or until separation is complete and liquid is transparent.
6. Carefully aspirate and discard supernatant without disturbing beads.
7. Add 150µL of **80% EtOH** without disturbing beads.
8. Move samples to different positions on magnet to wash thoroughly.
9. Carefully add an additional 150µL of **80% EtOH**.
10. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
11. Carefully aspirate and discard supernatant without disturbing beads.
12. Repeat steps 7-11 for a total of two washes.
13. Spin capped tubes in mini-centrifuge for 3 seconds to draw all liquid to the bottom of the tube.
14. Place samples on magnet.
15. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
16. While on magnet, aspirate and discard all residual liquid without disturbing beads.
17. Allow beads to air-dry for 5 minutes or until beads no longer appear wet and shiny.
    - **Note:** Do not over-dry.
18. Once completely dry, carefully remove tubes from magnet.
19. Add 21 µL of **Nuclease-free water** to each sample.
    - **Note:** If sample was split into multiple reactions, add 21 µL to the first pellet, resuspend, then transfer the 21 µL including beads to the next pellet to combine. Repeat for each corresponding sample.
20. Incubate sample for 5 minutes at room temperature.
21. Place sample on magnet.
22. Incubate on magnet for 30 seconds or until separation is complete and supernatant is transparent.
23. Carefully transfer 21 µL of eluted sample to a new 1.5 mL LoBind tube without disturbing beads.
24. Store samples on ice if proceeding to library quantification.

Optional Stopping Point: *If stopping here, freeze libraries at –80°C*

Next stopping point: ~20 minutes or ~2hrs if pooling immediately

Library quantification

Libraries can be quantified using a variety of methods. Protocol has been optimized using the D1000 ScreenTape for the Agilent4200 TapeStation, which quantifies both library concentration, molarity and size distribution. See Agilent4200 TapeStation manual for operation instructions.

The final library has a single peak around 242 bp and the concentration will be between 5-10 nM.

Table 9. FLI-Seq Library Sequencing Options

Sequencing	Comment
SE50 or SE75 with dual indexes	will *not* read through sgRNA sequence from the 3'-end
SE100 with dual indexes	will read through sgRNA sequence twice, reducing error rate

FLI-Seq libraries cannot be sequenced without addition of PhiX or other high-complexity libraries into the final pool. We recommend addition of 10% PhiX control into your sample.

Provided indexed primers are Illumina HT-indexed primers with the following sequences (index sequence in color):

```
AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGACGCTCTTCCGATCT – 505
AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGACGCTCTTCCGATCT – 506
AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGACGCTCTTCCGATCT – 507
AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGACGCTCTTCCGATCT – 508

CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT – 705
CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT – 706
CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT – 707
CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT – 708
```

Pool and sequence final library

Final library pooling concentration and conditions are typically set by the high-throughput sequencing provider. See provider information or Illumina website for additional details, particularly for color balance for successful sequencing.

Protocol End

Store final library at -80°C and avoid freeze-thaw cycles

APPENDIX A: Genomic DNA Isolation

Required Equipment and Materials

Reagents/Equipment Required
RNase A (100 mg/mL) (QIAGEN cat. #19101)
DNeasy® Blood and Tissue Kit (QIAGEN cat. #69504)
Phosphate buffered saline (PBS) (Corning cat. #21-040-CM)
Ethanol, 200 proof, for molecular biology
Tabletop microcentrifuge
Vortex
Thermomixer
1.5mL DNA LoBind Micro-centrifuge tubes
50 mL conical centrifuge tubes
DNA quantification method

Preparation

- **Note:** This protocol is a modified version of QIAGEN's DNeasy® Blood and Tissue Kit.
- **Note:** Volumes given at each step are recommended for 4 × 10^6 cells. Scale volumes up accordingly based on sample size.
- **Note:** Large samples [over 1 × 10^7 (10 million)] cells **must be** processed in 50 mL conical tubes until use of columns; columns have a capacity of 4 x 10^6 cells.
- **Preparative Note:** Preheat thermomixer with 1.5 mL or 50 mL tube adapter to **50°C**.
- **Preparative Note:** Prewarm **Qiagen Buffer AE** to **37°C**.

Procedure

- **Preparative Note:** If cells were previously frozen, thaw cell pellets before adding PBS.

1. Resuspend cell pellets in 200 µL PBS for each 4 × 10^6 cells.
   - **Note:** If working with 20 million (2 × 10^7) cells, use 1 mL PBS.
2. Add 18 µL **Proteinase K** for each 4 × 10^6 cells. Vortex well for 5 seconds.
   - **Note:** If working with 20 million (2 × 10^7) cells, use 90 µL of Proteinase K.
3. Add 4 µL **RNase A 100 mg/mL** for each 4 × 10^6 cells**.** Vortex for 15 seconds to mix and incubate for 10 minutes at room temperature.
   - **Note:** If working with 20 million (2 × 10^7) cells, use 20 µL of RNAse A.
4. Add 200 µL **Buffer AL** for each 4 × 10^6 cells in two increments as follows:
   - **Note:** For 20 million (2 × 10^7) cells, add 1 mL Buffer AL, 500 µL at a time.

   4.1 Add **half** of the total volume of Buffer AL. Close tube immediately and mix thoroughly by vortexing for 20 seconds.

4.2 Add remaining volume of Buffer AL. Close tube immediately and mix thoroughly by vortexing for 20 seconds.

- Critical step: Solution should be homogeneously cloudy **without** visible chromatin precipitates.

5. Incubate samples at **50°C** for at least 30 minutes with interval mixing at 1,250rpm.
   - **Note:** For large samples with volumes exceeding 1.5 mL, keep samples in 50 mL conical tubes.
   - **Note:** Lysate should be clear following incubation. Lysate may be slightly yellowish. If solution is not clear, continue 50°C incubation on thermomixer for another 15-30 minutes. Vortex every 5-10 minutes.
6. Add 200 µL **100% EtOH** to lysate for each 4 × 10^6 cells. Mix thoroughly by vortexing.
   - **Example:** If working with 20 million (2 x 10^7) cells, use 1 mL of 100% ethanol.

Double bind sample to columns

1. Place one column for each 4 × 10^6 cells into a clean 2 mL collection tube.
   - **Example:** If working with 20 million (2 x 10^7) cells, you will use 5 columns.
2. Transfer all liquid (up to 700 µL) of lysate-ethanol mixture to corresponding labeled filter-columns in collection tubes.
3. Centrifuge at 6,000 × g for 1 minute. If residual sample is left, centrifuge until all liquid has passed through filter-column.
4. **Collect flow-through** by transferring to a new 1.5 mL LoBind tube, or pool flow-throughs from each sample into a 50 mL conical if multiple columns were processed.
5. Carefully add an additional 100 µL of **100% EtOH** for each 4 × 10^6 cells to the sample flow-through from the previous step and vortex thoroughly.
6. Bind this flow-through to the column a second time by transferring 700 µL of liquid to corresponding labeled filter-columns in collection tubes.
7. Centrifuging at 6,000 × g for 1 minute. If residual sample is left, centrifuge until all liquid has passed through filter-column.
8. Transfer each filter-column to a new collection tube. Discard used collection tubes.
9. Add 500 µL **Buffer AW1** to column, rinsing interior rim of column to remove salt.
10. Centrifuge at 6,000 x g for 1 minute.
11. Place column in a new collection tube. Discard flow-through and used collection tube.
12. Add 500 µL **Buffer AW2** to column.
13. Centrifuge at 15,000 × g for 2 minutes.
14. Transfer each filter-column to a new labeled 1.5 mL LoBind tube. Discard used collection tubes.
15. Remove wash buffer from inner rim of column using a P10 pipet and tracing around the inside rim immediately above the filter to remove any residual ethanol.
16. Open columns' caps and allow to air dry for 3 minutes.

Elute sample from columns

17. Elute all samples by adding 67 µL of **prewarmed Buffer AE** directly to each filter.
18. Incubate for 2 minutes at room temperature.
19. Centrifuge at 6,000 × g for 1 minute.
20. Add an additional 67 µL of **prewarmed Buffer AE** directly to filter.
21. Incubate for 2 minutes at room temperature.
22. Centrifuge at 15,000 × g for 2 minutes.
23. Combine elutions from all samples.
    - **Note:** Final elution volume is ~130 µL for each 4 x 10^6 cell sample.
24. If proceeding to next step, store all samples at room temperature.

***Optional Stopping Point*:** *If stopping here, freeze isolated gDNA at –20°C*

Next stopping point: ~1-2 hrs

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gDNA quantification

1. Isolated genomic DNA can be quantified ysing a variety of methods. Protocol has been optimized using Agilent's Genomic DNA ScreenTape, see manufacturers protocol for details.
   - **Note:** Ideal concentration should be between **200-300 ng/µL.**
   - **Note:** To gauge efficiency of DNA isolation, estimate 6.7 pg of DNA per cell for a diploid genome.

     Example: For 4 × 10^6 cells, the total DNA estimated would be:

     $$4 \times 10^6 * 6.7 \times 10^{-12} = 26 \times 10^{-6} \text{ g}$$

     $$= 26 \text{ µg of total DNA}$$

     Efficiency will be:

     Total DNA isolated / Total DNA estimated = % efficiency