**Initial notes**

**\*\*\*Before beginning, please read the entire KAPA Hyper Prep Kit protocol that this document is based on\*\*\***

* What is true for all library preparation methods is just as true here: the vast majority of researcher time and effort should go into obtaining the best quality DNA fragmented to the optimal range. Take your time to get it right here and it will pay off with easier library preparation and better quality data.
* This protocol assumes enough starting material to begin sonication with 1 µg of DNA. However, the Kapa kit is suitable for library construction from 1 ng – 1 μg of double-stranded DNA, so in cases where 1 µg in unrealistic, this same protocol can be used with increased numbers of indexing PCR cycles.
* A refinement of size selection is possible after adapter ligation but it is easiest to get fragments to approximately the correct range before the enzymatic steps of library prep begin.
* The optimal size range of library inserts will vary based on whether data will be de novo assembled, mapped to a reference, or captured. Please consult with the lab manager before making fragmentation decisions.
* SPRI beads and PEG/NaCl solution should only be used at room temperature.
* Fresh 80% ethanol should be diluted each day.
* Always ensure that KAPA Hyper Prep Kit components have been fully thawed and thoroughly mixed before use. The KAPA Hyper Prep End Repair & A-tailing Buffer and Ligation Buffer may contain precipitates when thawed at 4°C. These buffers must be thawed at room temperature and vortexed thoroughly before use.
* Reaction master mixes prepared from the enzymes and buffers for end repair and A-tailing, as well as for ligation, are viscous and require special attention during pipetting. Keep all enzyme components and master mixes on ice as long as possible during handling and preparation.
* Always thaw the adapter stub on ice. Do not warm in hands or leave at room temperature for long periods.
* Safe stopping points: The protocol can be paused after sonication, post-sonication size selection, and post-ligation bead clean-up. Do not stop after end repair.

**Special notes for full-plate reactions**

*A high-throughput protocol is in development, but until that is prepared, here are tips for how to efficiently do this protocol with a full (or nearly full plate)*

Use the Benchsmart for all bead cleaning steps. This instrument is designed to work with 96 tips, but we can manually remove tips from the box when working with samples that are less than a full plate.

In this case, exclusively use the tips to mix during bead cleaning. Do not vortex.

Always be in the habit of orienting plates so that the letters are to the right and the numbers on top. Really, the only big mistake we can make is inverting a plate 180°, so if we are used to always orienting plates in the same direction, we will minimize the chances of that occurring.

Space sonication tubes out in 6 rows on two plates, with an empty row between them. The plates can balance each other in a plate centrifuge for a spin-down. Then use a 200 µL multichannel pipette to transfer 100 µL of each row into the corresponding row of a semi-skirted plate. Samples that have volume below this value can be topped off in the plate to be equal volume with the others. The plate can be frozen until ready to proceed to double-sided selection. Keep the original 0.2 mL tubes in a PCR rack with lid and/or photograph for later reference.

The ERAT mix needs a larger than usual multiplier since volumes are so low. Master mix for a full plate may need a multiplier of 115 or 120. Then distribute 35-36 µL into each well of your strip tube for distribution.

The ligation master mix multiplier can be 105-110 and still have sufficient volume. Distribute 195-200 µL into each well of your strip tube for distribution.

For distributing 2X ReadyMix, add 200 µL into each well of your strip tube. You can return any unused mix to the stock tube if you have excess remaining once it is aliquoted. You can also re-use that same strip tube over multiple plates if its condition remains good.

If setting up the PCR reaction immediately following post-ligation cleaning, use the same tips that just moved 20 µL of your adapter-ligated product off the beads to move 10 µL from there to your PCR plate. Then switch to a clean set of tips to use the Benchsmart to add 5 µL of indexing oligo mix to the PCR reaction plate and mix.

Seal plates with clear or foil sticky mats and use a hard sealing tool to ensure that the seal is thorough. Foil seals can be punctured with a pipette tip which is useful for keeping track of your location when performing QC assays. However, be sure to stretch the puncture hole to be larger than the pipette tip to prevent a vacuum forming that will distort pipetting accuracy.

**Sonication**

**Changes for Mills squirrel project: start with 8 minutes of total sonication ON time (40% amplitude, 15s on/off pulse)**

*Before using the qSonica instrument for the first time, consult with the lab manager for proper training.*

1) In a qSonica 0.2 mL tube, dilute a 100µL aliquot of each sample to 10 ng/μL (by qubit) or 15 ng/µL (by nanodrop) using 1x LTE (10mM Tris, 0.1 mM EDTA), 1x TE or EB(T) (10mM Tris with or without 0.05% tween). Make sure tubes are completely closed. Spin down samples to keep all liquid at the bottom. Keep samples very cold before sonicating. (It is okay to freeze them; then they can be thawed just before beginning sonication.)

[Note: this protocol can use less starting material if 1 µg is not available. It is best to keep the mass of material within each 18 sample batch as similar as possible. The sonication tube volume should always be identical within each batch)

2) Ensure that all tubing is properly connected. Add ~1.5 L of cold deionized water to the qSonica bath compartment (if empty). Turn on the water cooler and wait 5-10 to allow the system to cool to 4**°**C. (If the bath is filled with room temperature water, it will take 15-20 minutes to cool.)

3) Turn the water adjustment dial to “-“ to add water to the reservoir in back. Fill this to ~50% full. The water level inside the bath should be ~2 cm above the titanium horn. If not, add more water to achieve this level.

4) Turn on the power supply (“|” icon on the top right). Ensure that the sonication bath is empty and that the cabinet door is latched closed. Select the “degas” program which will run for 10 minutes.

5) While degassing, load your samples into the blue 18-place tube holder. (If you have < 18 tubes to sonicate, fill empty spaces with blank tubes containing water.) Cover with the white donut and screw on the top section. Leave the assembly on ice or at +4C until ready for use.

6) Sonication times will vary by DNA size, genome size, tube volume, and extraction method. The following is just an example protocol. Before you begin, please consult with the lab manager for the best trial conditions to select based on other recent results. Additional user validated protocols can be found here: <https://www.sonicator.com/pages/publications-and-protocols-chromatin-dna-shearing>

(Note: in all cases qSonica and users report the “Total Sonication ON Time”. A protocol using a pulse such as 15s on/15s off will take twice as long to complete)

7) After degrassing, use the setting on the power supply to set the sonication conditions. Example:  
 Timer = 2:00 (total sonication on time)  
 Pulse = 15s ON / 15s OFF  
 Amplitude = 40%

8) Attach the sample rack to the lid of the sonicator bath. Make sure the lid is plugged in.

9) Assess the bath water level by eye and adjust using the water adjustment dial if it is too high or too low. The water level in the bath should match that of the sample tubes as closely as possible. However, if they are not a perfect match it is better if the water level of the bath is slightly below the water level of the sample tubes. (Otherwise excessive splashing may occur.)

10) Close the cabinet door and use the red start/stop button to start the sonication run. Check that you see only minor splashing during the first ON cycle. (A slight misting is fine, but large droplets are not.) You can adjust the water level while sonication is proceeding if small adjustments need to be made.

11) Partway through then sonication protocol, stop sonication, remove the tubes rotator from the instrument, and vortex the tubes (easiest to do while they are still in the rotator.) Then remove the tubes from the blue 18-place tube holder and spin down any splashing in your tubes. This will result in more even sonication and very little residual HMW DNA. (Note: this means that if you actually want 8:00 minutes of ON time for sonication, set the instrument for 2:00 and run this program x4, spinning down in between each session.)

12) Run the sonicator program again. You may have to make small adjustments to the water level again.

13) After sonication is complete, open the cabinet and remove the sample rack. Spin down tubes and either proceed directly to bead cleaning or store frozen until ready to proceed.

14) When optimizing, after sonicating, take an aliquot (~5 µL) of sample to run on an agarose gel to assess the sizing pattern. This is not necessary once the sonication parameters have been worked out for your project. If you assess some samples in a set but not others, spike in the same amount of liquid you remove (EB or water) to keep the volumes equal.

15) Tips for gel electrophoresis:

* Pour a 1.5-2.0% gel to better visualize smaller fragments
* Use fresh running buffer rather than recycled (which may contribute background which may be difficult to distinguish from your sonicated material
* Flank your sonicated samples on both sides of the row with ladder (instead of just using one). This will make it easier to assess the samples in the middle of the row.

16) With most library types, an ideal sonication result is to have the brightest part of the smear overlap with the 300-500 bp range and to have only trace amounts of material greater than 1000 bp. A good example:A screenshot of a computer

Description automatically generated with medium confidence

In this case further sonication would move some of the 500-1000 bp pieces into the 300-500 bp window, but will also make some of the currently 300-500 bp pieces too small. So, in this example, it is better to just proceed rather than to spend too much time on additional sonication and gel checks. The double-sided bead cleaning will further refine the sizing.

However, in a case like this, it would be best to give most of these samples 1-2 minutes of further sonication time due to how much material is > 500 bp:

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Description automatically generated

17) Once general conditions are worked out for a project, continue with the next set of tubes to sonicate. When you are done for the day, turn off the chiller pump and use the “0” button on the power supply to turn it off. Consult with the lab manager about when and how to empty the water from the bath.

18) Optional: assess all project samples on a gel. (note: only those starting with 500 ng+ will be easily visible.) Add extra sonication time to any that are outliers.

19) Completed samples may be stored at -20C while sonication for a project is on-going.

20) When all samples in set are sonicated to the desired size, spin down and use a multichannel to transfer the same volume from each to a plate or set of strip tubes. 100 µL is ideal but other volumes are fine so long as all samples are the same volume. If any are slightly lower than the others, spike-in water or EB to make all the volumes equal before double-sided selection.

Note: when storing sonication tubes before or after sonication, keep them in a box or rack with a lid. Then store this box/rack in a plastic baggie. That way if it is dropped, all your tubes will stick together and not scatter to the far corners of the lab.

**Post-Sonication Double-sided Size Selection**

The following protocol has been used successfully for a double-sided selection centered around 350bp, using 0.525x for right-side selection and 0.675x for left-side. However, the best bead ratio will be partially dependent on the shearing profile of your samples. Initial testing of size selection ratios on non-essential samples (or with special ladder) is recommended before beginning.

**This protocol follows the notation of an Rx/Lx double-sided clean-up, where R is the right-side ratio and L is the left-side ratio.**

1. Resuspend your aliquot of **low-ratio formula SPRI** (Sera-Mag) bead suspension by inverting or vortexing. Take the bead tube out to warm up to room temperature (< 30 min)
2. Standardize all reactions/extractions to the same volume (V) by adding water to your samples if necessary
3. Add SPRI bead suspension to samples as follows reaction to start the right-side clean-up:

A) Add an **R-fold volume** of SPRI bead suspension to each (ex: if V=100µL and R=0.525, add 52.5 µL low-ratio formula SPRI beads to each sample).

B) Pipette up and down 10 times to mix well and/or seal the tubes and vortex gently. Ensure that the beads are resuspended and homogenized in the liquid.

C) Let the tubes incubate for **15 minutes** at room temperature.

D) While the samples are incubating, label a new set of 0.2mL strip tubes for the supernatant in step 5.

1. Briefly (0.5s) spin down the contents of the tubes. Place them in a magnetic plate or stand and let sit for **3 minutes** (or until the supernatant is clear) to separate the beads from solution.

**\*\*\*Reminder: when you remove the supernatant from the beads in step 5, keep the liquid. Do not discard it!\*\*\***

1. Carefully pipette the supernatant without removing or disturbing the beads and **move it to the new, empty strip tubes. Be very careful not to carry over any beads at this step.** (The tubes with the beads can then be discarded.)
2. Add **low-ratio formula** SPRI (Sera-Mag) bead suspension to the sample in the new tube as follows to start the left-side clean-up:

A) If the second ratio is L, add an **L - R -fold volume** of low-ratio formula SPRI bead suspension to each reaction based on the original volume

(ex: if the right-side ratio (R) is 0.5x and the left-side ratio (L) is 0.675x, add 0.15 \* 100 µL or 15 µL).

B) Pipette up and down 10 times to mix well and/or seal the tubes and vortex gently. Ensure that the beads are resuspended and homogenized in the liquid.

C) Let the tubes incubate for **15 minutes** at room temperature.

1. Briefly (0.5s) spin down the contents of the tubes. Place them in a magnetic plate or stand and let sit for **3 minutes** (or until the supernatant is clear) to separate the beads from solution.
2. Pipette off the supernatant and discard without removing or disturbing the beads
3. Leave beads on magnet and wash with **200 µL** of freshly prepared **80% ethanol** (make a new ethanol dilution before every SPRI clean-up).

Let stand for **at least 30 seconds** and discard supernatant.

1. Repeat step 9.
2. Then use a small volume pipette (such as Rainin 20µL LTS pipette) to remove as much residual ethanol as possible without disturbing the beads. A toothpick can be used to soak up alcohol spots. Let the beads air-dry for **3-5 minutes** at room temperature without caps. (For large sets of samples, you may be able to resuspend the first tubes as soon as you are done removing residual ethanol from the final ones.) Avoid overdrying which appears as cracking in the beads
3. Elute as follows:

A) Remove the tubes from the magnetic rack. Add **10 µL** of **EB** to the wells

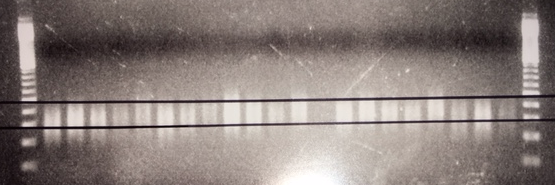
B) Pipette up and down 10 times to mix well and/or seal the tubes and vortex gently. Ensure that the beads are resuspended and homogenized in the liquid.

C) Let the tubes incubate for **10 minutes** at room temperature.

1. Briefly (0.5s) spin down the contents of the tubes. Samples can stay in the bead solution if you do not need to assess them before continuing to end repair.

14) Optional: if concentration assessment is required, place the plate back on the magnetic rack, let stand for **3 minutes** (or until the supernatant is clear) to separate the beads from solution. Then remove 1 μL of clear DNA solution to qubit these samples to know how much DNA is available as input material for End Repair and A-Tailing

15) Optional if sufficient material is available: run an agarose gel with 1 µL of sample in order to check that sizing is correct. (Here an insert size of 300-500 bp was targeted)



17) If optional assessment steps were performed, add water or EB to return the volume of the sample/bead solution to 12.5 µL

**End Repair and A-tailing**

**Changes for Mills squirrel project: use a 1/5th dilution of the Kapa kit**

**Only have the ERAT buffer and enzyme tubes in 4170 (keep the ligation components in the cold room)**

**Double check that you have one buffer and one enzyme tube (if there is more than one, spin down the contents and merge)**

**Check them off below as you add each**

1) While the double-sided selection is in progress, remove the End Repair and A-Tailing reagents from the yellow Kapa kit on the top shelf of the 4122 freezer. Keep the enzyme on ice, and let the buffer thaw at room temperature.

2) Make a master mix composed of:

End Repair & A-Tailing Buffer: 1.4 µL

End Repair & A-Tailing Enzyme: 0.6 µL

For a full plate, use a multiplier of 116 for the following recipe:

* End Repair & A-Tailing **Buffer**: 162.4 µL
* End Repair & A-Tailing **Enzyme**: 69.6 µL

Gently vortex and briefly spin before aliquotting. The master mix can be distributed into a strip tube in order to facilitate adding to samples with a multichannel pipette.

3) Add **2.0 µL of ERAT master mix** to each sample well to make a 12 µL reaction.

4) When adding the ERAT master mix, pipette each well up and down ~2-3 times to mix the eluted bead solution and the ERAT master mix. Pipette gently to avoid introducing excessive bubbles. [**Note for benchsmart users: you can use the final tips form the post sonication bead cleaning to mix instead of doing it manually]**

5) Seal the tubes, gently vortex\*, and briefly spin down in a centrifuge (< 30 s).

*\*Note: to gently vortex, start with the vortex on the lowest setting. Then gradually increase the speed until the liquid starts to dance around and mix but doesn’t tornado violently or move towards the top of the well.*

6) Incubate\* in a thermocycler programmed as outlined below:

20°C: 30 minutes

65°C: 30 minutes

4°C: hold

 \*A heated lid is required for this incubation. For PTC-200 cyclers, set the temperature of the lid at tracking 10 °C above the block. For all others, set the lid temperature to 75°C

7) Return end repair enzymes to the yellow Kapa gDNA box and retrieve the Ligation Enzyme, adapter stub, and Ligase Buffer. Leave the enzyme and stub on ice, and let the buffer thaw at room temperature.

8) After the End-repair and A-tailing reaction is completed and the sample has cooled to 4°C, proceed to Adapter Ligation

**Adapter Ligation**

**Changes for Mills squirrel project: use a 1/5th dilution of the Kapa kit**

**Only have the Ligation buffer and enzyme tubes in 4170 (return the ERAT components to the freezer)**

**Double check that you have one buffer and one enzyme tube (if there is more than one, spin down the contents and merge)**

**Check them off below as you add each**

1) Make a master mix composed of the following and keep it on ice:

Ligation Buffer: 6.0 µL

Ligation Enzyme: 2.0 µL

Nuclease-free water 2.5 µL

\*Adapter stub (50 µM) 1.5 µL

*\*Note: do not add the adapter stub to the ligation master mix until immediately before it will be aliquoted. (The ligase will start to make dimer as soon as the adapter stub is introduced into the master mix, even when kept on ice.)*

The master mix can be distributed into a strip tube to facilitate adding to samples with a multichannel pipette

For a full plate, use a multiplier of 110 for the following recipe:

* Ligation **Buffer**: 660 µL
* Ligation **Enzyme**: 220 µL
* Nuclease-free water: 275 µL
* Adapter stub (50 µM) 165 µL

2) Add **12 µL of Ligation master mix** to each sample well to make a 24 µL reaction.

3) When adding the ligation master mix, pipette each well up and down ~2-3 times to mix the ERAT reaction and the ligation master mix. Pipette gently to avoid introducing excessive bubbles.

4) Seal the tubes, gently vortex (just so that the liquid starts to move around), and briefly spin down in a centrifuge (< 30 s).

*Note: this is just to mix the eluted DNA and the enzyme. The beads do not need to be resuspended for the ligation reaction. They can remain at the bottom of the tube if they have settled there.*

5) Incubate at 20°C for 15-60 min. or overnight at 4°C.

*Note: The overnight incubation may result in higher ligation efficiency, but it may also result in more adapter dimers. However, the 0.67X bead cleaning should do a good job removing most dimer. Overnight ligations are recommended when starting with less sonication input material (< 500 ng), but can also be used when the timing is better to proceed with the Post-Libation Bead Clean-up the next day.*

6) Proceed to the next step. (Post-Ligation Bead Clean-up)

**Post-Ligation Bead Clean-up**

**Changes for Mills squirrel project: the volume is different here (24 µL) so we will reduce the amount of PEG solution added to 16 µL in order to keep the ratio the same (0.666667X)**

1) Before beginning the bead clean-up, warm a tube of PEG/NaCl solution to room temperature for < 30 minutes. If the ligation reactions were in cold storage, warm these to room temperature as well.

2) In the same tubes as the ligation reaction, perform a 0.67X bead-based cleanup by combining the following:

Adapter ligation reaction product: 24 µL

**PEG/NaCl solution** at room temperature: **16 µL**

3) Mix thoroughly by gently vortexing and/or pipetting up and down multiple times.

4) Incubate the tube at room temperature for **15 minutes** to bind DNA to the beads.

5) Place the tubes on a magnet to capture the beads. Incubate **5 minutes** or until the liquid is clear.

6) Carefully remove and discard the supernatant.

7) Keeping the tubes on the magnet, add **200 μL** of freshly prepared **80% ethanol**.

8) Incubate the tubes on the magnet at room temperature for **30 seconds**

9) Carefully remove and discard the ethanol.

10) Keeping the tubes on the magnet, add **200 μL** of freshly prepared **80% ethanol**.

11) Incubate the tubes on the magnet at room temperature for **30 seconds**

12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads. To accomplish this, use a small volume (10µL) tip to remove ethanol remaining at the bottom of the tube. Then use wooden toothpicks to blot up any dots of ethanol on the sides of the tubes.

13) Dry the beads at room temperature for **3 minutes or less**, until all of the residual ethanol has evaporated from the tube but the beads themselves are still damp and shiny. *Caution: over-drying the beads may result in reduced yield.*

14) Remove the tubes from the magnet. Then thoroughly resuspend the beads: in **22 μL** of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) a.k.a. Buffer EB or EBT

15) Incubate the tubes at room temperature for **10 minutes** to elute DNA off the beads.

16) Place the tubes on a magnet to capture the beads. Incubate for **3 minutes** or until the liquid is clear.

17) Transfer **20** µL of the clear supernatant to a new set of tubes and seal.

SAFE STOPPING POINT: samples can be stored here until ready to proceed to indexing PCR

**Indexing PCR**

**Changes for Mills squirrel project: we’ll use 8-10 cycles of PCR depending on the concentration of the samples after dilution for sonication**

Before starting indexing PCR, make a detailed plan of which sample will be matched with which well of the indexing oligo plate and which color plate. Then make a very clear map that you can bring with you into the lab to be certain that the correct library will be matched with the chosen index well. Obviously working in sets of 8 and using the multichannel makes it far less likely that mistakes will occur. For larger projects, use the Benchsmart to ensure correct matching of sample well to indexing oligo mix.

*Note: the GSL indexing oligo plates contain a unique P5 and a unique P7 indexing oligo pre-mixed in each well, both at 5µM.*

1) Take the Kapa 2x Ready Mix and indexing oligo plate out to thaw on top of ice (or leave at +4C for ~30 minutes).

2) Label a new plate for indexing PCR for each sample. If you wish to use up all the library product, set up two parallel reactions for each sample.

3) Add 15 µL Kapa 2x Ready Mix to each well of the empty indexing PCR plate.

4) Then add 10 µL of adapter-ligated product to each reaction well, ideally, add 8 at a time using the LTS 20 µL multichannel. Gently pipette up and down once or twice to mix.

*Note: if you just used the Benchsmart for post-PCR bead cleaning, re-purpose your final set of tips to load 10 µL of adapter-ligated product into the PCR plate and mix.*

5) Finally, add 5 µL of the corresponding indexing oligo plate., ideally add 8 at a time using the LTS 20 µL multichannel. Gently pipette up and down to mix. Then seal tubes well.

6) Briefly spin down the tubes and place in a cycler **with a heating lid.**

7) Amplify using the following cycling protocol:

Initial Denaturation: 98 °C for 45 seconds

Denaturation: 98 °C for 15 seconds \

Annealing: 60 °C for 30 seconds | x 8 - 10 cycles\*

Extension: 72 °C for 60 seconds /

Final Extension: 72 °C for 5 minutes

Hold at 10 °C

\**7 cycles is sufficient for most samples starting sonication with 500-1000 ng. (Don’t use fewer cycles than 6 since that is the minimum to ensure that DNA becomes full-length sequence-ready libraries after indexing PCR.) If you start with less input, Table 3 of the Kapa Hyper Prep kit is a useful resource. Generally, about 25% of initial sonication starting material enters the end repair reaction. So, starting with 200 ng of DNA during sonication, we can estimate that there is ~50 ng remaining after size-selection*

**Post-PCR Bead Clean-up**

1) Before beginning the bead clean up, warm a tube of **low-ratio SPRI bead solution (SeraMag)** to room temperature.

2) In the same tubes as the PCR reaction, perform a 0.6X bead-based cleanup by combining the following:

Indexing PCR product: 30 µL

Water or elution buffer: 70 µL

**Low-ratio SPRI bead solution** at room temperature: **60 µL**

3) Mix thoroughly by gently vortexing and/or pipetting up and down multiple times.

4) Incubate the tube at room temperature for **15 minutes** to bind DNA to the beads.

5) Place the tubes on a magnet to capture the beads. Incubate **5 minutes** or until the liquid is clear.

6) Carefully remove and discard the supernatant.

7) Keeping the tubes on the magnet, add **200 μL** of freshly prepared **80% ethanol**.

8) Incubate the tubes on the magnet at room temperature for **30 seconds**

9) Carefully remove and discard the ethanol.

10) Keeping the tubes on the magnet, add **200 μL** of freshly prepared **80% ethanol**.

11) Incubate the tubes on the magnet at room temperature for **30 seconds**

12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads. To accomplish this, use a small volume (10µL) tip to remove ethanol remaining at the bottom of the tube. Then use wooden toothpicks to blot up any dots of ethanol on the sides of the tubes.

13) Dry the beads at room temperature for **3 minutes or less**, until all of the residual ethanol has evaporated from the tube but the beads themselves are still damp and shiny. *Caution: over-drying the beads may result in reduced yield.*

14) Remove the tubes from the magnet. Then thoroughly resuspend the beads: in **30 μL** (or more\*) of **elution buffer** (10 mM Tris-HCl, pH 8.0 – 8.5, a.k.a. Buffer EB or EBT) or **nuclease-free water**\*\*

*\*Note1: if you know or suspect your samples will all have high yields, eluting in 50 µL or more will help to keep them in preferred range of the qubit/plate reader, bioanalyzer, and GSL*

*\*\*Note2: water is the best choice for most samples that will be captured since for some probe technologies, the concentrated salts could interfere. Use commercially-purified water to ensure that the pH is not too low. If samples will be directly sequenced, EB(T) is best for long-term preservation since it is buffered.*

15) Incubate the tubes at room temperature for **10 minutes** to elute DNA off the beads.

16) Place the tubes on a magnet to capture the beads. Incubate for **3 minutes** or until the liquid is clear.

17) Transfer ~**5 μL** of the clear supernatant to a plate or a set of **low bind/siliconized** tubes and seal. This will be used for quality control assays. (At a minimum you will need 1 µL for qubit or nanodrop, and 2-3 µL for bioanalyzer or agarose gel)

18) The remaining **20 μL+** will be moved to a second set of **low bind/siliconized** tubes or a plate and sealed. This will be where your libraries will be stored until submitting for sequencing. So label these well.

18) The remaining **~5 µL** can be used for quality control. (At a minimum you will need 1 µL for qubit or nanodrop, and 2-3 µL for bioanalyzer or agarose gel)

**Quality control, GSL submission and/or pooling:**

1) Qubit or nanodrop all samples using 1 µL of final library product. If any sample values are too low, they can be re-amplified using IS5 and IS6 oligos.

* Nanodrop is recommended when library quantities are high (> 50 ng/µL) and researchers are pooling the libraries themselves.
* Use Qubit/plate reader when the GSL is pooling since they require that value with your submission

Very little DNA is required for sequencing. The GSL requests 10 µL at 10 nM which is around 4 ng/µL at an average length of 600 bp. However, it may be difficult for us to accurately assess library quantities and sizing that are so low.

2) To reamplify any samples that are too low:

a) Set up the following master mix:

25 µL 2x Kapa HiFi HotStart ReadyMix

5 µL 10x Kapa primer mix (or 2.5 µL each of IS5 and IS6 at 10 µM)

20 µL final library product

b) Use the same indexing PCR cycler conditions at 2 or more cycles, depending on how many are required to achieve a more robust product but not overly amplify the libraries.

c) Clean using the 0.6X low-ratio SPRI bead protocol

3) If submitting to the GSL for pooling, run all samples on a bioanalyzer DNA 1000 chip. Any samples with an adapter peak around 150 bp may need to be re-cleaned. Use the region table to determine the average size and molarity of the library hill.

If self-pooling, running 50-100 ng of library product on an agarose gel will usually suffice in assessing general sizing and whether libraries are free of dimer.

4) Optional: prepare samples to be pooled for capture or direct sequencing by calculating the amount needed to combine equimolar amounts of each library per pool.

*Note: if all libraries have roughly the same size distribution, mass (total ng) can be a proxy for molarity if you assessed by gel rather than bioanalyzer.*

5) Chat with the lab manager for recommendations on pooling