LIBRARY PREPARATION & GENE CAPTURE

reporter： Lifang PENG

2019.01.07

N.B.

It is very important to avoid potential cross-contamination. The following tips should be followed:

- Clean your bench with bleach (84) every time after you did DNA extraction or fish dissection. Clean your pipettors, rack and bench space with bleach once a while.
- Portion all reagents into appropriate amount, so not to contaminate the whole tube of reagents.
- Never use tips without filter.
- **Centrifuge the tube every time before you open a lid.**

- When pipetting, do it smoothly. Keep the tip submerged just under the solution, so not push the liquid to overflow.
- When adding samples, set the tip against the wall of the tube.
- Move your tubes to an up row or switch the orientation of your tubes after adding samples to keep track which one has been added to.
- Keep focused

Library Prep Using the “With-Bead” Method

Target'enrichment'–'library'preparation'

1). Shearing the genomic DNA

2). Blunt-end repair

3). Adapter ligation

4). Fill-in

5). Pre-hybridization PCR “with-beads”

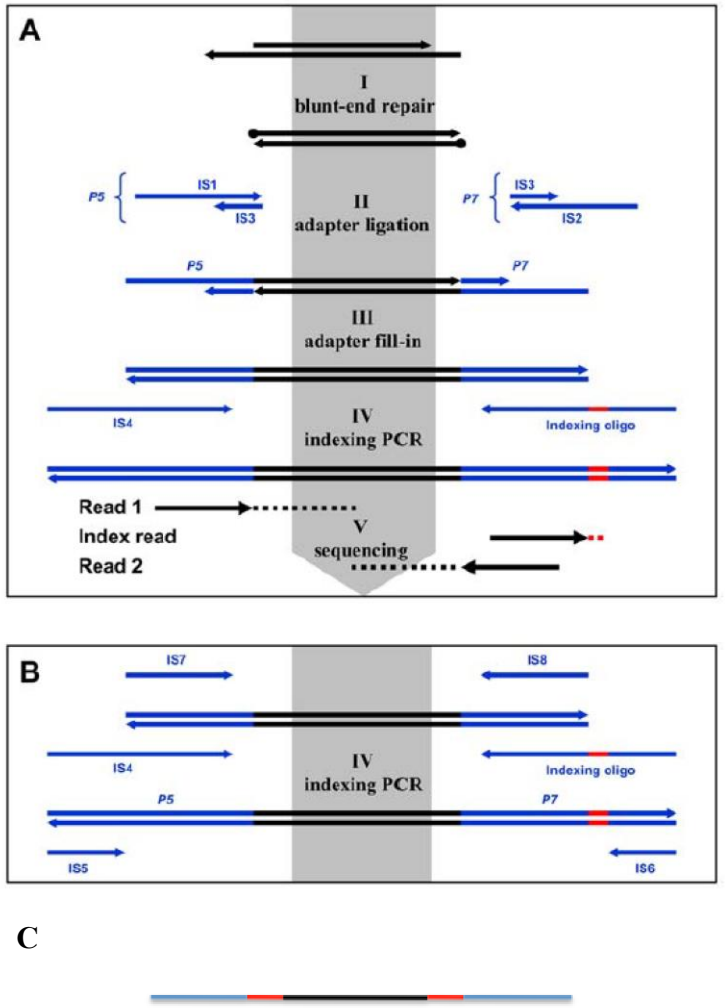


Fig.2. Schematic overview of the protocol and alternative amplification schemes (Meyer and Kircher, 2010). C shows the inline index (red).

DNA Clean-Up Using MagNA Beads

sheared samples： MagNA buffer= ？

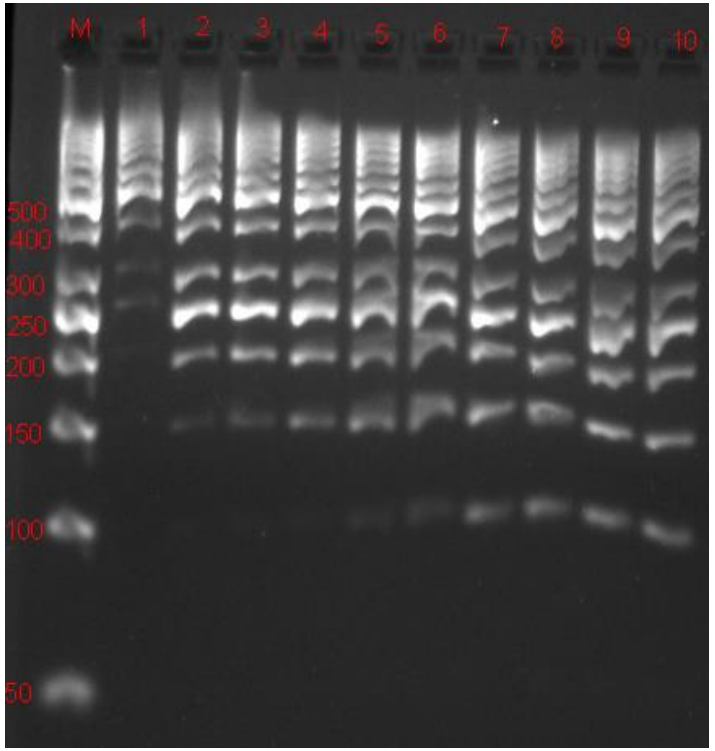


Fig.1. 4% agarose gels showing DNA ladder cleaned up with different amount of MagNA Buffer added

lane descriptions are listed in this table

Lane	1	2	3	4	5	6	7	8	9	10
Sample(μL)	20	20	20	20	20	20	20	20	20	20
MagNA buffer (μL)	10	15	18	24	30	36	42	48	54	60
MagNA buffer/Sample	0.5x	0.75x	0.9x	1.2x	1.5x	1.8x	2.1x	2.4x	2.7x	3.0x

DNA Clean-Up Using MagNA Beads

sheared samples： MagNA buffer= ？

1). Shearing the genomic DNA

microTUBE

regular pcr tubes

microTUBE holder

Covaris M220

1). Shearing the genomic DNA

Table 1. Parameters for shearing DNA using 0.2 ml PCR tubes.

Lane	PIP	DF (%)	CB	D (sec)	Repeats	TUBE Holder
1	50.0	20	200	60	2	altered
2	**50.0**	**25**	**200**	**60**	**2**	**altered**
3	50.0	30	200	60	2	altered
4	50.0	25	200	90	1	altered
5	**50.0**	**25**	**200**	**90**	**2**	**altered**
6	Control, using microTUBE and default parameters for 500bp DNA					unaltered
7	Control, unsheared DNA					
8	50.0	25	200	90	2	unaltered
9	**50.0**	**30**	**200**	**60**	**2**	**unaltered**
10	**50.0**	**35**	**200**	**90**	**2**	**unaltered**

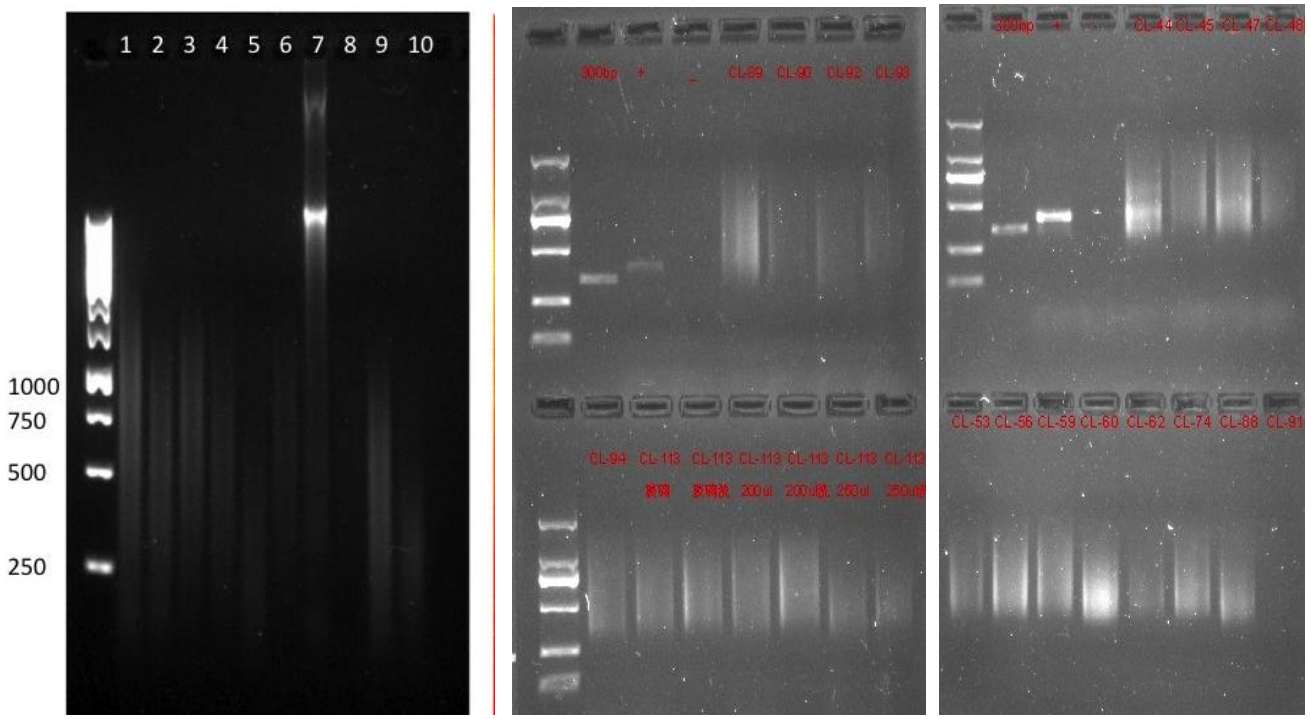


Fig. 2. Size of sheared DNA with different parameter setting and microTUBE holder (left); libraries made from sheared DNA (right).

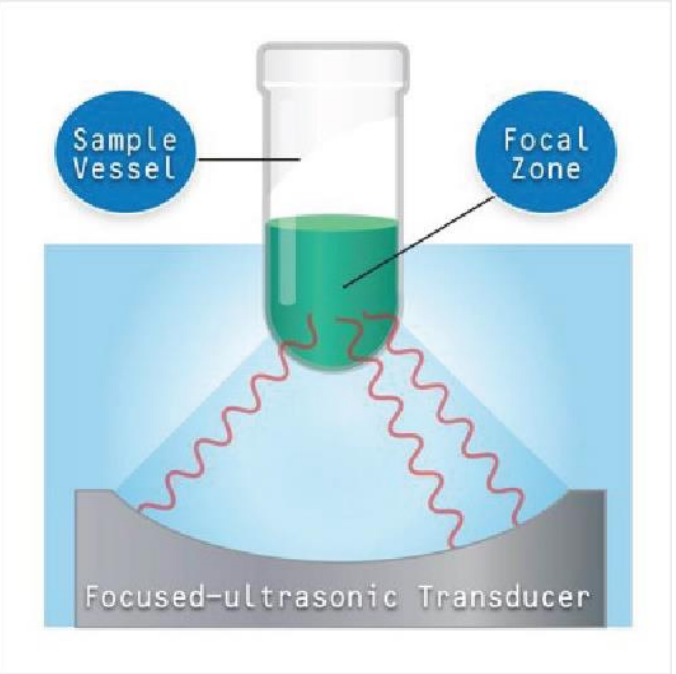
1). Shearing the genomic DNA

1. Add 270 μL samples to the full tube for each shearing. (0.3 – 1 μg genomic DNA)

2. Set the temperature as 18 – 22 ˚C.

3. Add a 30 second delay between any repeat treatments.

4. We choose the parameters used in lane5 for shearing DNA to 250bp, the parameters used in lane2 for shearing DNA to 500bp using the **ALTERED** microTUBE Holder (table 1, Fig. 2)



Skip the shearing step if using samples with highly degraded DNA.
e.g., eDNA, ancient DNA

1. Check the size distribution of the sheared DNA on a mini agarose gel.

2. Dry down the sheared DNA using MagNA beads the protocol “DNA Clean-Up Using MagNA Beads”.

2). Blunt-end repair

1. Prepare a master mix for the number of samples needed as the follow. Add 20 µL of the master mix to each sample.

Reagent	Volume (µL) per sample × *n*	Final concentration in 20-µL reaction
Buffer Tango (10×)	2	1×
dNTPs (10 mM each)	0.2	100 µM each
ATP (100 mM)	0.2	1 mM
T4 polynucleotide kinase (10 U/µL)	1	0.5 U/µL
T4 DNA polymerase (5 U/µL)	0.4	0.1 U/µL
H_2O	16.2	

2). Blunt-end repair

2. Incubate the samples in a thermal cycler for 15 min at 25 °C followed by 5 min at 12 °C.

3. Clean up the reaction according to the MagNA beads protocol. Keep the dried beads.

***Proceed immediately to the ligation step.**

3). Adapter ligation

1. Prepare a master mix for the number of samples needed. Add 40 µL of the master mix to each sample tube. Mix the samples well by pipetting.

Reagent	Volume (µL) per sample	× *n*	Final concentration in 40-µL reaction
T4 DNA ligase buffer (10×)	4		1×
PEG-4000 (50%)	4		5%
Inline Adapter mix IS1 (50 µM each)	1		2.5 µM each
Inline Adapter mix IS1 (50 µM each)	1		
T4 DNA ligase (5 U/µL)	1		0.125 U/µL
H_2O	29		

3). Adapter ligation

2. Spin down the liquid by brief centrifugation, then incubate for 30 min at 22°C in a thermal cycler.

3. Cleanup the reaction using the MagNA bead method. Keep the dried beads.

*** Proceed immediately to the next step.**

4). Fill-in

1. Prepare a master mix for the number of samples needed.

Reagent	Volume (µL) per sample	× *n*	Final concentration in 40-µL reaction
Bsm buffer (10×)	4		1×
dNTPs (10 mM each)	1		250 µM each
Bsm polymerase, large fragment (8U/µL)	1.5		0.3 U/µL
H_2O	33.5		

4). Fill-in

2. Add 40 μL of master mix to the samples. Incubate the samples for 20 min at 37 °C.

3. Cleanup the samples using the MagNA beads method.

4. Add 20 μL of TE buffer to each sample tube, keep the beads within the tube. Transfer the sample with the beads to a new tube labeled as “sample name + lib”.

5). Pre-hybridization PCR “with-beads”

1. Prepare a master mix as the follow for the number of samples needed.

Reagent	Volume (μL) per sample	× *n*	Final concentration in 25-μL reaction
KAPA HiFi taq Ready Mix (2×)	13.75		1×
Primer IS7 (10 μM)	0.55		0.3 μM
Primer IS8 (10 μM)	0.55		0.3 μM
Total	14.85	***No need to prep mix for extra samples***	

Put the empty sample tubes on an **iced box**. Add 13.5 μL of master mix to empty tubes, and then add 11.5 μL of “lib” samples. Mix well.

5). Pre-hybridization PCR “with-beads”

2. Amplify the samples using the following thermal profile:
   98 °C for 45 sec, **12 ~ 18** cycles of 98 °C for 15 sec, 60 °C for 30 sec and 72 °C for 45 sec, then followed by 72 °C for 1 min, and hold at 4 °C for 10 min.

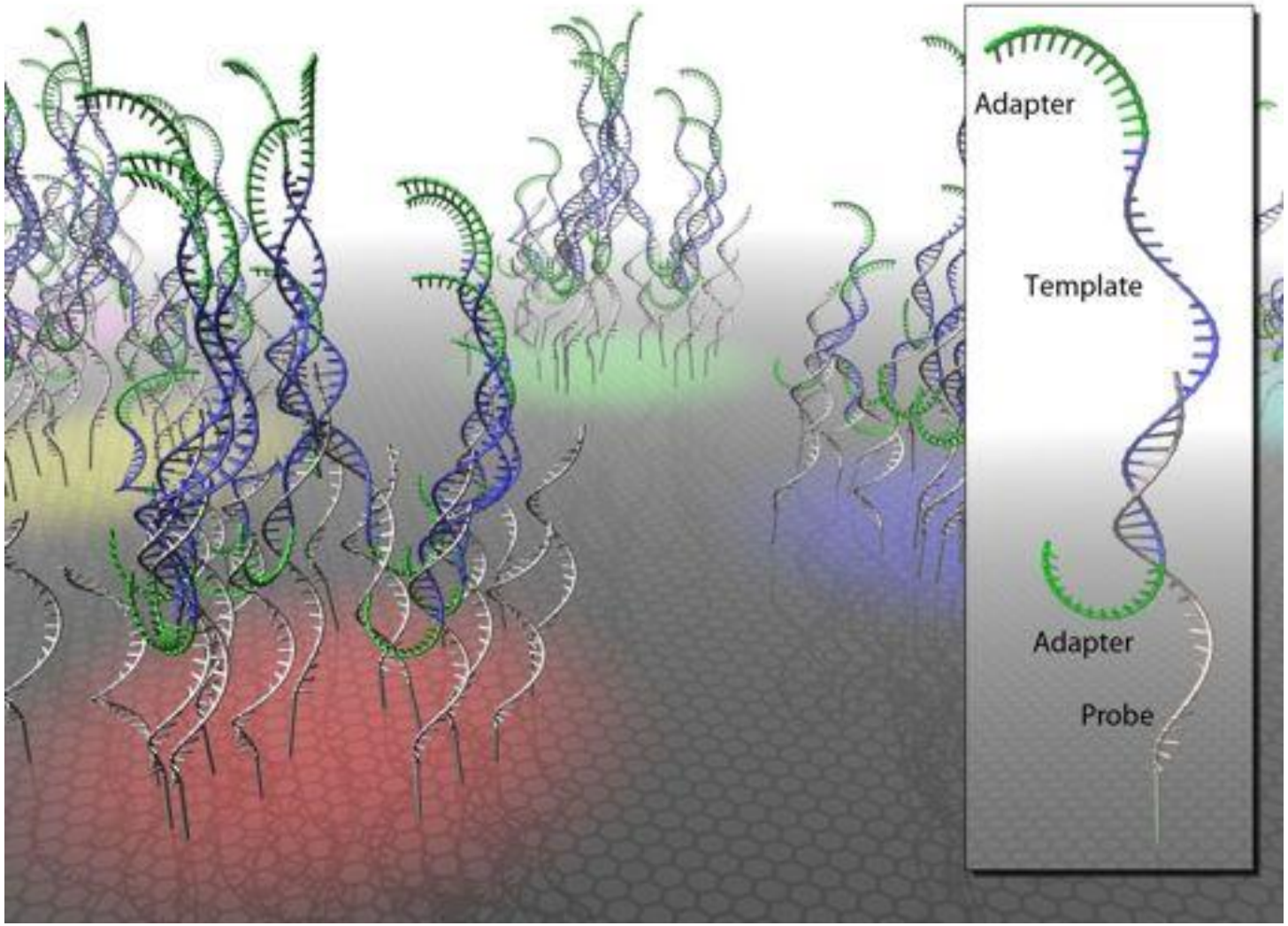
3. Cleanup the PCR product using the MagNA bead method. Elute the DNA using 25 μL of TE buffer and transfer the supernant to a new tube labeled as “sample name + preH”.

4. Gel electrophoresis to check the amplified products, use 1 μL product.

5. Measure the concentration using Nanodrop3300. Store the preH product in -20 C.

Gene capture

What is gene-capture?

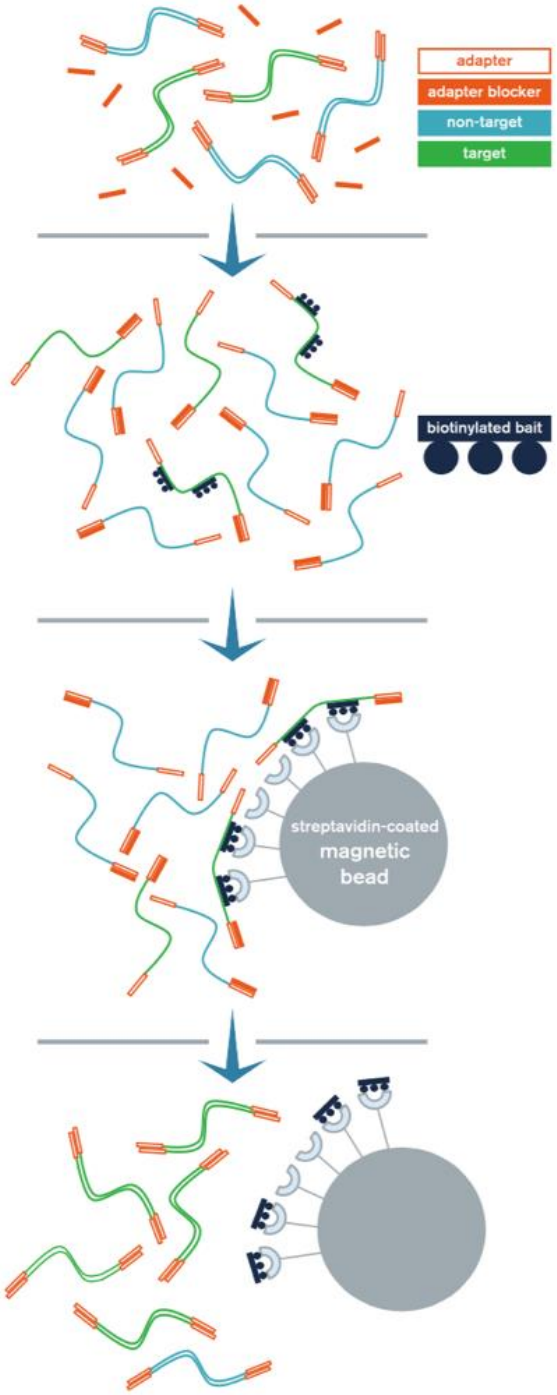


1. Sequencing library, adapter blockers , and other hybridization reagents are combined

2. Libraries are denatured and cooled to allow blockers to hybridize to adapters, and then baits are introduced and allowed to hybridize to targets for several hours

3. Bait-target hybrids are bound to streptavidin-coated magnetic beads and sequestered with a magnet

4. Most non-target DNA is washed away, and the remaining library is amplified



Gene capture

- Hybridization
- Bind to beads and wash
- Post-hybridization indexing PCR

Hybridization

1. Set the following program on a thermal cycler:

95 °C → Hybridization Temp. → Hybridization Temp.

5 min | 5 min | forever

According to “myBaits-Manual-v4

Hybridization

2. Prepare lib master mix as follow for the number of samples needed:

Reagent	× *n*	Volume (µL) per sample
Block#1/Human Cot1 (1 µg/µL)		2.5
BO1.P5short.F (200 µM)		0.25
BO3.P7.part1.F (200 µM)		0.25

Add **3 µL** of the mixture and add **7 µL** of "PreH" sample (adjust according to conc., usually 100ng ~ 500ng in total) to each tube

Set aside in a refrigerator until step 5

Hybridization

3. Prepare Hybridization master mix for the desired number of samples as follow:

Reagent	× *n*	Volume (µL) per sample
HYB#1/20X SSPE		10
HYB#2/0.5 M EDTA		0.4
HYB#3/50× Denhardt's		4
HYB#4/1% SDS		4

Set aside in a refrigerator until step 5.

Hybridization

4. Prepare the bait mix for the number of samples needed as follow:

Reagent	× *n*	Volume (µL) per sample
SUPERase•In (20U/µl)		1
RNA Baits (MYselect) *** don't dilute**		1
H_2O		4

Set aside until step 5.

Hybridization

5.

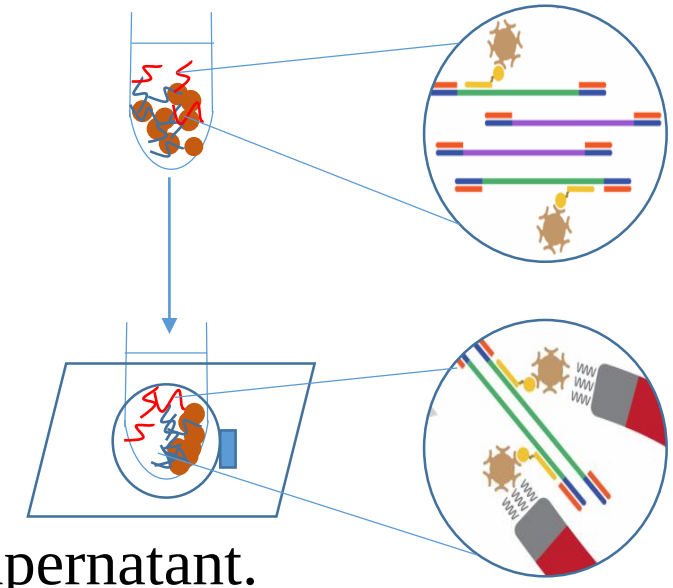
95 °C → Hybridization Temp. → Hybridization Temp.

5 min | 5 min | forever

Bind to beads and wash

- Please don’t stop the pcr program, we will still use it.
- Open the hybridization machine before you do the experiment.

Bind to beads and wash



1. Add *n* × 10 μL of MyOne beads to 200 μL tubes.
2. Pellet beads using a magnetic particle stand and discard the supernatant.
3. Add 200 μL Binding Buffer to beads to wash.
4. Repeat step 3 twice for a total of three washes.
5. Resuspend the beads in *n* × 20 μL Binding Buffer, add 1 μL 10% Tween.
6. Add 180 μL Binding Buffer to empty tubes labeled with sample names, and then add 20 μL resuspended beads to those.

Bind to beads and wash

7. Incubate for 2 min at last hybridizing temp, e.g., 50 °C.
8. Transfer the hybridization solution to the Binding Buffer/Beads and incubate 30 minutes at last hybridizing temp, e.g. 50 °C on a rotator.
9. Add 186 µL 50 °C Wash Buffer 2 to the beads, mix the beads gently using pipettor. Incubate for 10 minutes at 50 °C on a thermal cycler.
10. Repeat step 9 two times for a total of three 50 °C washes.
11. Wash one more time with TE buffer at room temperature.
12. Add 35 µL TE buffer to beads, label as “sample name + 1st cap”. Put in -20 °C freezer.

Pre-hybridization indexing PCR (for the second Gene Capture)

1. Prepare a master mix as the follow for the number of samples needed.

Reagent	Volume (µL) per sample	× *n*	Final concentration in 25-µL reaction
KAPA HiFi taq Ready Mix (2×)	13.75		1×
Primer IS7 (10 µM)	0.55		0.3 µM
Primer IS8 (10 µM)	0.55		0.3 µM
Total		***No need to prep mix for extra samples***	

Do a second gene capture use the product of “sample name + preH2” as template.

Post-hybridization indexing PCR

1. Prepare a master mix as the follow for the number of samples needed.

Reagent	Volume (µL) per sample	× *n*	Final concentration in 25-µL reaction
KAPA HiFi taq Ready Mix (2×)	13.75		1×
Primer IS4 (10 µM)	0.55		0.2 µM
Total		***No need to prep mix for extra samples***	

Add 13 µL of master mix, 0.5 µL P7 indexing primer and 11.5 µL well-mixed.

2. Cleanup the PCR product using the MagNA bead method. Elute the DNA using 25 µL of TE buffer and transfer it to a new tube labeled as “sample name + Ind”.

3. Load 1 µL of PCR product to a mini agarose gene to check the size of the captured library.

Pooling multiple samples for sequencing

1. Determined the DNA concentration of “Ind” samples using Nanodrop 3300. **Accuracy is very important for pooling samples equimolarly.**

2. Pool all samples in equimolar ratios.

3. Quantify the pooled library using nanodrop 3300 before sequencing.

Thanks !