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Prepare Samples for Miseq

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ABSTRACT

(MiSeq nano V2 chip 300 cycle), MiSeq multiplexes sequences, allowing you to DNA sequence multiple samples at once. This is particularly useful for genotyping fish with mosaic genotypes, such as F0 knockouts.

@FishFloorUCL

Design Miseq Primers

1 Design primers surrounding your site of interest

- Optimal amplicon size **200bp**
- As far as possible, the site of interest should be in the middle of the designed amplicon. (This is so that it has the best chance of being read by both the forward and reverse reads)
- If possible, try and avoid designing primers in non-coding regions of DNA since these regions

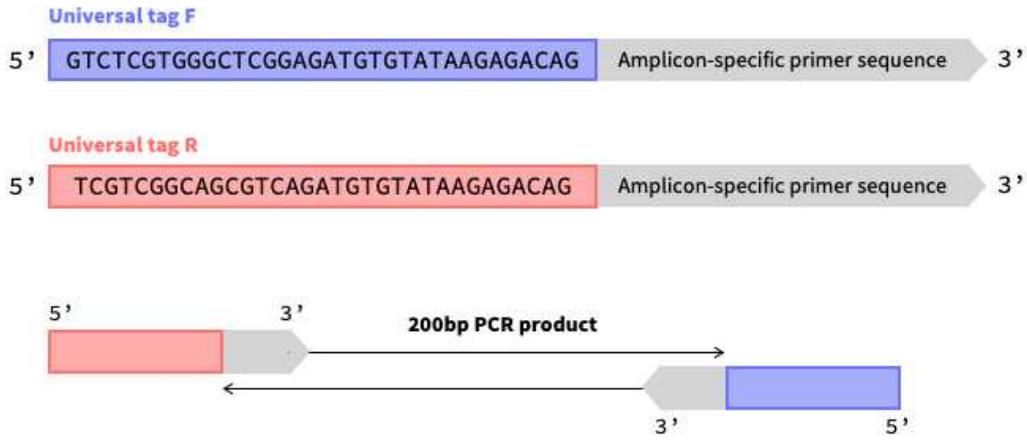
are more likely to have variation and SNPs, which might affect the efficacy of your primer.

I usually use the [NCBI Primer-Blast] tool. Enter ~300bp of DNA sequence around your region of interest under 'PCR Template' and set the 'Range' for the forward and reverse primer so that your site of interest is more or less in the middle of the PCR product.

If you previously used a set of primers to check your TALLEN/CRISPR efficiency using Melt Curve Analysis, or if you have your primer sequence from CHOPCHOP, you can use the same sequence to design the primers for MiSeq. Anecdotally this seems to work well for most people on their first try.

2 Add miseq tags/overhangs to your primer sequence.

- Universal forward (F) tag: 5' **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG** 3'
- Universal reverse (R) tag: 5' **GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG** 3'



These overhang sequences correspond to the Illumina nextera transposase overhangs and will be compatible with Illumina kits using Nextera XT

3 (Optional) Test primers (including tag) for secondary structure formation (e.g., dimers and hairpins) using this online IDT OligoAnalyzer tool

NB. It is unlikely that you will have high scores due to the length of the primers and amplicon restrictions. Just pick the best

4 Order primers - standard desalted primers are fine for this application.

5 If the primers arrive as dry pellets, reconstitute the primers in autoclaved distilled or nuclease-free water to create a stock solution and working solution.

- [M] 100 micromolar (µM) **stock solution** by adding nuclease-free water. If x = nMoles of your primer (check the tube), resuspend the dry pellet in 10x µL of water
- [M] 10 micromolar (µM) **working solution**

(Optional) Test Primers

11m 30s

6

11m 30s

Note

Testing your primers is optional. Although, it is recommended, especially if you are having trouble getting your PCR to work or if you have a lot of samples and want to ensure your primers work before running the entire experiment.

For optimal Miseq results, it is best to use a high-fidelity polymerase. However, at this stage, you can use a cheaper polymerase. Feel free to use a DNA polymerase and PCR protocol that works for you and follow the instructions with the kit. I usually use standard *Taq DN*A Polymerase.

Taq DNA Polymerase recombinant **Invitrogen - Thermo Fisher Catalog #10342**

Run a PCR

I have designed a handy **master mix calculator** for *Taq*, Platinum *Taq* and Pre-mix Schier PCR mix.

Here is an example of a PCR mix for a single well with a 10 µL **total volume**

	A	B
	Autoclaved, distilled (nuclease free) water	6.46 µL
	10X PCR Buffer, -Mg	1 µL
	50 mM MgCl2	0.3 µL
	10 mM (each) dNTP Mix	0.2 µL
	10 µM forward primer	0.5 µL
	10 µM reverse primer	0.5 µL
	Taq DNA Polymerase	0.04 µL
	Template DNA (from 100µL HotShot)	1 µL

Note, *Taq* is unstable and should be taken out of the freezer for the shortest amount of time possible. It is sensible to prepare PCR mix on ice.

Suggested PCR programme:

1. 95 °C , 00:05:00

2. 95 °C , 00:00:30 *(Denaturation step)*
3. Temperature gradient* around 60 °C , 00:00:30 *(Annealing step)*
4. 72 °C , 00:00:30 *(Extension step)*
5. Repeat steps 2-4, 25-35 times
6. 72 °C , 00:05:00 *(Final extension step)*
7. 10 °C hold

*For your test run, you should use a gradient PCR to test the ideal temperature for your primers (Note. By default, CHOPCHOP designs primers for an optimal melting temperature of 60°C). We usually run a gradient PCR for five different temperatures, with 60°C being optimal, then varying 1°C-1.5°C above and below.

7 Run on a 1% agarose gel

Expected result

Assuming a 200bp amplicon, you should expect a final PCR product of 267bp on your gel. If you don't see a band in the expected region, you may need to re-design your primers.

Prepare samples for miseq

11m 30s

8 **Amplify up PCR products** to analyse by MiSeq.

11m 30s

Note

Note **you can only submit up to 96 samples per amplicon** on one MiSeq run.

We recommend using a high-fidelity **Taq polymerase**. We recommend using Taq DNA Polymerase Platinum **Invitrogen - Thermo Fisher** or Phusion® High-Fidelity DNA Polymerase from New England Biolabs (M0530), but any high-fidelity polymerase will do.

I have designed a handy master mix calculator.
Here is an example of a PCR mix for a single well with 10 µL total volume

	A	B
	Autoclaved, distilled (or nuclease free) water	7 µL
	10X PCR Buffer, -Mg	1 µL
	50 mM MgCl2	0.3 µL
	10 mM (each) dNTP Mix	0.2 µL

	A	B
	10 µM forward primer	0.2 µL
	10 µM reverse primer	0.2 µL
	Platinium Taq DNA Polymerase	0.1 µL
	Template DNA	1 µL

Note, *Taq* is unstable and should be taken out of the freezer for the shortest amount of time possible. It is sensible to prepare PCR mix on ice.

Suggested PCR programme:

1. 95 °C , 00:05:00 *(Initial Denaturation Step)*
2. 95 °C , 00:00:30 *(Denaturation step)*
3. 60 °C * 00:00:30 *(Annealing step)*
4. 72 °C , 00:00:30 *(Extension step)*
5. Repeat steps 2-4, 25-35 times
6. 72 °C , 00:05:00 *(Final extension step)*
7. 10 °C hold

*Or T_m of your given primer pair

9 **Run on a 1% gel** to confirm a single band at the anticipated weight. This is just to check the PCR worked. To save time, just run a couple of your samples.

10 Clean up your samples

30m

Traditional column-based PCR clean-up kits would work fine, but we recommend using ExoSAP, ExoSAP-IT™ PCR Product Cleanup Reagent **Thermo Fisher Catalog #78201.1.ML** . Although it is more expensive (~£30 per plate), it is much more practical, especially for many samples.

1. Transfer 5 µL of your PCR product into a new plate
2. Add 2 µL of ExoSAP reagent to each well
3. (Vortex optional) Spin down & run the ExoSAP programme thermocycler.
   - 00:15:00 at 37 °C – Degrade the remaining primers and nucleotides
   - 00:15:00 at 80 °C – Inactivate ExoSAP

11 **Check the concentration of your clean PCR product.** This can be done using Nanodrop or Qubit. I would recommend Qubit due to its superior accuracy and reliability. We recommend only testing a few of your samples to save time and money.

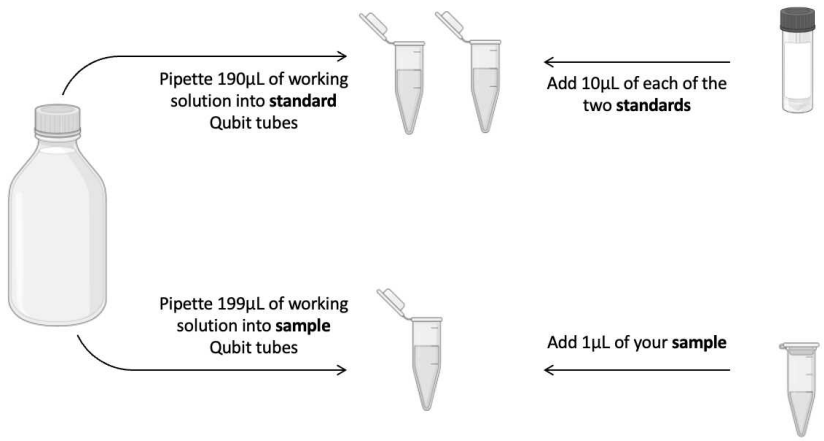
We usually use the following kit from Thermo Fisher, which comes with a working solution.

Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33231**

Setup and label the lids of the required number of assay tubes for your **samples** plus **two standards** (Use thin-wall, clear, 0.5-mL Qubit Assay PCR tubes (Cat. No. Q32856)).

1. Add 190 µL Qubit™ 1X dsDNA working solution to each **standard** tube
2. Add 10 µL of each Qubit standard to the appropriate **standard** tube
3. Add 199 µL Qubit™ 1X dsDNA working solution to each **sample** tube
4. Add 1 µL of each sample to the appropriate tube

All tubes should have a final volume of 200 µL



Read concentration using the Qubit machine and note them down.

12 **Prepare your samples** for miseq:

You need to submit a minimum of 6 µL **at a final concentration of 15-25 ng/µL**

Label your plate/tubes with your name, the date and '*For Miseq*' and leave the samples with the **Payne lab in the Cancer Insititute or in the Fish Facility B03 fridge in the anatomy building**, whichever is most convenient for you. Once you have dropped off your samples, email Alexandra Lubin, letting her know.

Note

You could save time and plastic by combining **clean-up** with dilution to **15-25 ng/µL**.

Step 1: Measure the concentration of the PCR product before clean-up

Step 2: In a new plate/tubes, add your PCR product, ExoSAP and nuclease-free water (if necessary). Volumes will differ according to the concentration of your samples. Calculate how much you need to dilute your PCR product according to the concentration you measured. Add 2 µL ExoSAP for every 5 µL you add of your PCR product and top up with nuclease-free water where needed. The following table shows some examples:

	A	B	C	D
	Concentration	PCR prodoct (vol)	ExoSAP (vol)	nuclease-free water (vol)
	< 15-25 ng/µL	5 µL	2 µL	0 µL
	30-50 ng/µL	5 µL	2 µL	3 µL
	45-75 ng/µL	2.5 µL	1 µL	~6 µL

Step 3: (Vortex optional) Spin down & run the ExoSAP programme thermocycler.

- 00:15:00 at 37 °C – Degrade the remaining primers and nucleotides
- 00:15:00 at 80 °C – Inactivate ExoSAP

The resulting plate/tubes will be ready to submit for miseq.