

ITS2 MiSeq Sample Prep

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This protocol was developed to amplify single sequence products via the MiSeq platform to gauge within-sequence diversity of the ITS2 region. However, this protocol can be applied to any amplicon sequencing application. It is important to start with a large template amount to include all the sequence diversity that exists. Cycle checking avoids over-amplification of products, which can result in anomalous sequences and over/under-representation of specific products. PCR clean-up following the initial amplification removes “short” primers so that the barcoded MiSeq primers can be added without resulting in concatenated products in the second PCR step. Since the identity of each sample is preserved through the barcode sequence, samples can be pooled for sequencing.

Protocol Summary

Step	Sample Volume/Notes
ITS2 initial PCR	3µl of DNA extract, 30 µl total rxn (Can lower to 2µl & 20µl once you get comfortable)
Visualize on gel	Use 2µl of PCR product
PCR cleanup 1	Elute in 30µl
Denovix	1µl for measuring
Normalize all samples to 10 ng/µl (optional)	Variable
Barcoding PCR	2µl of cleaned product, 20µl total rxn
Visualize on gel	Use 2µl of PCR product
Pool samples based on band intensity	Variable
PCR Cleanup 2	Elute in 40µl
Gel extraction	Use taped wells, SYBR Green gel, run 20 µl of library on gel, cut out band
Incubate gel band overnight	20µl of sterile, nuclease free water
PicoGreen for library concentration	Transfer liquid from extraction into new tube. Use 2µl for PicoGreen read out

Overview:

- I. PCR amplification of ITS2 region
- II. Barcoding of ITS2 amplicon
- III. Pooling your samples & gel extraction
- IV. P5-P7 test to check that gel extraction worked
- V. Submit for sequencing!

Protocol:

- I. PCR amplification of the ITS2 region
 1. Dilute your DNA samples down to 10 ng/ μ l each using the same solution your DNA was isolated in.
 2. Get ice.
 3. Collect the following reagents:
 - a. ExTaq enzyme (KEEP ON ICE **ALWAYS** – DEGRADES AT ROOM TEMP)
 - b. dNTPs (2.5mM)
 - c. ExTaq Buffer (10x)
 - d. MilliQ water or molecular grade water
 - e. ITS2 forward primer (stock is 100 μ M)
 - f. ITS2 reverse primer (stock is 100 μ M)
 - i. Make a 10 μ M primer stock that combines the forward & reverse primers by combining the following:
50 μ l ITS2 forward (100 μ M)
50 μ l ITS2 reverse (100 μ M)
400 μ l MilliQ water or molecular grade water
gives you a 500 μ l primer stock of 10 μ M forward + reverse
 4. Put all the reagents on your bench to thaw at room temperature, except for the ExTaq enzyme of course – KEEP ON ICE **ALWAYS**.
 5. While the reagents are thawing, label PCR tubes (the very tiny, attached ones) with all your sample names on the top AND sides (marker can get removed by the PCR heat later).
 6. Once reagents are thawed, mix via flicking or vortexing and then spin down for 2 seconds at your bench.
 - a. Only flick the ExTaq enzyme, don't vortex, it's fragile
 7. Label a new tube “MM” (short for “Master Mix”). This tube needs to be able to hold at least 27 μ l x the number of samples you have.
 8. Write down your PCR ingredient list & then do your math for the recipe, adding in an additional half of a sample for pipette irregularities. If it's your first time, please get a second set of eyes on the master mix math to ensure that you have not made a mistake.

Master Mix Recipe

<u>Reagent</u>	<u>(1x)</u>
H ₂ O	18.6μl
10x ExTaq buffer	3.0μl
2.5 mM dNTPs	2.4μl
10μM F+R primer	3.0μl (can also start with 1μM stock – I find 10μM works better)
<u>ExTaq enzyme</u>	<u>0.15μl</u>

Total: 27μl/reaction plus 3μl (10ng/μl) DNA sample = **30μl total**

Note: this really adds up to 30.15μl but the ExTaq enzyme is negligible so don't math it

Example math for 8 samples:

<u>Reagent</u>	<u>(8.5x)</u>
H ₂ O	158.1μl
10x ExTaq buffer	25.5μl
2.5 mM dNTPs	20.4μl
10μM F+R primer	25.5μl (can also start with 1μM stock – I find 10μM works better)
<u>ExTaq enzyme</u>	<u>1.28μl</u>

Total: 229.5μl total (27μl/reaction x 8.5 reactions)

Note: this really adds up to 230.78 but the ExTaq enzyme is negligible so don't math it

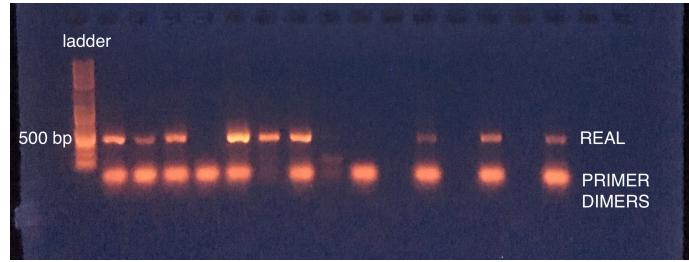
9. Add in each ingredient to your “MM” tube in the order above (least expensive → most expensive just in case you mess up).
10. Once all your ingredients have been added, flick to mix and then spin down.
11. Add 27μl of MM to each of your labeled PCR tubes using the same tip. Be careful not to produce bubbles.
12. Carefully add in 3 μl of your sample DNA (10 ng/μl) ensuring that DNA has entered and left the tip into its correct respective tube. Change tips for each sample.
13. Flick & spin down PCR tubes, do a quick visual check that they all have the same volume.
14. Put PCR tubes in PCR machine.

ITS2 PCR Profile

26x 95°C for 40s
 59°C for 2min
72°C for 1min
 72°C for 7min

15. Check the time that the PCR will finish & set an alarm.
16. While the PCR is running – make a 1% agarose gel.
17. When the PCR is finished – run 2μl of each sample on the gel at 180mV, 270mA for 20 minutes. Take VERY SPECIFIC notes of the order in which you loaded the samples.

18. Take a picture of the gel & upload. Your bands should look like this:
19. If 26 cycles didn't work, try again with 30 cycles. If that didn't work, try again with 35 cycles. NEVER DO MORE THAN 35 CYCLES FOR PCR – NOT REAL AMPLIFICATION.



Most of these amplifications were successful ("REAL" bands). The 4th, 8th, and 9th lanes didn't work & I didn't load anything in the 10th, 12th, and 14th lanes.

Other troubleshooting options:

- Play around with primer concentration. If 10µM stock didn't work well – do a 1µM stock, or vice versa.
 - Try less or more DNA template.
 - Try adding more ExTaq buffer (has good things for PCR in it).
 - Try cleaning your genomic DNA using the Zymo DNA clean & concentrator kit.
 - If all else fails, try re-extracting the DNA from your tissue sample.
20. For each successfully amplified ITS2 sample, clean it using the GeneJET PCR Purification Kit. Elute in 30µl of elution buffer.
 21. (Optional) for each cleaned sample, nanodrop & dilute in elution buffer as necessary to reach 10ng/µl for each sample. If you don't do this step, you can just add 2µl of each sample for barcoding without checking the concentration – you may get less uniform results but it should still work.

II. Barcoding of the ITS2 amplicon

1. Get ice.
2. Collect the following reagents:
 - a. ExTaq enzyme (**KEEP ON ICE ALWAYS – DEGRADES AT ROOM TEMP**)
 - b. dNTPs (2.5mM)
 - c. ExTaq Buffer (10x)
 - d. MilliQ water or molecular grade water
 - e. MiSeq barcoded adapters 96-well plate (1µM)
3. Thaw reagents at room temp, besides keeping ExTaq enzyme on ice. Flick & spin down all reagents. For the MiSeq barcoded adapters plate, **ALWAYS** spin it down in the plate spinning machine before you lift the lid off.
4. Label a new "MM" tube (must be able to hold 15µl x the number of samples you have) & do the master mix math: multiply by number of your samples + ½ of a sample, same as in amplicon PCR. So, if you have 8 samples, multiply the following master mix numbers by 8.5 & add to "MM" tube:

Master Mix Recipe

Reagent	(1X)
H2O	11.4µl
10x ExTaq Buffer	2µl
2.5mM dNTPs	1.6µl
ExTaq	0.1µl

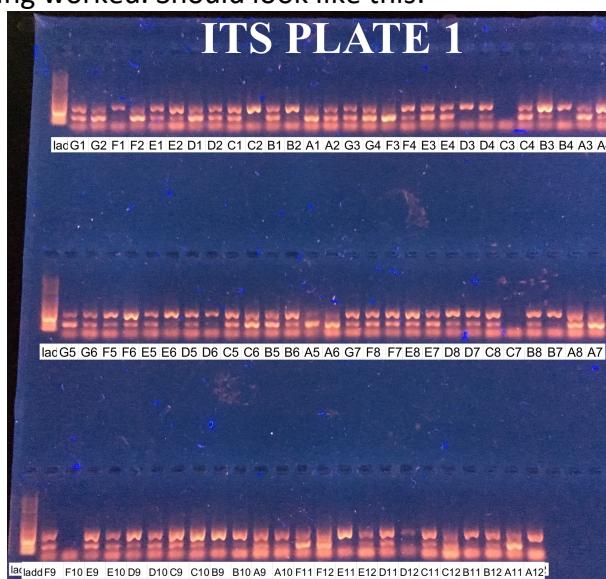
Total: 15µl/reaction (will be 20µl total when you individually add barcodes & amplicon)

5. Grab a new 96-well PCR plate. Make sure you know exactly which samples will be in which wells. Aliquot 15µl from "MM" tube into each well that you will be using for your samples.
6. Use a multi-channel pipette to transfer 3µl of each well of the MiSeq F & R 1µM barcoded adapter plate to the same well on your sample plate. Pipette up & down to mix when you release the adapters into the sample plate.
7. Individually add 2µl of your 10ng/µl (or unknown concentration) ITS2 amplicon to each well carefully.
8. Seal your PCR plate **tightly** with clear plastic lid. Spin down in the plate spinning machine.
9. Start PCR:

Barcode PCR Profile

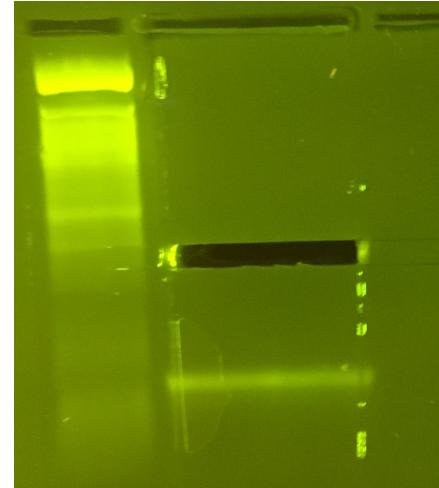
5x
95°C for 40sec
59°C for 2min
72°C for 1min
72°C for 7min

10. Check 2µl of each sample on a gel (can try again with 1-2 more cycles if the band didn't work the first time). The bands will be a bit higher up (*i.e.* heavier) than before if the barcoding worked. Should look like this:



III. Pooling your samples & gel extraction

1. "Eyeball" each barcoded band and decide how much of that sample to pool into final pooled library. A 'normal' band would have 5 μ l of that sample pooled, a 'bright' band would have 3 μ l pooled, and if a band is faint I would pool 10 μ l of that sample. For example, in the gel above, I would say G1 would have 5 μ l of sample added, C2 would have 3 μ l of sample added, and A1 would have 10 μ l added.
2. If you had 96 samples to pool & 5 μ l per sample was pooled on average, you would now have a tube with ~500 μ l of pooled library. Remember that you can't pool more than 96 samples because that's the number of unique MiSeq barcodes we have.
3. Clean 40 μ l of the pooled library using the GeneJET PCR Purification Kit. Elute with 40 μ l of elution buffer. (You run the risk of clogging the column if you clean too much of your pool, so play around with this step for your pool.)
4. Make a 60ml 2% agarose gel, adding 6 μ l of SYBR green (should live in the enzyme box in the freezer). Use a 9-well comb and tape together two wells so you can run more library & cut out the band more easily.
5. Clean out the gel box where you will be running your gel & fill it with brand new running buffer.
6. Start by running 20 μ l of the cleaned pool in each of two lanes on your 2% gel at 70 V for at least 1.5 hours. Illuminate your gel on a blue light box (the UV box will damage your DNA). You may need to run for longer than 1.5 hours if you do not yet have sufficient separation between your target and & other non-specific bands, see right:
 - a. If your band is super smeared, you have too much product, run 10 μ l on the gel next time.
7. Using a sterilized (add 100% ethanol & flame it) razor blade, cut out target band (~500 bp). Use sterilized tweezers to collect in a 1.5ml tube. You should be throwing out a gel that now looks like this on the right:
8. Soak the gel band in 20 μ l MilliQ water (or other nuclease-free water) at 4°C overnight.
 - a. Alternatively, you can use a commercial gel extraction kit.
9. The next day, collect all the liquid that you can in a fresh tube. You can throw out the left behind gel bit.



IV. P5-P7 test to check that gel extraction worked

1. Set up PCR:

Reagent	(1x)
H ₂ O	6.6μl
10x Titaq Buffer	1μl
2.5mM dNTPs	1μl
P5 IC2 10μM	0.2μl
P7 IC1 10μM	0.2μl
TiTaq	0.2μl
Total:	9μl/reaction plus 1μl of library

2. Run PCR:

P5-P7 PCR Profile

95°C for 5 min
12x 95°C for 40sec
63°C for 2min
72°C for 1min

3. Run 2 μl on 1% agarose rose with gel red (180V for 20 min)
4. If successful amplification, PicoGreen for concentration. Protocol for PicoGreen assay is in the TagSeq protocol document.

V. Submit for sequencing!

1. Each genomics center will have their own instructions. I would submit as much library as possible to avoid having to send more. Example spreadsheet for sending to Tufts is available in another document.

Example Primer Sequences

It's fairly simple to create your own barcoded primers. The target amplicon primers are slightly modified to include a linker that any of the remaining barcode primers can bind to. Barcodes can then be used for any amplicon target, so long as amplicon primers are properly modified. Dual barcoding is cost efficient – more samples with less barcodes – ex. 20 forward and 20 reverse barcodes can label up to 400 unique samples. See <https://wikis.utexas.edu/display/GSAF/Illumina+-+all+flavors> for more primer examples and barcodes.

Primer Name	Barcode	Sequence
ITS2-F-miseq	n/a	<u>Adaptor + Linker + Pochon ITS2 Forward Primer</u> TCG TCG GCA GCG TC A GAT GTG TAT AAG AGA CAG GTGAATTGCAGAACTCCGTG
ITS2-R-miseq	n/a	<u>Adaptor + Linker + Pochon ITS2 Reverse Primer</u> GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACAG CCTCCGCTTACTTATATGCTT
Hyb_F13_i5	AGTCAA	<u>Illumina (Nextera) Primer + Barcode + Forward Adaptor</u> AAT GAT ACG GCG ACC ACC GAG ATC TAC AC AGTCAA T CGT CGG CAG CGT C
Hyb_R43_i7	GCTGTA	<u>Illumina (Nextera) Primer + Barcode + Reverse Adaptor</u> CAA GCA GAA GAC GGC ATA CGA GAT GCTGTA GTC TCG TGG GCT CGG