Bombus mixtus Molecular Methods

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HOTSHOT DNA Extraction Protocol

This protocol should be used to extract DNA for downstream applications such as microsatellite genotyping, or other PCR-based methods where the quantity/quality of input DNA is low. It should not be used for metabarcoding or other protocols meant to measure species diversity.

Based on protocol from Truett et al., 2000.

Reagents/Supplies:

- Tissue samples
- NaOH
- EDTA
- 96 Well Plate or 0.2 uL PCR tubes
- Plate Seal (PCR compatible)

Equipment:

- Thermocycler

1. Prepare lysis reagent and neutralizing buffer.
    a. Lysis reagent: 25mM NaOH, 0.2 mM EDTA
        i. Some notes: NaOH is often sold in units of N (normality) instead of M (molarity). For compounds with different ionic charges, these measures are not equivalent, but for NaOH they are—so 1N NaOH is the same as 1M NaOH. EDTA is often sold as a powder—my recommendation is to create a stock concentration (mine is 20 mM). You can then dilute when mixing your lysis reagent. This is much easier because the mass of EDTA added to 50mL of a 20mM solution can be weighed on a scale more easily than the (very small) quantity you would add directly to a 0.2mM solution. Also...weighing things on the scale is harder than pipetting!
    b. Neutralizing buffer: 40mM Tris-HCl
        i. Some notes: you can purchase 1M Tris-HCl from most companies. It will last a very long time.

2. Prepare tissues samples in 0.2 uL PCR tubes (or a 96 well plate). For bumble bee samples, I typically use a half a leg (if available), although smaller samples (such as a tarsal segment) are also effective.

3. Add 75uL of lysis reagent to each tube/well.

4. Incubate tubes/plates on the thermocycler for 60min at 95°C. Set a hold temperature of 4°C.

5. Add 75uL of neutralizing buffer to each tube/well.

6. Store tubes/plate in the refrigerator or -20°C freezer.

Bombus mixtus Microsatellite Genotyping Protocol

For microsatellite amplification of 15 loci in *Bombus mixtus.*

Primers can be purchased through IDT and/or Thermofisher. A full table of primers is included below. My recommendation is to purchase 6FAM labelled primers through IDT, as they are much cheaper. Other dye tags (NED, PET, VIC) must be purchased through Thermofisher, as they are under copyright. It is possible to get an institutional discount through UBC (up to 50% off) but it may take some time/lots of emails.

In the following table, note the addition of PIG-tails ('GTT') to the 5' end of each reverse (unlabeled primer). Forward primers should be 5' labeled with the dye tag indicated in the second column. Also note the addition of a 'C' bp to the 5' end of the BTMS0126 forward primer (added because a 'G' base pair quenches the fluorescence of 6FAM.

Locus	Dye Tag	Plex	Approx. Size Range	Forward	Reverse
BL15	VIC	2	144-178	CGAACGAAAACGAAAAAGAGC	**GTT**TCTTCTGCTCCTTTCTCCATTC
BTMS0086	VIC	1	274-280	CTCGCGCTTGTCGAATCAAT	**GTT**AGAGAAATTGCATGCGGTCG
BTMS0057	VIC	1	96-135	TGCTTGAACCGAAATAGAGGG	**GTT**CACCGGCATTTTACACACCA
B126	PET	2	150-182	CGATTCTCTCGTGTACTCC	**GTT**GCTTGCTGGTGAATTGTGC
BTMS0136	PET	1	155-185	GCATTCGGGTATTGCGTTCTTTA	**GTT**CGTTTATCTGCTTCTCTCGTTCG
BTMS0066	NED	1	124-174	TTAACGCCCAATGCCTTTCC	**GTT**CATGATGACACCACCCAACG
BL13	NED	2	160-200	CGAATGTTGGGATTTTCGTG	**GTT**GCGAGTACGTGTACGTGTTCTATG
BTMS0062	NED	1	235-329	CTGGGCGTGATTCGATGAAC	**GTT**CTGTCGCATTATTCGCGGTT
BTMS0083	NED	2	288-324	CGACTCGTTCGAGCGAAATTA	GTTTTTGCCAGGCTCCGAAT
BT10	6FAM	1	139-193	TCTTGCTATCCACCACCCGC	**GTT**GGACAGAAGCATAGACGCACCG
BTERN01	6FAM	2	96-124	CGTGTTTAGGGTACTGGTGGTC	**GTT**GGAGCAAGAGGGCTAGACAAAAG
BTMS0059	6FAM	2	341-361	AGTTCGACAGACCAAGCTGT	**GTT**GGCTAGGAAAGATTAGCACTACC
BTMS0126	6FAM	2	164-190	**C**GGTGATCGCTTAAAGCTC	**GTT**GCCAACTACGTTCAATATCG
BTMS0104	6FAM	1	250-283	TCCTCTGTTCAGCACACGAT	**GTT**TTCGAAGCCTCGATGTCGT
BTMS0072	6FAM	2	223-254	TCCGACATTCCGAGAGCAACC	**GTT**ACTCCGTTATTCCCTTTCTCCT

Preparation of primers:

Final primer concentrations in PCR mix are as follows. Note, this is the concentration of each primer (e.g., 0.1 uM of BTMS0104 forward primer AND 0.1 uM of BTMS0104 reverse primer).

Plex 1:
BTMS0104: 0.1 uM
BTMS0062: 0.6 uM
BTMS0086: 0.6 uM
BT10: 0.2 uM
BTMS0136: 0.2 uM
BTMS0057: 0.2 uM
BTMS0066: 0.2 uM

Plex 2:
BTMS0126: 0.2 uM
BTERN01: 0.2 uM
BL13: 0.2 uM
BL15: 0.2 uM
B126: 0.2 uM
BTMS0072: 0.4 uM
BTMS0083: 0.4 uM
BTMS0059: 0.4 uM

For simplicity, mix 10X primer pools for each plex (i.e., all 14 primers for plex 1 [forward and reverse] should be mixed at 10X the final concentration.

PCR Prep & Protocol:

Reagents/Supplies:

- Genomic DNA (see HOTSHOT extraction)
- Plex 1 and plex 2 primer pools (10X concentration)
- Qiagen Multiplex PCR Plus Kit (highly recommended)
- 96 Well PCR Plate (or 0.2 uL PCR tubes)
- Plate Seal (PCR compatible)

Equipment:

- Thermocycler
- Vortexer
- Minicentrifuge
- Plate centrifuge

1. For each 96 well plate of samples, you will need 2 plates for PCR (plex 1 plate and plex 2 plate). Start by preparing two mastermixes (one for each plex). Prep enough mastermix for 100 reactions per 96 well plate (you can increase this to 105 reactions if you are especially worried about losing volume to pipetting error).
    a. Each 10 uL reaction contains:
    5 uL Qiagen Multiplex Mastermix (2X)
    1 uL primer pool (either plex 1 or plex 2 pool, depending on which plex you are prepping)
    4 uL genomic DNA (from extraction)

    (Hint: for a single 96 well plate, combine 500uL Qiagen Multiplex Mastermix with 100uL plex 1 primer pool and repeat for plex 2)

2. Pipette 75uL of plex 1 mastermix into each of eight (8) 0.2 uL PCR tubes. These should be placed in a tube rack so that they can be accessed by a multichannel pipette. Using a multichannel pipette with 8 pipette tips, transfer 6uL of mastermix from the PCR tubes into the columns of a 96 well plate. Some tubes may run out before others—use the "dregs" to fill in any plate wells which didn't receive sufficient mastermix. Alternatively, if you enjoy suffering, you can individually pipette into each of the 96 wells. You do you.

3. Using a multichannel with 12 pipette tips, transfer 4 uL of sample from each well of your DNA plate into the corresponding well in your PCR plate. Switch pipette tips between rows.

4. Seal plate and spin down.

5. Repeat steps 2-4 for plex 2.

6. Carry out the following thermocycling protocol (same conditions for both plexes):

    5 min @ 95°C
    35 cycles of:
        30 sec @ 95°C
        90 sec @ 57°C
        30 sec @ 72°C
    30 min @ 68°C
    Hold at 4 °C

7. Dilute each plate for AFLP submission. Add 9 uL of sterile, lab grade water to each well of a new 96 well plate. Transfer 1 uL of PCR product to each well (note: you'll submit two plates, for plex 1 and plex 2).

8. Seal plate.

9. Enter plate/sample data in a genotyping request at https://dnalims.sequencing.ubc.ca/

10. Place each plate in a plastic bag with the corresponding sample request (it's also good to write the order number ON the plate itself).

11. Submit plates to the mini-fridge in the lobby of the UBC Pharmaceutical Sciences Building.