Dye terminator sequencing of COI for the 3730xl DNA Analyzer (Applied Biosystems)

The cycle sequencing reaction recipe will depend on the sequencing clean-up method. Recently, CCDB switched to a semi-automated AutoDTR™ method from EdgeBio®. This sequencing clean-up method is less sensitive to PCR product concentration and allows longer high quality reads and further reduction of BigDye™ in a sequencing reaction, due to increased sensitivity. However, Sephadex® clean-up is a cheap and reliable method that could be used in any low or medium throughput facility.

Cycle sequencing reaction setup for Sephadex® clean-up

Set up a sequencing reaction according to the table below. Use 0.5 to 1.2 µl of unpurified PCR product per sequencing reaction.

	1 Reaction	96-well Plate: 104 Reactions
Dye terminator mix v3.1	0.25 µl	26 µl
5 X Sequencing Buffer	1.875 µl	195 µl
10% trehalose	5 µl	520 µl
10 µM Primer	1 µl	104 µl
H_2O	0.875 µl	91 µl
Total	9 µl	936 µl
PCR product	0.5 - 1.2 µl	

Note: 5X Sequencing buffer is: 400 mM Tris-HCl pH 9.0 + 10 mM $MgCl_2$ or 5X ABI sequencing buffer.

Aliquot 1/8 of total mix volume (115 µl) in 8-tube PCR strip (if making more than one plate, pour the mix into a disposable container) and dispense desired volume (9 µl) in 96-well plate and then add 0.5 - 1.2 µl of PCR product.

Addition of trehalose makes possible the freezing of aliquoted sequencing mixes. Currently, CCDB uses a batch strategy for making sequencing plates. Mixes are aliquoted directly into 96-well plates, using the Biomek® FX robot; plates are covered with PCR film and stored at -20°C for up to 3 months. Each batch is labeled, recorded in the system and tested to assure performance.

Run the sequencing reactions in a thermocycler under these conditions: Initial denaturation at 96°C for 2 min, followed by 30 cycles of 96°C for 30 sec, annealing at 55°C for 15 sec, and extension at 60°C for 4 min, followed by indefinite hold at 4°C.

The annealing temperature can be varied according to the primer specificity, but 55°C works well for most COI sequencing reactions.

Sephadex® clean-up

- Measure dry Sephadex® G-50 (Sigma-Aldrich, Cat. No. G5080-500g) with the MultiScreen® Column Loader (Millipore, Cat. No. MACL09645) into the Acroprep™ 96 Filter plate with 0.45 µm GHP membrane (PALL, Cat. No. PN5030).
- Hydrate the wells with 300 µl of molecular grade water.
- Let the Sephadex® hydrate overnight in the fridge *or* for 3 to 4 hours at room temperature before use.
- Put Acroprep™ plate together with MicroAmp® Optical 96-well Reaction Plate (Applied BioSystems®, Cat. No. N801-0560) and secure with at least 2 rubber bands.
- Make sure the two sets weigh the same (adjust weight by using different rubber bands.
- Centrifuge at 750 g for 3 minutes – this is to drain the water from the wells. Discard water from MicroAmp® plates (these plates could be reused for the same procedure without autoclaving).
- Add the entire volume of the sequencing reaction to the center of Sephadex® columns.
- Add 25 µl of 0.1 mM EDTA pH 8.0 to each well of the new (or autoclaved) MicroAmp® plate.
- To elute DNA attach MicroAmp® plate to the bottom of the Acroprep™ plate – secure them with tape and with rubber bands.
- Make sure the sets weigh the same (adjust weight by using different rubber bands).
- Centrifuge at 750 g for 3 minutes.
- Remove MicroAmp® plate and cover its top with Septa.
- Place MicroAmp® plate into the black plate base and attach the white plate retainer.
- Stack assembled plate in 3730xl DNA Analyzer (Applied Biosystems) - don't forget barcode and plate record.
- Discard Sephadex® from Acroprep™ plate.

Note: Acroprep™ plates could be reused up to 4 times (rinse them twice with deionized water and air dry).

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Cycle sequencing reaction setup for EdgeBio® AutoDTR™ 96™ clean-up:

Set up a sequencing reaction according to the table below. Use 1.5 to 2 µl of unpurified PCR product diluted 4 times (see details below) per sequencing reaction.

	1 Reaction	96-well Plate: 104 Reactions
Dye terminator mix v3.1	0.2 µl	20.8 µl
5 X Sequencing Buffer	1.9 µl	197.6 µl
10% trehalose	5 µl	520 µl
10 µM Primer	1 µl	104 µl
H_2O	0.9 µl	93.6 µl
Total	9 µl	936 µl
PCR product	0.5 - 1.2 µl	

Note: This dilution of BigDye™ works better in larger reaction volumes. To accommodate for this without increasing the cost, use the same 1/8 aliquot (115 µl), but dispense 9.5 µl instead of 9 µl! The original mix includes 104 reactions therefore it is sufficient to accommodate for a minor volume change.

Overview and Important Changes:

The AutoDTR™ 96 method requires some changes in both materials used and methodology employed. The most significant change is in the type of plate. The new cycle sequencing plates are PROGENE® non-skirted plates (Cat. No. 24-PCR-96LP-FLT-C).

- When pipetting to/from these plates, use a "holder" plate (Corning™, COSTAR® EIA/RIA plate, Cat. No. 9017) which provides structure to the non-skirted plate.
- When making pre-made cycle sequencing plates, this assemblage (i.e. non-skirted plate + costar plate base) is to be used.
- Because the non-skirted plates are not made of the same plastic, they cannot be heat sealed. Instead, use the aluminum sealing film to seal the non-skirted plates (ensure the edges are well sealed using a roller). Make sure that the "tabs" remain on the film but are tucked under the plate before loading onto the thermocycler.
- The Plate Clamp 96 (Promega Cat. No. V8251) is recommended for automated protocols and designed to ensure that nonskirted PCR plates are uniformly flat for liquid transfer on a robotic platform.

Note: Amount of PCR product should be reduced if using 1/20 dilution of BigDye™.

Once PCR amplification has been completed and evaluation of success determined using E-gel®, the PCR plate must be prepared for cycle sequencing. Our typical yield is ~100 ng/µl. The optimum range of PCR product concentration for 1/20 dilution is ~20-40 ng/reaction.

To accommodate for this, PCR products should be diluted approximately 4 times. Dilution of PCR product can be done two ways:

- Manually, by adding 15 µl of molecular grade water to the PCR plate. Do this by adding water to a solution basin and transfer the water using the 5-50 µl multichannel pipette and 30 µl filter tips.
- Using the Biomek® FX.

Note: Be careful not to cross-contaminate your samples when dispensing water. Use one box of 30 µl tips for each PCR plate you are diluting. Once the water has been added, seal the plate and spin at 1000 g for 1 min.

Cycle sequencing reaction setup:

- Add PCR product to either pre-made cycle sequencing plates or sequencing mixes. This may be done two ways: manually using the 1-10 µl multichannel pipette, or using the Biomek® FX.
- The amount of PCR product to add to a cycle sequencing reaction is critical for optimum success. The following volumes should be added for each given situation: vertebrates – 1.5 µl of diluted PCR product; invertebrates – 2 µl of diluted PCR product.
- Seal the cycle sequencing plates using aluminum sealing film and ensure that the tabs of the film are left on and tucked under the plate.
- Load plates in Eppendorf thermocyclers and run the cycle sequencing program "FAST-SEQ-EDGE": 96°C – 1 min; 15 cycles of 96°C – 10 sec, 55°C – 5 sec, 60°C – 1 min 15 sec; 5 cycles of 96°C – 10 sec, 55°C – 5 sec, 60°C – 1 min 45 sec; additional extension 60°C – 15 sec; 15 cycles of 96°C – 10 sec, 55°C – 5 sec, 60°C – 2 min; final extension 60°C – 1 min.

Note: this thermocycling program is suitable only for fast ramping thermocyclers.

AutoDTR™ 96™ cycle sequencing clean-up:

Please refer to EdgeBio® web-page for more detailed information on AutoDTR™ Dye Terminator removal kits and related products:
http://www.edgebio.com/catalog/dye-terminator-removal-products-AutoDTR™ -96-c-28_1005.html

Insert non-skirted plates into Plate Clamps, place assembly on top of holder plates. Load labware and reagents on a deck of Biomek™ FX. Prepare Binding Mix (mix 74 ml of 100% ethanol with 26 ml of AutoDTR™ 96 Binding Solution) and Elution Buffer* (5 mM DTT, 0.1 mM $NaHCO_3$). Run automated method on Biomek FX.

The following steps are performed automatically:

- Load new tips, transfer 10 µl of Sequencing reaction into AutoDTR™ plate, wash tips in a Wash Station.
- Add 24 µl of Binding Mix to AutoDTR™ plate, pipette-mix 20 times, wash tips in a Wash Station.
- Incubate for 5 min (if doing four plates, this step is optional).
- Aspirate all liquid from AutoDTR™ plate, discard into Wash Station, wash tips in a Wash Station.
- Add 50 µl of Binding Mix to AutoDTR™ plate, pipette-mix 8 times.
- Aspirate all liquid from AutoDTR™ plate, discard into Wash Station, wash tips in a Wash Station.
- Add 50 µl of Binding Mix to AutoDTR™ plate, pipette-mix 8 times.
- Aspirate all liquid from AutoDTR™ plate, discard into Wash Station, wash tips in a Wash Station.
- Pause the robot and spin the plates upside down at 1000xg for 3 min.
- Incubate plates for 10 min at room temperature to dry.
- Add 40 µl of Elution Buffer, pipette mix 20 times.
- Transfer 35 µl into receiver plate, wash tips in a Wash Station; discard tips.

After the method is completed, remove MicroAmp® plate from a deck, cover the top of plate with Septa. Place MicroAmp® plate into the black plate base and attach the white plate retainer. Stack the assembled plate in the 3730xl DNA Analyzer (Applied Biosystems).

References

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*US patent 6232076. Stabilizer of dye sequencing products.