PCR PLATE AND DATA SUBMISSION PACKAGE
SAMPLING INSTRUCTIONS

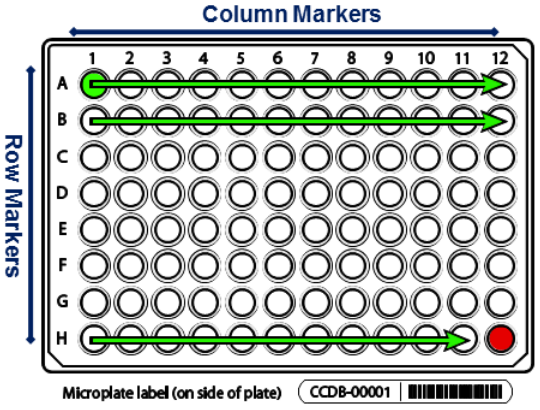
This Submission Package is aimed to facilitate the submission of ***PCR products*** and collateral information for processing at the **Canadian Centre for DNA Barcoding (CCDB)**. The kit contains:

- **Red-coloured microplates** filled with dry storage preservation medium, for housing PCR products;
- A digital CCDB Record file (MS Excel spreadsheet, attached to the automated notification email);
- These Sampling Instructions (enclosed in the package, copy sent by email);
- The Biological Material Analysis Agreement (BMAA) (enclosed in the package, copy sent by email).

Filled plates, together with the signed BMAA and filled CCDB Record files, should be returned for analysis to the **Centre for Biodiversity Genomics** — the hosting institution of CCDB. Specimen data should be submitted separately to the **Barcode of Life Data Systems (BOLD)**; a synopsis is provided below.

MICROPLATE

Each microplate contains wells pre-filled with dry storage media for aliquoting PCR products that are arranged in a 12×8 format. The sampling array starts with well **A01**. Well **H12** should be **left empty** for control, so each plate will accommodate 95 PCR aliquots. See below for details on the aliquoting procedure.



Each plate will be individually numbered, and will be shipped with the label pre-affixed to the plate. Each label contains a unique **barcode** and human-readable **identifier (CCDB Number)**. This CCDB number should be entered into the corresponding *CCDB Record* (see page 4).

Note: Before adding PCR products into a plate, make sure the **label is attached to the side corresponding to row H**. Always work with the plate label facing towards you. Pay special attention to the position of **row** (A through H) and **column** (1 through 12) markers: they should be on the **left** and **top** margins of the plate, respectively.

IMPORTANT: Only designated red-coloured PCR plates can be used for sending PCR products to the CCDB. Do not store or ship PCR plates together with tissue or whole genomic DNA extracts.

DIGITAL SPECIMEN DATA REQUIREMENTS

Prior to molecular analysis at the CCDB, accompanying data must be submitted in a compliant format via two different channels: the **CCDB** and the **Barcode of Life Data Systems (BOLD)**.

1. The **CCDB Plate Record** named **CCDB-00000_Record.xls**, is emailed to the recipient and used to record the location of samples in the corresponding sample container(s). Each sample must be assigned a **Sample ID**, which is a unique identifier connecting the sample with its source specimen. See section H for more details. Each container will have a corresponding plate record and up to 10 plate records can be included in the CCDB Plate Record file.

2a. A **BOLD Specimen Data Submission** is the first step in the process of creating records on BOLD. There will be one specimen data submission for each batch of containers. For more details on the specimen data submission protocol, please refer pg. 15 of the BOLD handbook accessible through this link: https://boldsystems.org/resource-hub/documentation/

Note: The 'Sample ID' field within this specimen data spreadsheet should be identical (including letter case) to the Sample IDs entered in the CCDB Plate Record and without any duplications.

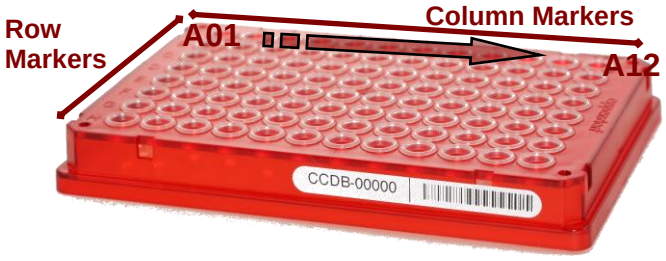
2b. A **BOLD Specimen Image Submission** is an additional requirement for some analytical services (see http://ccdb.ca/pricing/ for details) and should complement the specimen data submission. For details on the image submission protocol, please refer to pg. 26 of the BOLD handbook accessible through this link: https://boldsystems.org/resource-hub/documentation/

SAMPLING PROCESS: PLATE PREPARATION

IMPORTANT: The plates will be shipped to you sealed with clear film or aluminum foil. Before beginning the sampling process, remove the seal completely and ensure that no film or foil residue remains around plate wells.

To begin the sampling process, position the plate on a flat surface with the plate label facing towards you.

NOTE: Column markers (1–12) should be at the top and the **row markers** (A–H) should be on the left side of the plate.



SAMPLING PROCESS: PCR PRODUCT PREPARATION

Basic recipe for Polymerase Chain Reaction (PCR)

PCR reagents per 12.5 µl reaction:

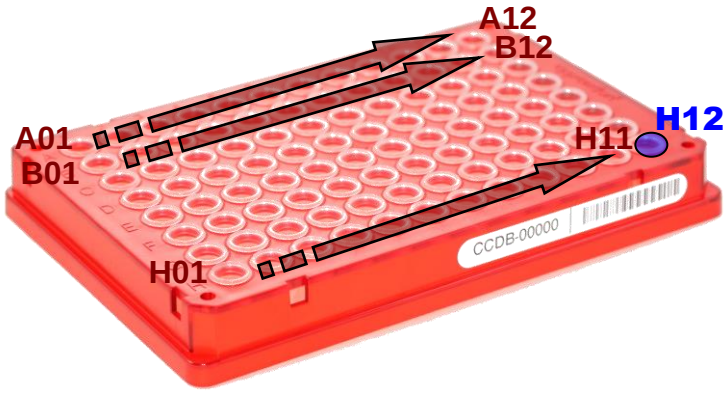
# of reactions	1	100
10% trehalose	6.25 µl	625 µl
ddH_2O	2 µl	200 µl
10X buffer	1.25 µl	125 µl
50 mM $MgCl_2$	0.625 µl	62.5 µl
10 µM primer A	0.125 µl	12.5 µl
10 µM primer B	0.125 µl	12.5 µl
10 mM dNTPs	0.0625 µl	6.25 µl
Polymerase (5 U/µl)	0.06 µl	6 µl
Total	**10.5 µl**	**1050 µl**
DNA template	2 µl per well	

Prepare PCR products according to CCDB PCR protocol: http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Amplification.pdf. **Do not cleanup PCR products.** The optimal concentration of PCR product is 100-150 ng/µl. If it is within recommended range, then add 8-10 µl of PCR product to each well.

IMPORTANT: Normalize PCR product concentration if necessary. If concentration exceeds recommended range, add less PCR product. For example, if PCR product concentration is 300-400 ng/µl, apply 5 µl of PCR product. Do not dilute PCR product prior to transfer.

Upon arrival of the PCR plates in the CCDB, PCR products will be resuspended in molecular grade water and incubated for 30 min at room temperature prior to gel check and sequencing.

SAMPLING PROCESS: THE PROCEDURE



Start aliquoting PCR products with well **A01** (row 1) and proceed in alphanumerical order to **A12** (left to right). When done with the first row, proceed to the second row (**B01**) and repeat the process until all 12 rows are filled. **Do not leave empty wells in the middle of the plate**.

IMPORTANT: Do not fill the last well, H12! It should be left empty as a negative control.

As you proceed with sampling PCR products into wells, keep a full record of Sample ID's in the Data Input worksheet of the corresponding CCDB RECORD workbook. For details, refer to instructions on page 4 of this manual and in the CCDB Record Data Input Sheet.

SAMPLING PROCESS: THE PROCEDURE

IMPORTANT: Replace tips between each PCR product sample transfer. Filter tips are strongly preferred.

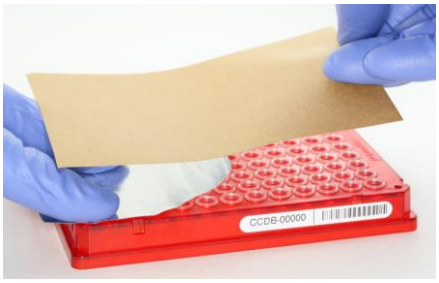
When transferring PCR products from individual tubes, it is recommended to organize tubes in a 12×8 rack in the same order as they will be transferred into the plate. Each tube should be repositioned, e.g., to another rack, as you retrieve sample from it. This will help minimize human error during the transfer process.

To visualize well contents (e.g., to ensure that PCR products have been added to all wells) examine the plate from below. When sampling into the last row (Row H), remember to leave the last well (**H12**) empty. If PCR transfer takes more than 1 day, the plate can be sealed with aluminum foil (see below) and kept at 4°C for several days. Make sure to centrifuge the plate at 1000×g for 1 min prior to opening and subsequent transfers. If your facility does not have a plate centrifuge, dry the samples completely (as described below) before sealing the plate.

Before sealing, leave the plate to dry either overnight at room temperature or incubate for several hours at 37-56°C.

NOTE: Dry PCR product will concentrate at the bottom of the plate forming a film or sticky residue.

IMPORTANT: Do not seal the plate until ALL wells are completely dry. Make sure that no liquid or condensate is present in the wells.



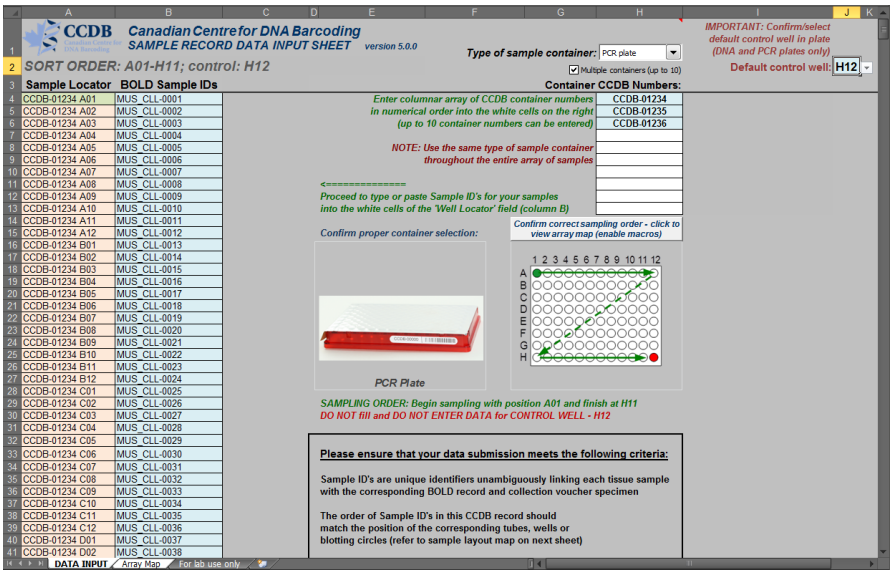
When the drying is complete, cover and seal the plate with aluminum foil (provided). Carefully seal the edges with a back side of aluminum foil.

When stacking multiple plates, use spacers to avoid puncturing foil with the wells of the plate above.

Please provide information on PCR product concentration range and the volume of diluted PCR product added to each plate. This can be written in marker on the foil seal or supplied separately (e.g., by e-mail).

SAMPLING PROCESS: KEEPING A RECORD

Open the MS Excel file titled **CCDB-00000_Record.xls**. By default, it will start with the tab (worksheet) called "DATA INPUT". Please follow the worksheet filling instructions typed in ***green italics*** and ensure that no **warning messages** remain in the header of the worksheet indicating missing information:



1. Select **"DNA plate"** from the dropdown field under 'Type of sampling container'. Make sure that the image appearing below matches the container you plan to fill.

NOTE: *Selecting the container* ***"DNA plate"*** *allows you to choose the sampling order: either A01-H12, control H12 (preferred standard) or H01-A11, control A12 (transposed, used in some molecular labs). To change the sort order, select the default control well in the top right corner of the DATA INPUT worksheet.*

2. If intending to submit multiple containers within the same record file, mark the checkbox **"Multiple containers..."** located below the container dropdown field.

3. Enter the CCDB container number(s) into the white cell(s) under **"Container CCDB Number(s)"** (type digits only, do not add prefixes). This will unhide the fields for entering Sample ID numbers.

4. Confirm that the correct sampling order is followed: refer to the container map image and the well coordinates indicated in the **"Sample Locator"** field.

5. As you place the samples into each container, enter their Sample ID numbers into the corresponding white cells of the column **"BOLD Sample IDs"**. Each CCDB Record should contain 95 entries per plate, corresponding to 95 samples. If preferred, the entire spreadsheet could be populated all at once, e.g., by pasting a column of data. In this case, please ensure that all measures are taken to ensure complete correspondence between the actual position of samples and the CCDB Record**.**

NOTE: *Do not attempt to paste more than one column of data and do not enter data for the control well(s).*

6. Make sure that your data submission adheres to the requirements outlined in the 'DATA INPUT' worksheet. Watch for **error messages** appearing in red colour on yellow background in the field to the right of the corresponding CCDB numbers and Sample ID records and correct your entries accordingly.

NOTE: All coloured (non-white) cells in the CCDB Record workbook are write-protected *to secure formulas and cross-links. Please type/paste your data only into white cells.* ***Avoid moving*** *(cutting and pasting) data between cells; use the copy-paste-delete procedure instead. When pasting data from another spreadsheet, make sure to* ***paste 'values'*** *or* ***'unicode text'*** *using the 'paste special' function of MS Excel.*

7. When data entry is completed, rename the file to incorporate the CCDB numbers included in it. For example, rename it to **CCDB-01234_Record.xls** for a single container or to **CCDB-01234-01236_Record.xls** for a set of several containers. This numbering format simplifies the archival and retrieval of these files after when they are submitted to the CCDB.

8. To visualize the correspondence between the data recorded and the position of samples in the microplate, refer to the next tab (worksheet) titled '**Array Map**'. If errors were detected when entering Sample ID information, an additional map will be displayed below the general layout map to help localize problematic sample entries. Please ensure that all error messages disappear before submitting the CCDB Record.

NOTE: *If the CCDB record sheet is filled prior to sampling, the 'Array Map' sheet can be printed as a reference to use when sampling into the container.*

DIGITAL SPECIMEN DATA SUBMISSION

1. The **CCDB Plate Record** file(s) and signed **BMAA** should be emailed to the CCDB via lims@ccdb.ca.

2. Templates for BOLD data submissions can be found at the following link:
https://boldsystems.org/resource-hub/templates/

a. The **Specimen Data** file can be submitted directly to BOLD using the specimen data submission protocol. Please refer to pg.15 of the BOLD handbook for instructions:

b. Specimen Images and the corresponding **Image Data** file can be submitted directly to BOLD using the online image submission protocol. Please refer to pg. 26 in the BOLD handbook for instructions:

For the BOLD handbook including detailed information on the BOLD data structure and submission procedures, please refer to the following link:
https://bench.boldsystems.org/libhtml_v3/static/BOLD4_Handbook_FinalVersion_Feb2023.pdf

Any questions concerning specimen data or image submissions should be directed to the BOLD Support Team support@boldsystems.org.

SUBMISSION OF BIOLOGICAL MATERIALS

Fill all 95 samples in each container before proceeding to the next one in the batch. Do not ship back partly filled containers, unless specifically arranged with the CCDB. Whenever a container is transferred to another person for sampling, please notify the CCDB.

NOTICE: Unless explicitly negotiated otherwise, all biological materials submitted to the Canadian Centre for DNA Barcoding (CCDB) at the Centre for Biodiversity Genomics (CBG) fall under the standard provisions of their associated Biological Material Analysis Agreement (BMAA).

All data submitted to the Barcode of Life Database and generated by the CCDB will comply with the Data & Resource Sharing Policies. Full texts can be requested from the CCDB.

A synopsis of the conditions relevant to this transaction with the CCDB is contained in the BMAA included in this submission package. Please acknowledge that you have read and agreed to these conditions by signing the BMAA in electronic or hard copy form and returning it to the CCDB with the first batch of samples.

DISCLAIMER: It is the sender's responsibility to ensure that (1) biological materials are shipped to the CCDB in compliance with any applicable shipping regulations, (2) they have been obtained under appropriate collection, and (3) animal care permits in their country of origin and the necessary export/import documentation required by Canadian and International customs and conservation authorities has been provided, including, but not limited to:

a) Export permit and/or zoosanitary certificate from the country of origin (if applicable)
b) CITES export certificate from the country of origin (if applicable)
c) Canadian Food Inspection Agency import permit (if applicable)

The CCDB cannot be held responsible in the event the provider fails to supply proper shipping documentation, causing the shipment to be held up or confiscated by customs, or any penalties resulting thereof.

After the sampling process has been completed, please return the samples by courier or registered mail to the following address. Please indicate a nil value on the shipping invoice.

Sample Submission
Canadian Centre for DNA Barcoding
Centre for Biodiversity Genomics
University of Guelph
50 Stone Road East
Guelph, Ontario, Canada N1G 2W1
Phone: +1 (519) 824-4120 ext. 56393

CCDB DISTRIBUTION OF DATA

Once CCDB processing is complete and all final sequences have been uploaded to BOLD, a **Sequence Upload Notification** will be sent to the external collaborator via the email address used during submission correspondence. The Sequence Upload Notification will contain the following information:

- The number of sequences uploaded
- Root plate name(s) associated with the data
- BOLD project name(s) associated with the data
- Contaminants, records flagged by BOLD, or other important information

The Sequence Upload Notification will contain the following attachments:

- Fasta file of uploaded sequences
- Taxon ID tree
- Spreadsheet of specimen information for sequences included in the taxon ID tree
- Specimen Images for sequences included in the taxon ID tree, if available
- BOLD Identification Engine results
- BOLD Sequence Submission Report
- Fasta file of sequences that contain a stop codon(s), indel(s), or identify as a contaminant, if any

In addition to the files attached to the Sequence Upload Notification, a copy of the CodonCode project, created by the CodonCode Aligner software used to perform sequence editing and assembly, as well as the fasta file of uploaded sequences will be attached to the associated BOLD Project for long term storage and review. All trace files will be uploaded to their corresponding specimen record on BOLD and can be reviewed or downloaded at any time.

Note: Sequences that contain greater than 1% ambiguous bases of the total recoverable sequence length (i.e. >5 N's in a 500bp sequence) do not meet CCDB sequence quality standards and will ***not*** be uploaded to BOLD.

Note: Final sequences that contain a stop codon(s), indel(s), or identify as a contaminant (e.g. *Homo sapiens*, *Mus musculus*, Proteobacteria (i.e. *Wolbachia*), fungal on a non-fungal plate, Nematoda on a non-Nematoda plate, etc.) will ***not*** be uploaded to BOLD. The sequence(s) will be provided as an attachment in the Sequence Upload Notification.

Note: Sequences that do not match the expected taxonomy at genus level or higher will be uploaded to BOLD and BOLD will flag the sequence to exclude it from use by the BOLD Identification Engine. The act of flagging maintains the integrity of the BOLD reference database used by the BOLD Identification Engine to determine closest matches for sequence identifications.

Validation of the final data is required from the external collaborator to address any mismatches between final sequence identifications and the expected taxonomy. To minimize mismatches, only known taxonomic information for specimens should be provided in the submitted **Specimen Data** file (i.e. specimens identified to Order if only Order level taxonomy is known). Flagged specimens require the review of specimen identifications. If the cause of the mismatch is a result of contamination from a non-target sequence, the sequence will remain flagged. If the cause of the mismatch is a result of specimen misidentification, the specimen data will be updated on BOLD by the external collaborator. Once taxonomic revisions of flagged sequences are complete, specimens with final sequence identifications matching the expected taxonomy can be unflagged upon request by the external collaborator to the BOLD Support Team at support@boldsystems.org.

All inquiries regarding CCDB processing or the information and data provided in the Sequence Upload Notification can be directed to CCDB Support Team at support@ccdb.ca.