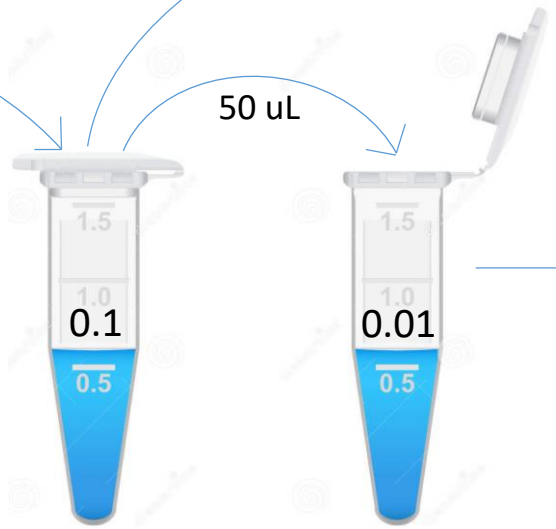
Isolating Fungi From Beetles

mycangium, head, fungi, etc.



50 uL

50 uL

50 uL

5 uL

0.1 Dilution

0.01 Dilution

0.001 Dilution

Primary Culture Plate Preparation

1. Prepare two tubes per each sample, label them “0.1” and “0.01”, fill each with 500 ul of water or PBS.
2. Suspend mycangium in the tube “0.1” and vortex.
3. Plate **50 ul** of the suspension on media. This is your 0.1 dilution.
4. Transfer **50 ul** of the initial suspension to the second tube (“0.01”) and vortex.
5. Plate **50 ul** of the second suspension on **second** media plate. This is your 0.01 dilution.
6. Plate **5 ul** of the second suspension on a **third** plate. This is your 0.001 dilution.

Entering Primary Cultures in Isolations DB

Culture Data Input Start Again

Primary Isolations | Isolates | Subcultures

plate_number	project	media	Scolytos_record	species	substrate_p	dillution	notes
14807	subculture	PDA-basic				0	Oxoplatypus_VA_OxoF2MycPick_orgi
14808	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery Scrape 2 Cr.Y.
14809	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery scrape 3 Oph
14810	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery wood plug 3 Oph
14811	subculture	PDA-basic				0	Oxoplatypus_VA_WoodAdjacentToGallery1
14812	subculture	PDA-basic				0	Oxoplatypus_VA_WoodAdjacentToGallery2
14813	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery wood plug 2 Oph
14814	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery wood plug 1 R2
14815	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery scrape 3 R3
14816	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery wood plug 3 R3
14817	subculture	PDA-basic				0	Oxoplatypus_VA_Gallery scrape 1 R3
14818	Zach's Isolations DB [	PDA-basic	25160	crassiusculus	mycangium	0.1	2017-02-02;
14819	Zach's Isolations DB [	PDA-basic	25160	crassiusculus	mycangium	0.01	2017-02-02;
14820	Zach's Isolations DB [	PDA-basic	25160	crassiusculus	mycangium	0.001	2017-02-02;
14821	subculture					0	
	subculture					0	

Plate # 14818

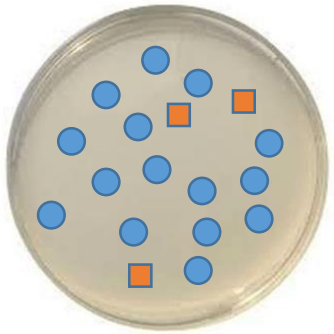
Plate # 14819

Plate # 14820

1. Label each of your primary culture plates with a new plate number, record under **plate_number** in Primary Cultures.
2. Assign a **project** to the plates, if your project is not available, add it to the projects table.
3. Record the **media** type on the plate, if not available, add to the media table.
4. When you isolate from a beetle, it should be given a **scolytos_record**, record the record number of the beetle the culture is from here. Record the **species** of beetle in the next column, add to OTUs table if needed.
5. Record where the fungi came from (beetle's mycangia, head, wash, gallery, etc.) in the **substrate_part** column.
6. Record the dilution factor (0.1, 0.01, 0.001) of the culture plate in the **dilution** column.
7. In the **notes**, add any relevant notes, especially the date the plates were made. Use YYYY-MM-DD format.

Morphotyping Primary Cultures and Counting CFUs

Plate # 14818



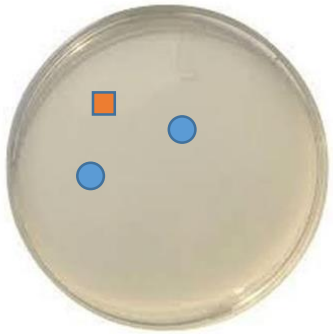
0.1 Dilution

Morphotypes:
- red_square_blue_border
- blue_circle

CFUs:
- 3
- 15

CFU Proportion:
- 10
- 75

Plate # 14819



0.01 Dilution

Morphotypes:
- red_square_blue_border
- blue_circle

CFUs:
- 1
- 2

CFU Proportion:
- 5
- 10

Plate # 14820

0.001 Dilution

Morphotypes:
- ~~red_square_blue_border~~
- blue_circle

CFUs:
- 0
- 1

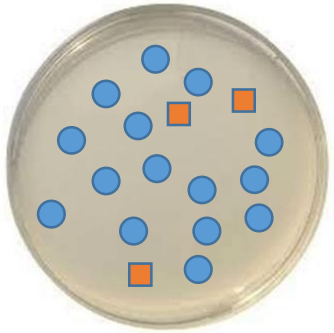
CFU Proportion:
- 0
- 5

Primary Culture Plate Morphotyping

1. After cultures have grown for a few days, describe colony types growing on plate. Be as descriptive as possible. These are **morphotypes**, they should be descriptive strings with underscores between words.
    1. It is extremely important that your morphotypes are consistent across your project. If you see the same fungus multiple times on multiple plates, name it the same for all. You can reuse names from other projects, they will not be cross referenced.
2. Count number of colonies on each plate for each morphotype. This is a count of colony forming units or **CFUs**.
3. Estimate the percentage of the plate that is covered by each morphotype. Record this value as the **CFU_proportion.**

Recording Isolates in Isolations DB

Plate # 14818



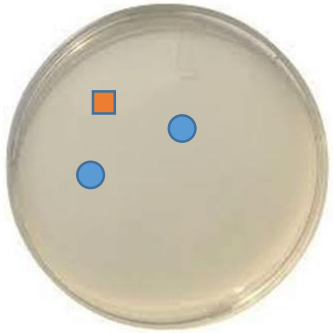
0.1 Dilution

Morphotypes: red_square_blue_border, blue_circle

CFUs: 3, 15

CFU Proportion: 10, 75

Plate # 14819



0.01 Dilution

Morphotypes: red_square_blue_border, blue_circle

CFUs: 1, 2

CFU Proportion: 5, 10

Plate # 14820

0.001 Dilution

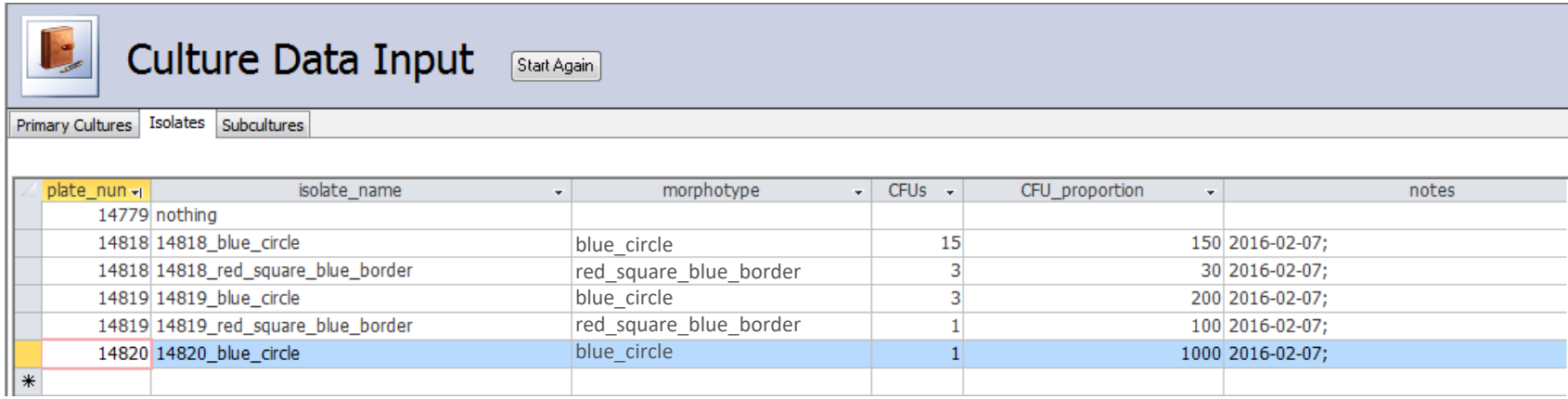
Morphotypes: ~~red_square_blue_border~~, blue_circle

CFUs: 0, 1

CFU Proportion: 0, 5

Isolates Database Entry

1. "Isolates" are morphotypes recorded from Primary Cultures (PC). As such, there will be duplicate entries for plates with >1 morph.
2. **Plate_number** is same as PC.
3. **Isolate_name** is "Plate#_morphotype".
4. Record **CFUs** and **CFU_proportion** counted on previous page.
5. Record date counted in **notes**.



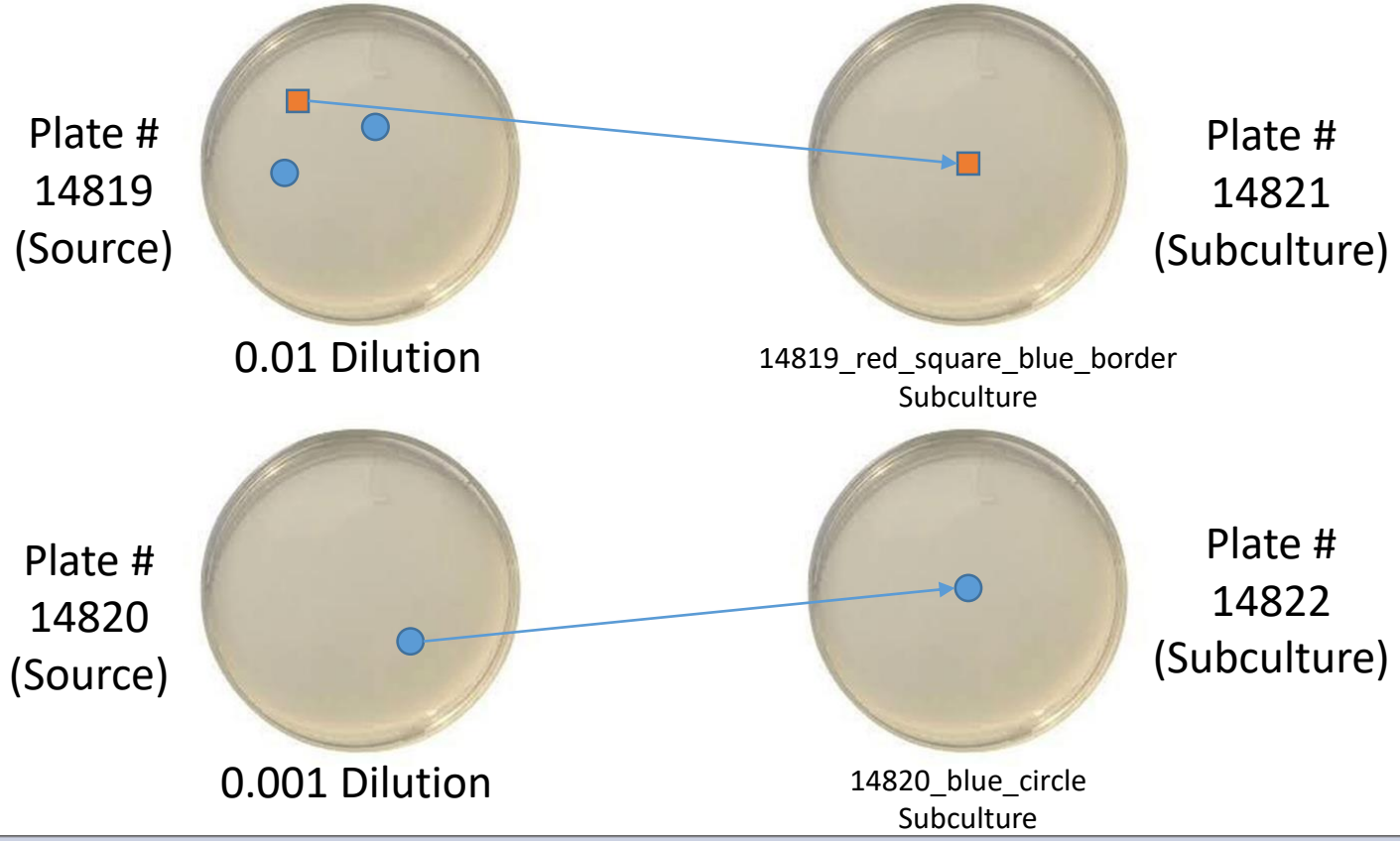
Culture Data Input

Start Again

Primary Cultures | Isolates | Subcultures

plate_nun	isolate_name	morphotype	CFUs	CFU_proportion	notes
14779	nothing				
14818	14818_blue_circle	blue_circle	15	150	2016-02-07;
14818	14818_red_square_blue_border	red_square_blue_border	3	30	2016-02-07;
14819	14819_blue_circle	blue_circle	3	200	2016-02-07;
14819	14819_red_square_blue_border	red_square_blue_border	1	100	2016-02-07;
14820	14820_blue_circle	blue_circle	1	1000	2016-02-07;
*					

Subculturing Isolates and Recording in DB



Subculturing Isolates and Entering in Database

1. To subculture an isolate, choose the lowest dilution Primary Culture plate you can find the morphotype on. This is your **source_plate**.
2. Take a small piece from the edge of the colony and culture it on a new plate. This is your subculture, give it a new **plate_number.**
3. Record the **isolate_name** of the colony from the previous page.
4. Record any relevant **notes**, including the date.
5. If this isolate will be archived, create a scolytos record for it and record the **Scolytos_archive_vial** on this form.

Culturing is now complete!* Now it's time to get some DNA!

Culture Data Input

Start Again

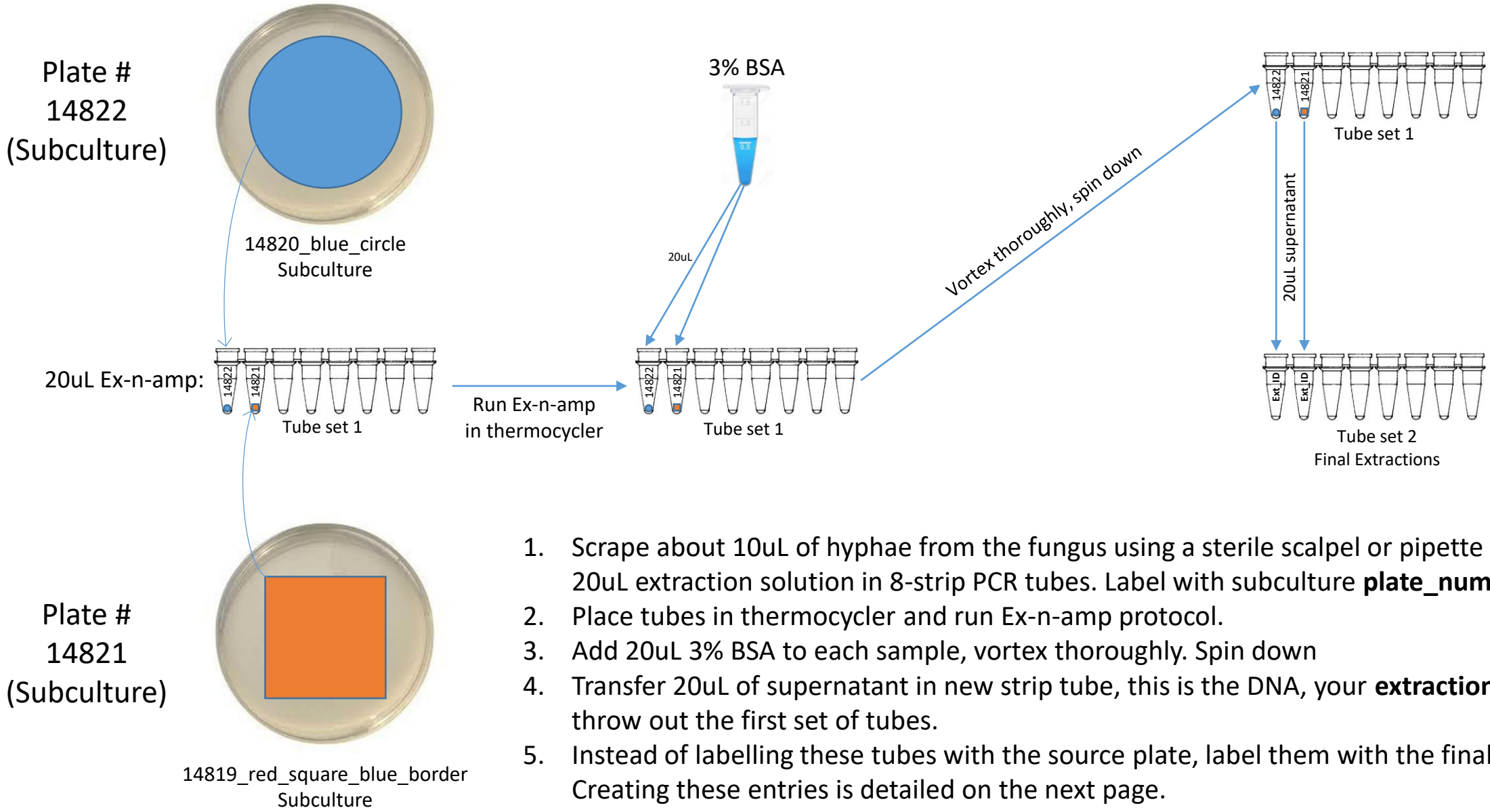
Primary Cultures | Isolates | Subcultures

plate_nun	media	source_plate	isolate_name	notes	Scolytos_archive_vial
14821	PDA-basic	14819	14819_red_square_blue_border	2017-02-07;	
14822	PDA-basic	14820	14820_blue_circle	2017-02-07;	
*		0			

Note about reviving fungi:
You may want to revive a fungus from the -80C. If you do, make a note that this is what you are doing and use the vial's source plate as your subculture **source_plate**

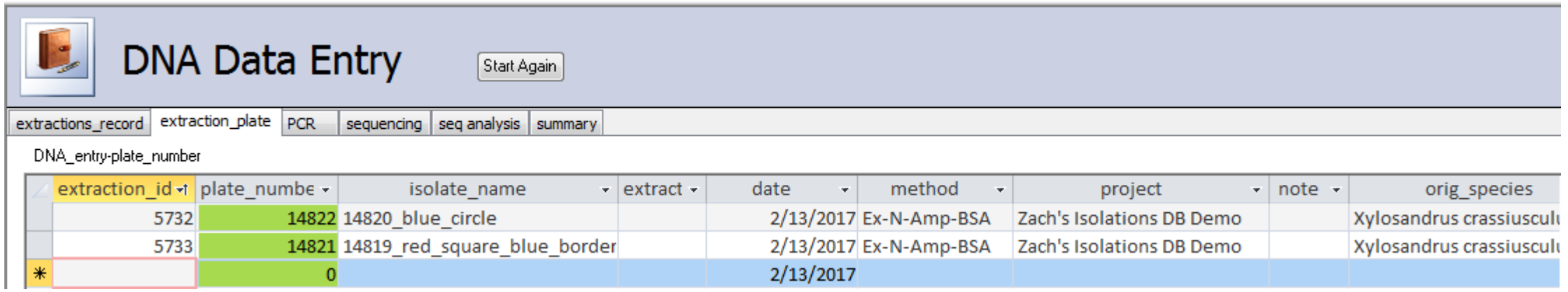
*Results may vary

Extracting DNA from Fungi

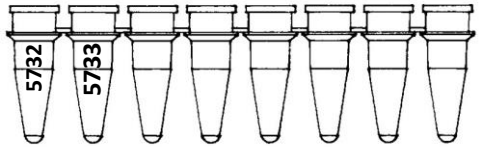


1. Scrape about 10uL of hyphae from the fungus using a sterile scalpel or pipette tip. Place in 20uL extraction solution in 8-strip PCR tubes. Label with subculture **plate_number**s.
2. Place tubes in thermocycler and run Ex-n-amp protocol.
3. Add 20uL 3% BSA to each sample, vortex thoroughly. Spin down
4. Transfer 20uL of supernatant in new strip tube, this is the DNA, your **extraction**. You may now throw out the first set of tubes.
5. Instead of labelling these tubes with the source plate, label them with the final **extraction_ID**. Creating these entries is detailed on the next page.

Recording extractions in the database



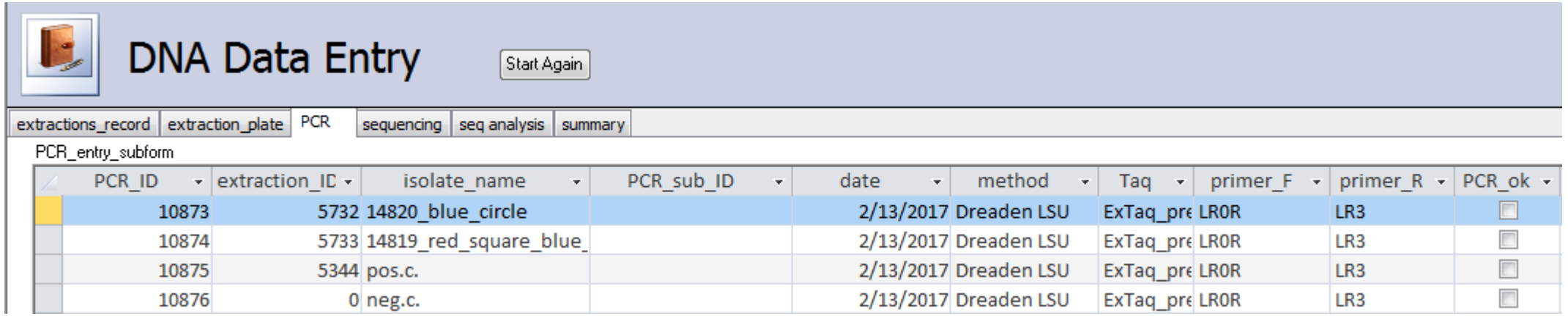
extraction_id	plate_numbe	isolate_name	extract	date	method	project	note	orig_species
5732	14822	14820_blue_circle		2/13/2017	Ex-N-Amp-BSA	Zach's Isolations DB Demo		Xylosandrus crassiusculu
5733	14821	14819_red_square_blue_border		2/13/2017	Ex-N-Amp-BSA	Zach's Isolations DB Demo		Xylosandrus crassiusculu
*		0		2/13/2017				



Tube set 2
Final Extractions

1. On the “Start Here” form in isolations, select “DNA from culture” for fungal DNA. If you are extracting from a beetle, you will choose “DNA from Scolytos sample.”
2. Create an **extraction_id** for each sample.
3. Record the subculture **plate_number** the sample came from. If you are extracting from a beetle, you will have a **scolytos_record** column in this position.
4. Record the **isolate_name** associated with the sample.
5. The **date** will automatically fill with today’s date. Change this if you run the extraction on a different date.
6. For most fungal extractions the **method** will be Ex-N-Amp-BSA, select an alternate if you did not use this method.
7. Record the **project** these samples are associated (should be the same from the Primary Cultures).
8. Record any relevant notes.
9. Record the original beetle species the fungi was isolated from under **orig_species**.

Creating PCR database entries



PCR_ID	extraction_ID	isolate_name	PCR_sub_ID	date	method	Taq	primer_F	primer_R	PCR_ok
10873	5732	14820_blue_circle		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☐
10874	5733	14819_red_square_blue_		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☐
10875	5344	pos.c.		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☐
10876	0	neg.c.		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☐

Creating PCR database entries

1. Create **PCR_ID**s for each of your samples and a positive and negative control.
2. Record the **extraction_ID** of each sample, including your positive control. Your negative control will have 0 as its extraction ID.
3. Record the **isolate_name** of each extraction you will be amplifying. Your positive control and negative control will have “pos.c.” and “neg.c.” as their isolate names respectively.
4. The **date** will autofill, change this if you have run or will run PCR on a different date.
5. Record the PCR protocol under **method**. This will depend on your primers and DNA.
6. Record the **Taq** you will be using, for most fungi this is “ExTaq_premix”.
7. Record your forward and reverse primer names under **primer_F** and **primer_R** respectively.
8. The remaining columns will be filled in after you run your PCR and gel.

Note: Many of these columns require you select a value from a drop down list. If your value is not on the list, add it to the appropriate table in the database. For instance, new PCR protocols should be recorded in the DNA_methods table. They will then be available to select in the **method** column.

Running PCR and gels

Running PCR

1. Create master mix, a common mix is listed below:
    - 12.5 uL PremixTaq
    - 9.5 uL PCR H20
    - 1 uL Forward primer
    - 1 uL Reverse primer
    - 1 uL DMSO
2. Multiply these values by the number of reactions you will have (including controls) + 2. Mix the resulting volumes together in an Eppendorf tube and label Master Mix or MM.
3. Label 8-strip PCR tubes with your **PCR_ID**s from the database. Transfer 25 uL (sum of reagent volumes for one reaction) of the master mix to each vial to be used.
4. Transfer 1 uL of extraction to its corresponding PCR vial.
5. Run in thermocycler using protocol you recorded as the **method**.

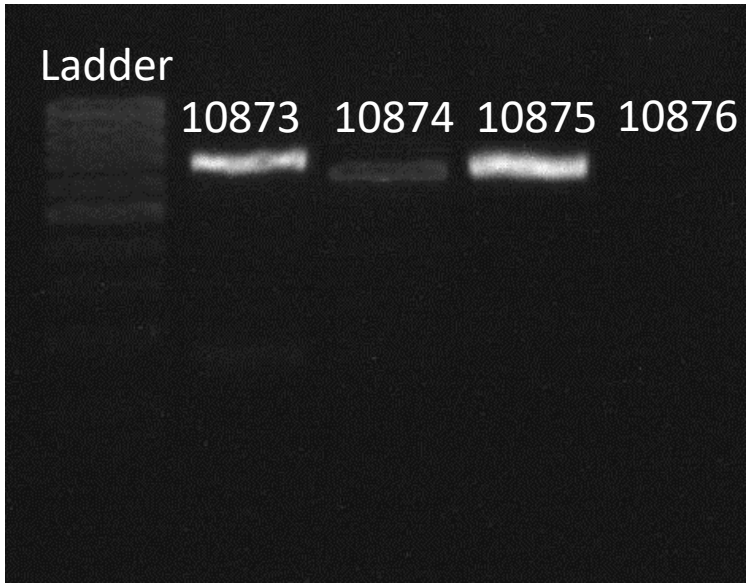
Running Gels

1. Determine how many wells your gel will need, and select the best apparatus for your needs. You will need enough wells for all PCR samples plus one extra well per row for the ladder.
2. Either in new tubes, or on a piece of parafilm, mix 1.5 uL SYBR Green+6x Loading Dye mix with 5uL of your ladder, transfer to the left well, repeating for each row.
3. Mix 1.5uL SYBR Green+6x Loading Dye mix with 5 uL of PCR product, then transfer to a well in the gel. Keep track of what sample is in each well.
4. When mixing dye and PCR product, always use a new pippete tip and clean vial or parafilm for mixing.
5. When all PCR producted is loaded in gel, fill with 1x TAE buffer until gel is submerged, and close apparatus.
6. Set electrodes to ~110V and runtime as needed. 45 minutes seems to work well for most gels, and additional time can always be added.
7. Use Enduro to take a photograph of the gel under UV light.

Recording gel results in the database

Please remember to record your gel results in the database. Also, save an image of each gel you run in your lab records with a way to connect the bands to a PCR ID.

1. Check the box in PCR_ok if your gel returns a single band for a sample.
2. In band_strength, record how strong the band appears on the gel, if it appears at all.



DNA Data Entry

Start Again

extractions_record | extraction_plate | PCR | sequencing | seq analysis | summary

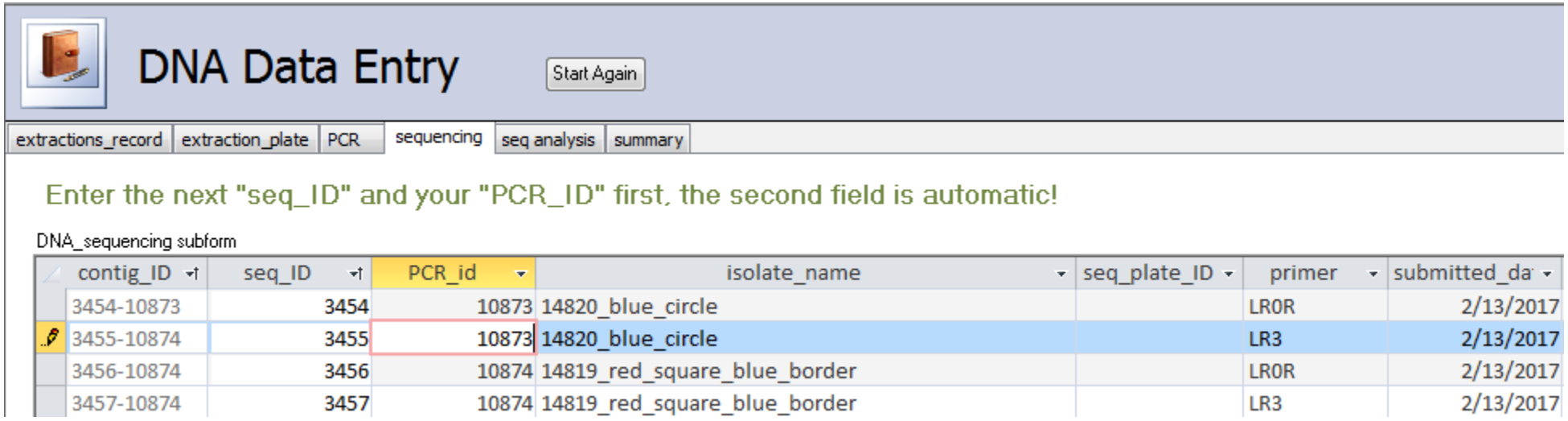
PCR_entry_subform

PCR_ID	isolate_name	PCR_sub_	date	method	Taq	primer_F	primer_R	PCR_ok	band_strength
10873	14820_blue_circle		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☑	strong
10874	14819_red_square_blue_		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☑	weak
10875	pos.c.		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☑	strong
10876	neg.c.		2/13/2017	Dreaden LSU	ExTaq_pre	LR0R	LR3	☐	none

Preparing a database entry for sequencing

Here, you will generate a database entry that connects your sequence to your PCR product and in turn, the rest of your work.

1. Decide if you will be sequencing one direction or both. If one, each PCR product submitted will have one sequence ID (**seq_ID**). If both, each will have two. Record the **PCR_id** in accordingly, the **contig_ID** will automatically.
2. Record the **isolate_name** the PCR product is associated with.
3. Record the **primer** the sequence reading will start at. If you will be sequencing both directions, one seq_ID will have the forward primer, the other will have the reverse.
4. The **submitted_date** will automatically select today as the date you submit your samples to be sequenced. Edit if needed.



contig_ID	seq_ID	PCR_id	isolate_name	seq_plate_ID	primer	submitted_da
3454-10873	3454	10873	14820_blue_circle		LR0R	2/13/2017
3455-10874	3455	10873	14820_blue_circle		LR3	2/13/2017
3456-10874	3456	10874	14819_red_square_blue_border		LR0R	2/13/2017
3457-10874	3457	10874	14819_red_square_blue_border		LR3	2/13/2017

Recording your sequence in the database

1. Once you have your sequences assembled, submit them to https://blast.ncbi.nlm.nih.gov to get an ID.
2. Record the **PCR_ID** of the sample the sequence came from, it will get a unique **Seq_analysis_ID**.
3. Record the **assembled sequence** itself, along with the **gene** the sequence was taken from.
4. Record the species of the first BLAST result as the **BLAST_ID**, including any specific modifiers of the strain or similar.
5. Record the Ident value in BLAST in the **summary** column, and the Query cover value in the **Query coverage** column.

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Ambrosiella beaveri genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence, is	473	473	100%	7e-130	100%	LC175290.1
☐	Ambrosiella nakashimae genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequen	473	473	100%	7e-130	100%	LC175285.1
☐	Ambrosiella hartigii strain CMW25525 28S ribosomal RNA gene, partial sequence	473	473	100%	7e-130	100%	KM495317.1
☐	Ambrosiella hartigii strain CBS404.82 28S ribosomal RNA gene, partial sequence	473	473	100%	7e-130	100%	EU984288.1