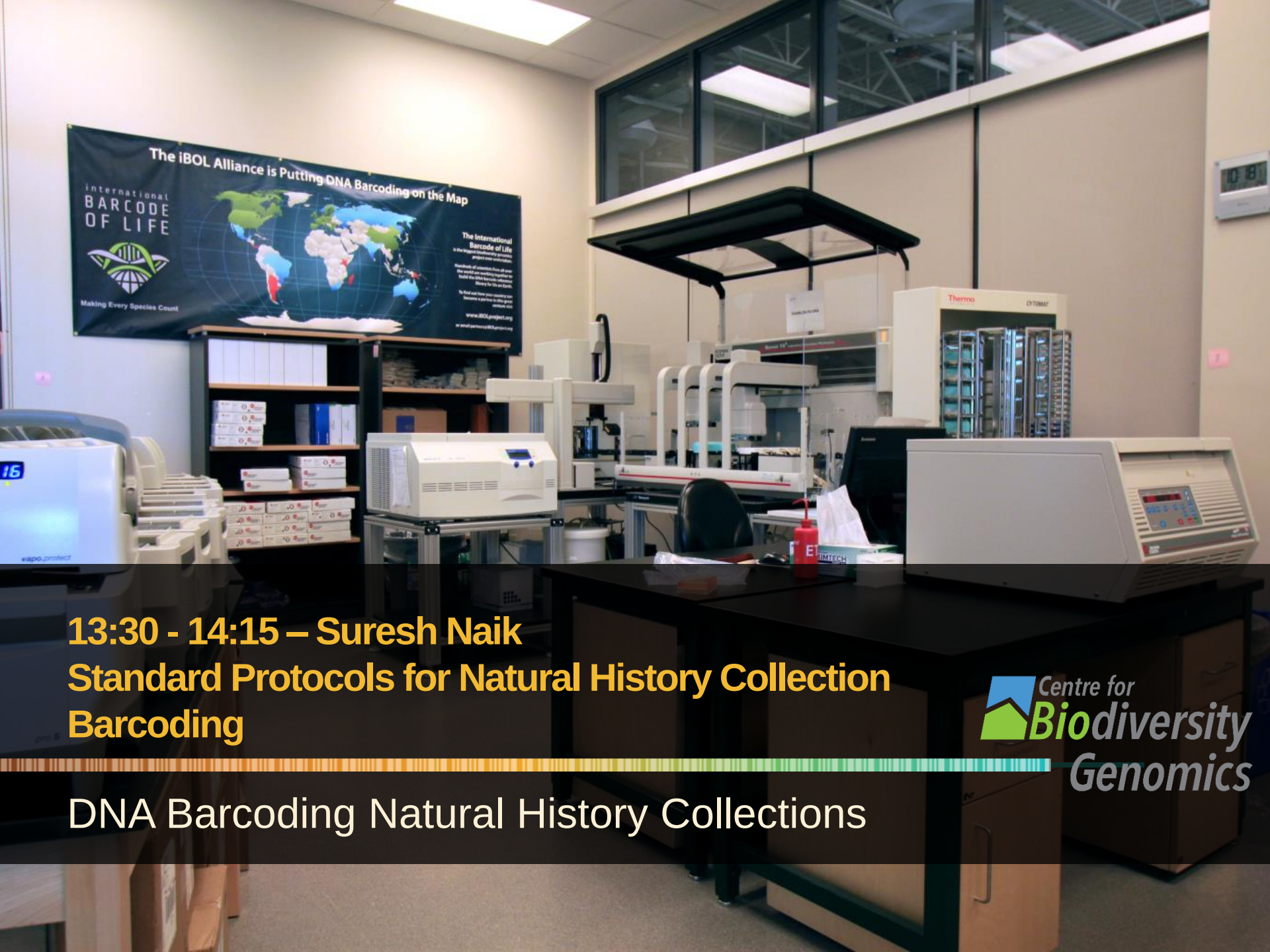
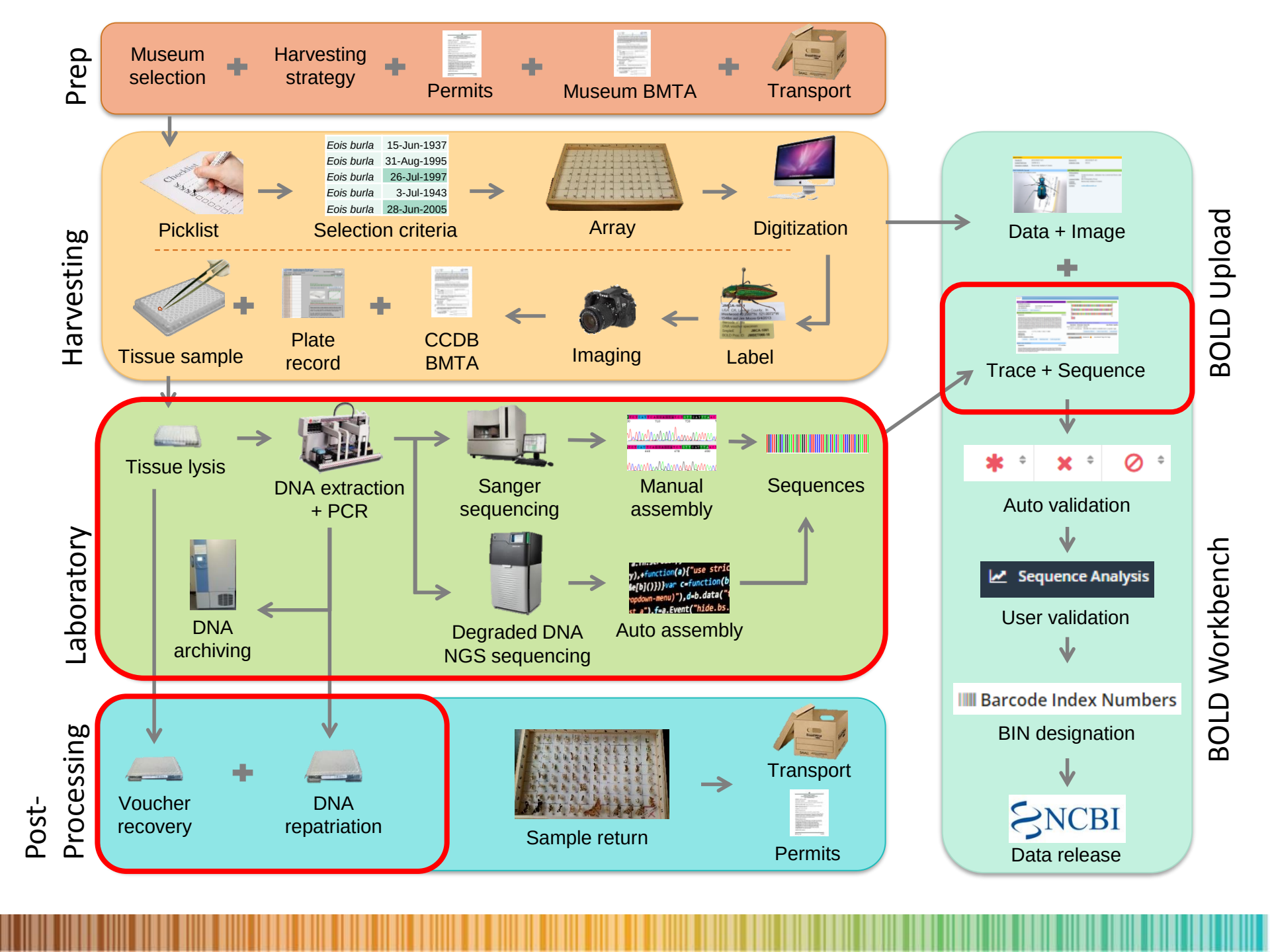


13:30 - 14:15 – Suresh Naik

Standard Protocols for Natural History Collection Barcoding

Centre for Biodiversity Genomics

DNA Barcoding Natural History Collections


Prep
Museum selection
Harvesting strategy
Permits
Museum BMTA
Transport
Harvesting
Picklist
Eois burla 15-Jun-1937
Eois burla 31-Aug-1995
Eois burla 26-Jul-1997
Eois burla 3-Jul-1943
Eois burla 28-Jun-2005
Selection criteria
Array
Digitization
Tissue sample
Plate record
CCDB BMTA
Imaging
Label
Laboratory
Tissue lysis
DNA extraction + PCR
Sanger sequencing
Manual assembly
Sequences
DNA archiving
Degraded DNA NGS sequencing
Auto assembly
Post-Processing
Voucher recovery
DNA repatriation
Sample return
Transport
Permits
Data + Image
Trace + Sequence
Auto validation
Sequence Analysis
User validation
Barcode Index Numbers
BIN designation
NCBI
Data release
BOLD Upload
BOLD Workbench

Standard Protocols

CCDB Workflow

Lysis & Extraction

PCR Amplification

PCR

Taxon Specific Protocols

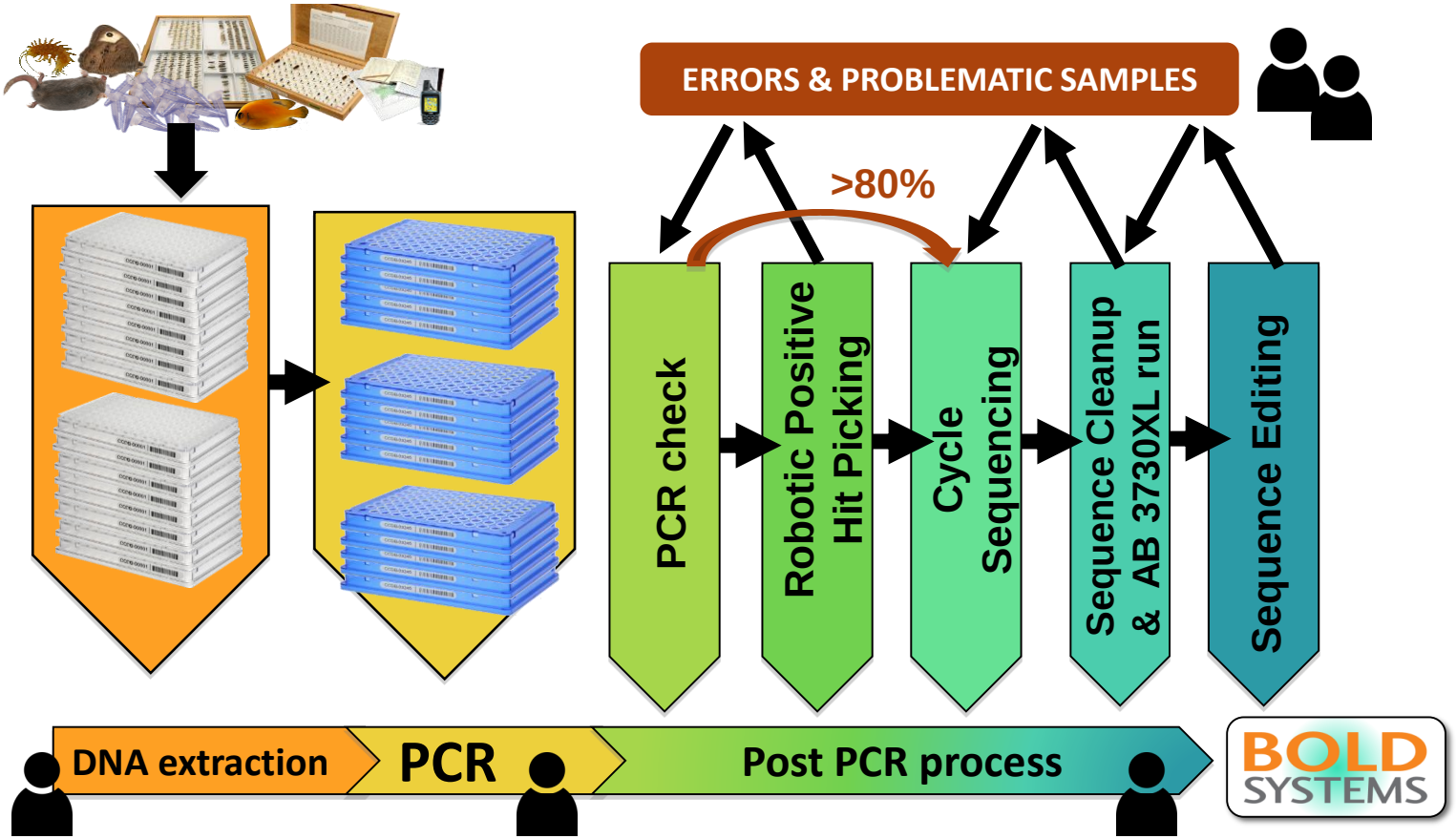
Sequencing

Failure Tracking

DNA Archiving

Quality Control

CCDB Workflow



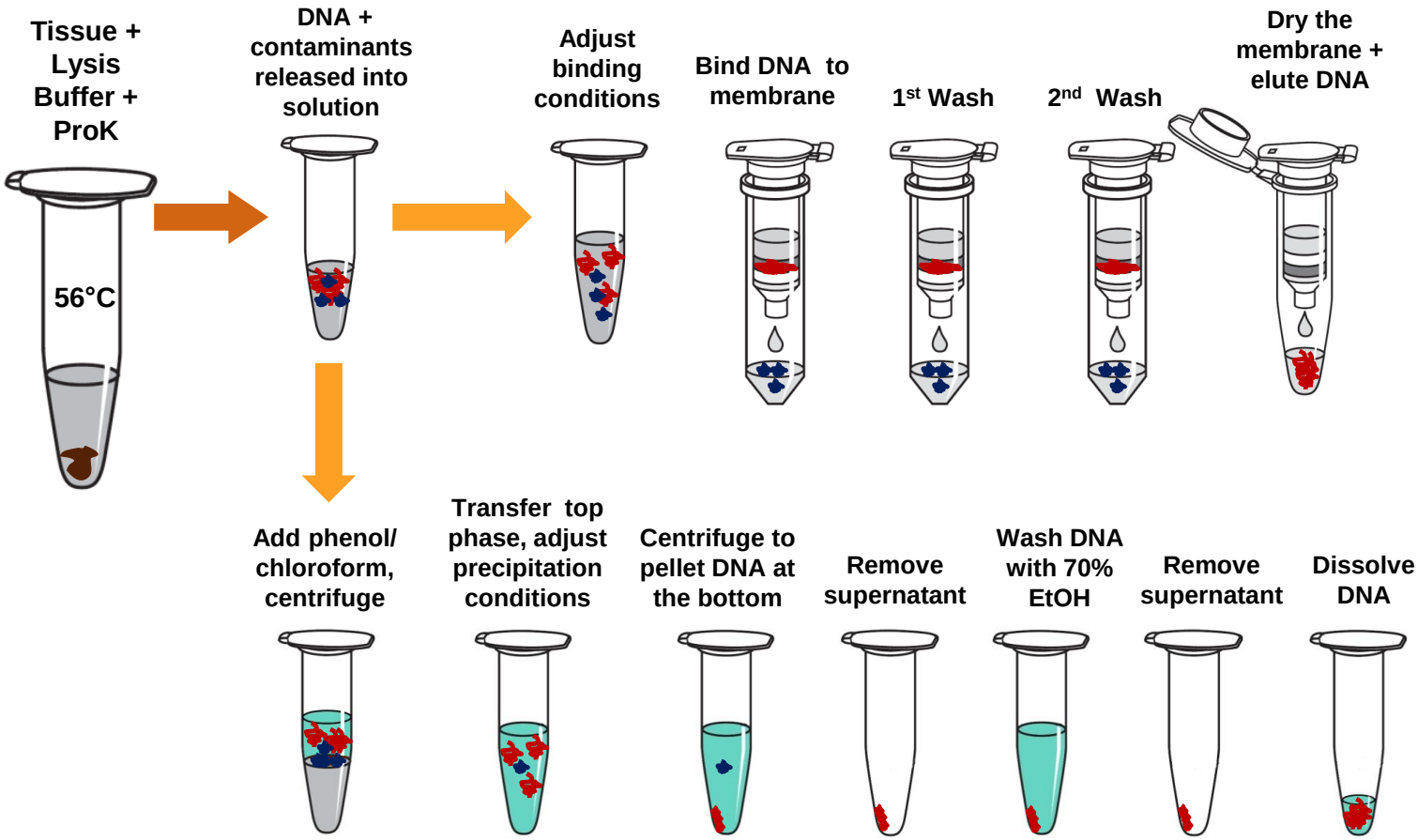
Lysis & Extraction

Key Steps in Quality Assurance

- Follow good lab practices (GLP)
- Maintain clean lab areas
- Separate reagent preparation, DNA and PCR working areas
- Use separate pipettes for post and pre-PCR stages
- Always perform visual checks
- If something goes wrong, exclude one factor at a time

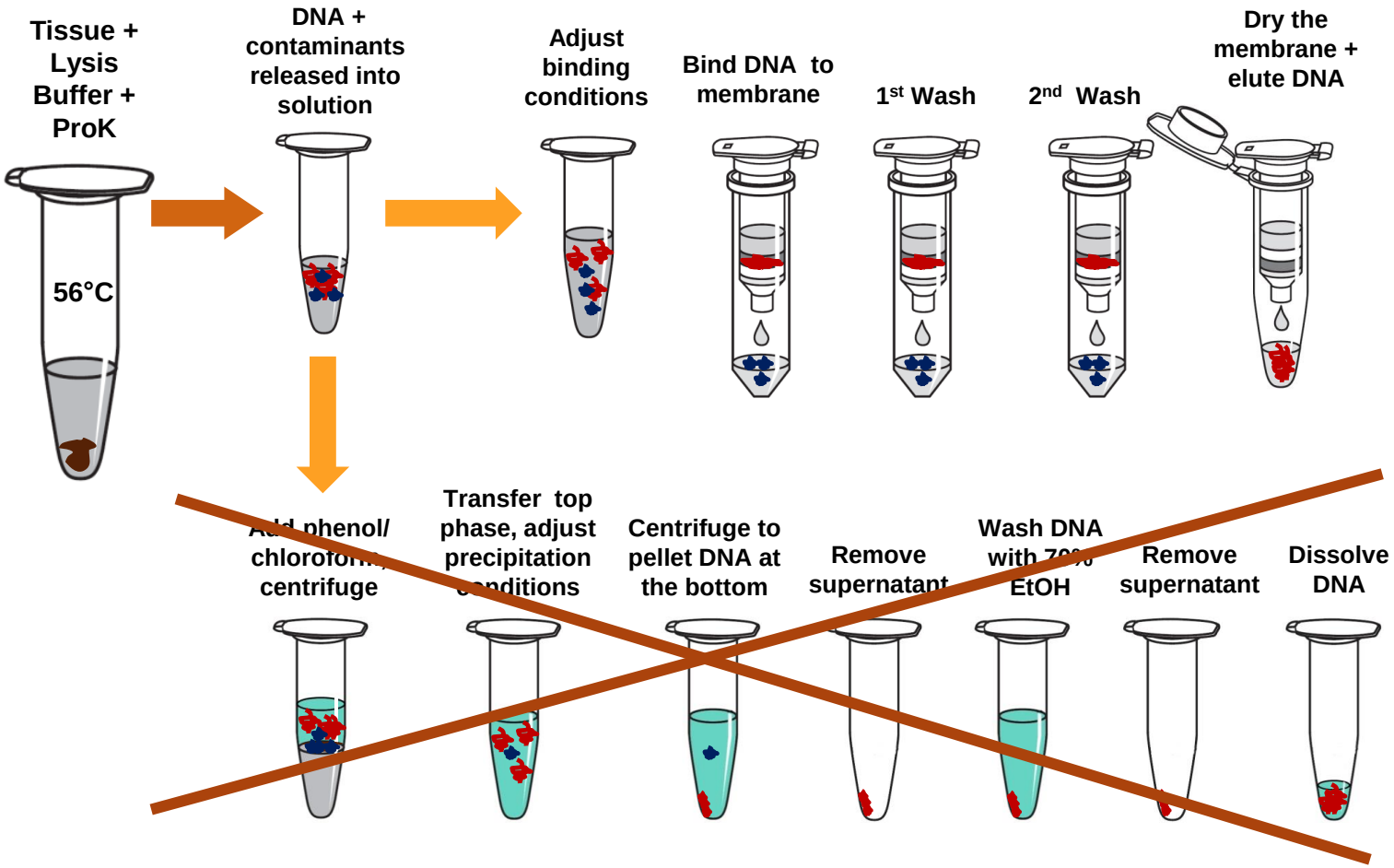
Lysis & Extraction

Overview



Lysis & Extraction

Overview



Lysis & Extraction

Extraction Protocols Used by the CCDB

Alkaline lysis

(Crude extraction)

Bind-wash-elute (BWE)

(membrane based)

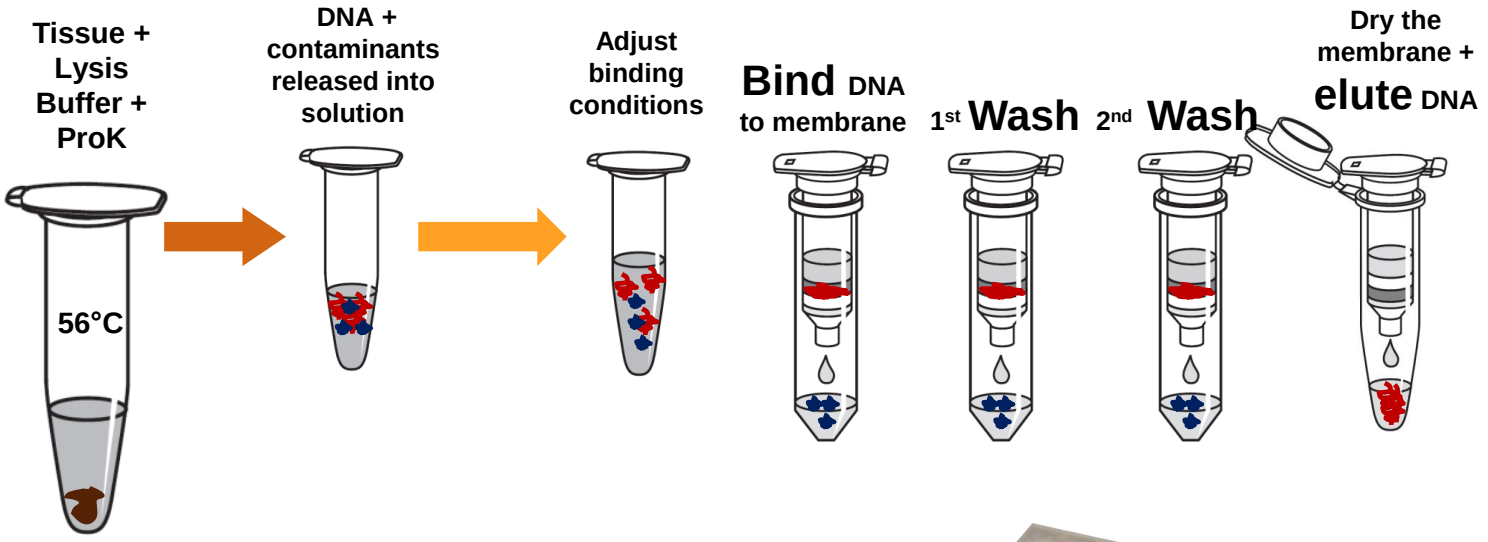
Lysis & Extraction

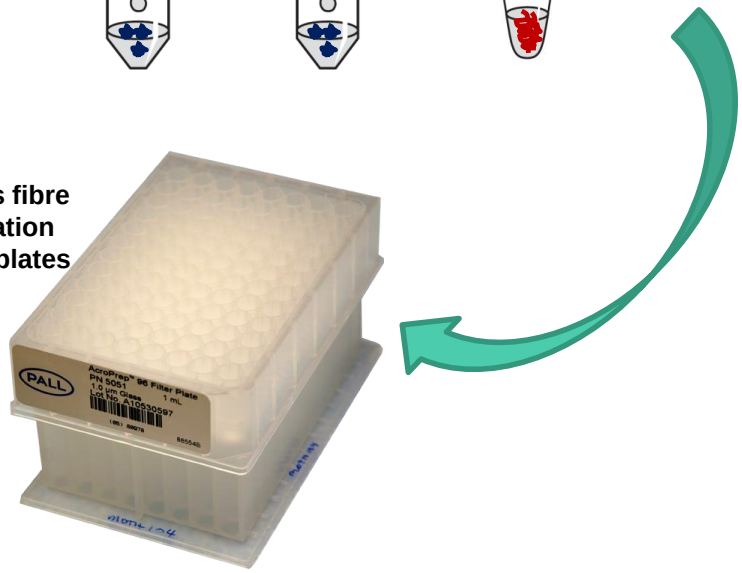
Alkaline lysis

- Simple, quick, inexpensive
- Suitable for very small organisms
- Does not produce stable DNA extracts
- Tissue amount is critical

Lysis & Extraction

Bind-Wash-Elute (BWE)





- Compatible with high- throughput pipeline
- Less errors
- Better quality of DNA

Lysis & Extraction

Glass Fiber DNA Extraction

Molecular Ecology Notes (2006) 6, 998–1002 doi: 10.1111/j.1471-8286.2006.01428.x

TECHNICAL NOTE

An inexpensive, automation-friendly protocol for recovering high-quality DNA

NATALIA V. IVANOVA, JEREMY R. DEWAARD and PAUL D. N. HEBERT
Biodiversity Institute of Ontario, Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Plant Mol Biol Rep (2008) 26:186–198
DOI 10.1007/s11105-008-0029-4

Semi-automated, Membrane-Based Protocol for DNA Isolation from Plants

Natalia V. Ivanova · Aron J. Fazekas · Paul D. N. Hebert

Lysis & Extraction

BWE - Lysis Buffers

- Invertebrate (**GuSCN**) ----------

- Vertebrate (**SDS**) ----------

- Plant (**CTAB**) ----------

- Algal Buffer (**KOAc**) ----------


Lysis & Extraction

BWE - Binding Conditions

- Binding mix (**3M GuSCN buffer, 50% ethanol**)


- Plant binding buffer (**5M GuSCN buffer**)





Lysis & Extraction

BWE - Invertebrate Extraction

- **Tissue source** – legs or whole specimens
- Invertebrate lysis buffer + Proteinase K
- Incubation overnight at 56°C
- Binding with BM buffer, wash with PWB and WB buffers
- 3 µm GF
- Elution volume – 30-40 µl




Lysis & Extraction

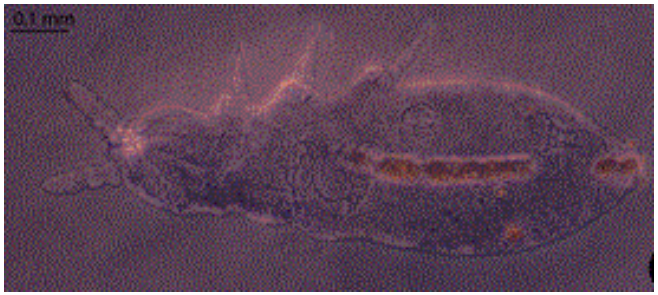
Special Case:Voucher Recovery

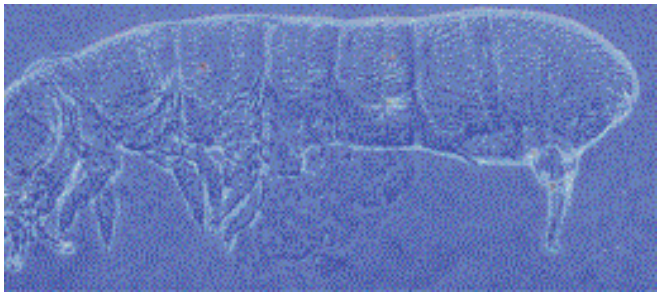
Molecular Ecology Resources (2010) doi: 10.1111/j.1755-0998.2010.2839.x

DNA BARCODING

Coupling non-destructive DNA extraction and voucher retrieval for small soft-bodied Arthropods in a high-throughput context: the example of Collembola

DAVID PORCO*, RODOLPHE ROUGERIE*, LOUIS DEHARVENG† and PAUL HEBERT*
*Canadian Center of DNA Barcoding, University of Guelph, 50 Stone Road East, N1G 2W1, Guelph, Ontario, Canada,
†UMR 5202 CNRS ''Origine, Structure et Evolution de la Biodiversité'', Muséum National d'Histoire Naturelle,
C.P.50 45 rue Buffon, 75005 Paris, France





Lysis & Extraction

Special Case:Voucher Recovery

1. Overnight incubation at 56°C
2. Apply lysate to PALL 0.45 µm Supor filter plate sitting on top of collection plate with PALL collar
3. Centrifuge at 3000×g for 2 min
4. Use clarified lysate for extraction
5. Add 95% EtOH to lysis and PALL plates
6. Collect vouchers under microscope

Lysis & Extraction

BWE - Vertebrate Extraction

- **Tissue source** – ideally muscle
- Vertebrate lysis buffer + Proteinase K
- Incubation overnight at 56°C
- Binding with BM buffer, wash with PWB and WB buffers
- 1 µm GF membrane
- Elution volume – 50-60 µl




Lysis & Extraction

BWE - Plant & Fungal Extraction

- **Tissue source** – silica dried tissue, herbarium samples, seeds
- Grinding – TissueLyser
- CTAB lysis for 1.5 hour at 65°C
- Binding with 5M GuSCN buffer, wash with BM and WB buffers
- 1 µm GF membrane
- Elution volume – 50-60 µl

Lysis & Extraction

BWE - Echinoderm, Cnidaria & Mollusk Extraction

- **Tissue source** – ethanol fixed soft tissue
- CTAB + Proteinase K lysis
- Incubation overnight at 56°C
- Binding with 5M GuSCN buffer, wash with BM and WB buffers
- 3 µm GF over 0.2 µm Bioinert membrane
- Elution volume – 50-60 µl



Lysis & Extraction

BWE - Algal DNA Extraction

J. Phycol. **29,** 251–254 (1993)

GEL PURIFICATION OF RED ALGAL GENOMIC DNA: AN INEXPENSIVE AND RAPID METHOD FOR THE ISOLATION OF POLYMERASE CHAIN REACTION-FRIENDLY DNA[1]

Gary W. Saunders

School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2005) **360**, 1879–1888
doi:10.1098/rstb.2005.1719
Published online 8 September 2005

Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications

Gary W. Saunders*

Centre for Environmental & Molecular Algal Research Department of Biology, University of New Brunswick Fredericton, NB, Canada E3B 6E1

Lysis & Extraction

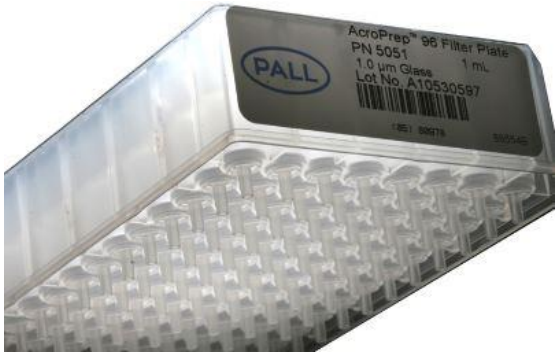
BWE - Algal DNA Extraction: Saunders Lab/CCDB

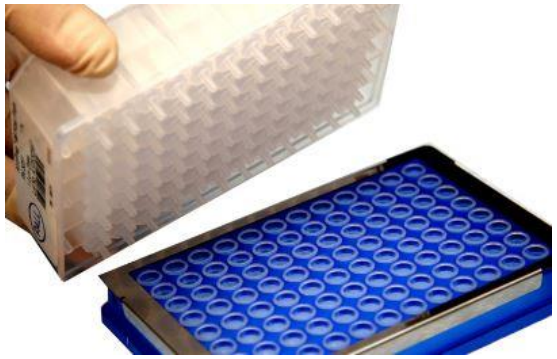
- **Tissue source** – silica dried tissue, herbarium
- Acetone extraction to remove polyphenols
- Grinding – TissueLyser
- Algal buffer + Tween 20 + Proteinase K, incubate 1 hour at room temperature
- Incubate on ice, centrifuge
- Transfer aliquot of lysate
- Binding with 5M GuSCN buffer, wash with BM and WB buffers
- 1 µm GF membrane
- Elution volume – 50-60 µl

Lysis & Extraction

BWE - Manual Extraction

- Scalable for 4-6 plates/day
- Centrifugation at 5000×g
- Better for old material
- Reduced chance of contamination





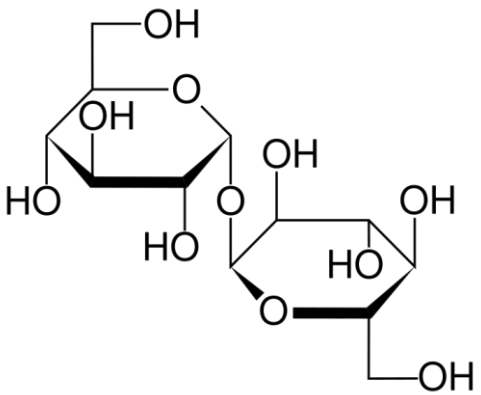
Lysis & Extraction

BWE - Automated & Semi-Automated Extraction

- 40 plates/day
- Vacuum manifold
- Centrifugation to remove WB
- Elution in centrifuge
- Robotic centrifuge integration
- Reduced errors

PCR Amplification

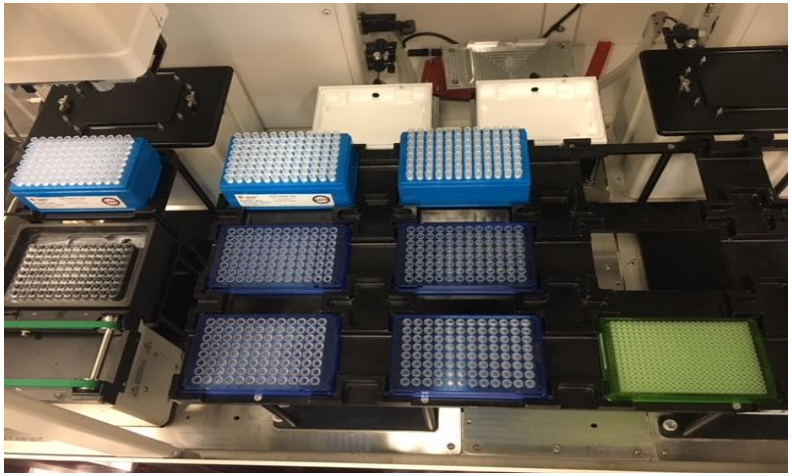
- Pre-made frozen plates with trehalose
- Platinum or KAPA Ab Taq polymerase
- Low dNTPs and primers concentration
- No PCR cleanup!

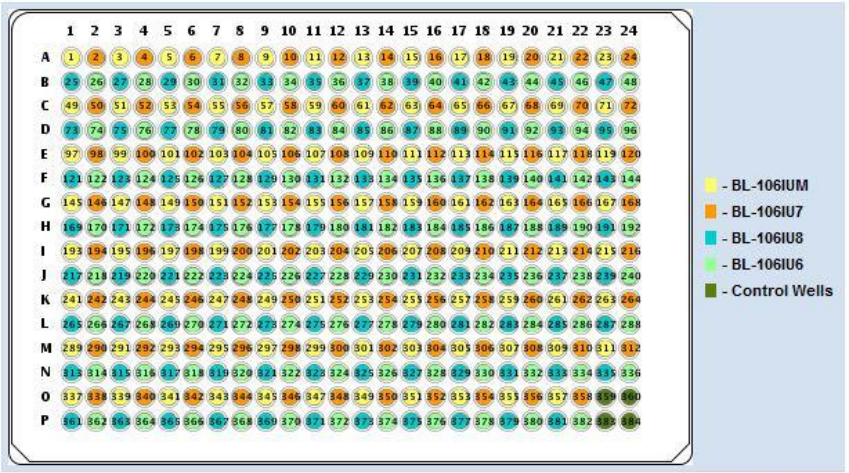


PCR Amplification

Consolidation into 384 Format Plate

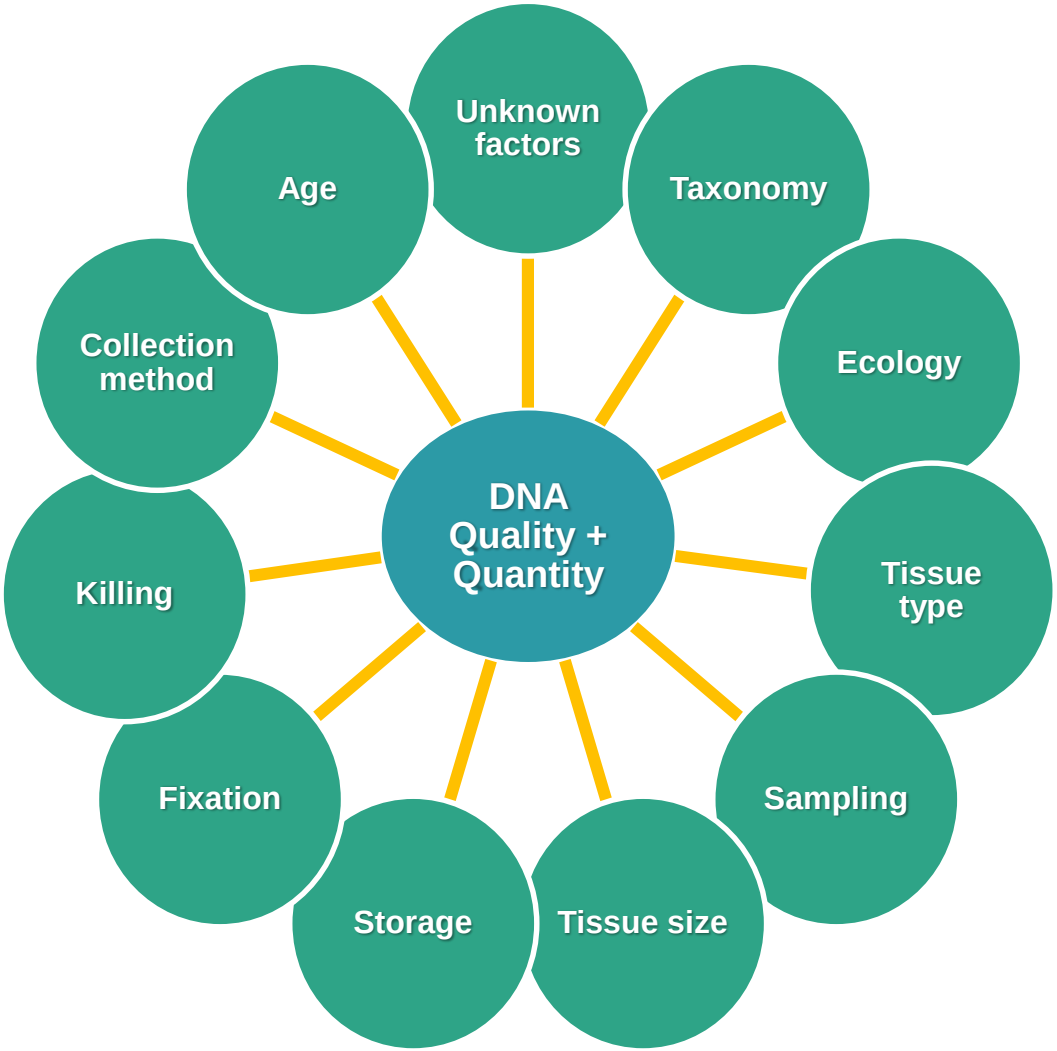
- Four 96-well plates are consolidated into one 384-well PCR plate
- Save on reagents by 50%
- Speeds up the process





PCR

Success depends on…



PCR

Major contributing factor… Age!

0-15 Years: full-length fragment (**Sanger sequencing**)

15-60 Years: two overlapping fragments of 300-400bp (**Sanger sequencing**)

60-240+ Years: 1 to 15 short overlapping fragments of approx. 100-200bp (**Sanger sequencing or NGS**); clean lab usually used.

* NOTE Each method is followed by subsequent failure tracking depending on initial results

PCR

Age: 0-15 Years

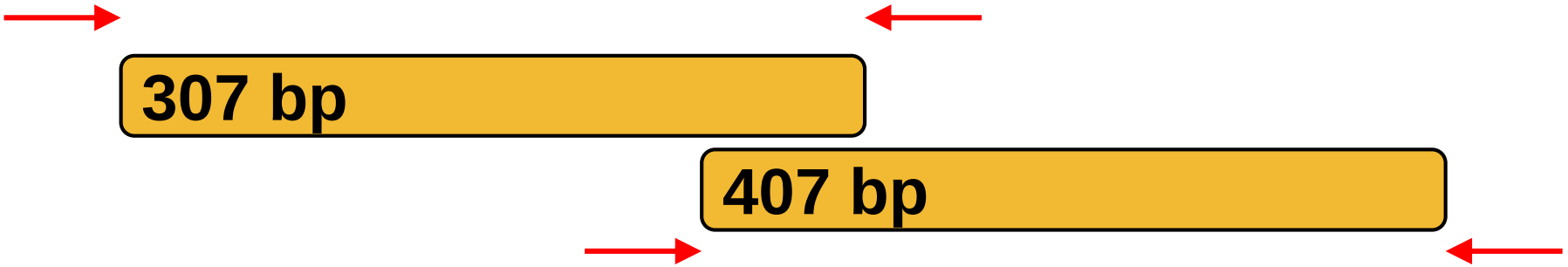
Step 1: Amplify full-length barcode using a single primer set:

Step 2: If fails, proceed to protocol for 15-60 year old material

PCR

Age: 15-60 Years

Step 1: Amplify two overlapping fragments using two primer sets, “Mini primers”

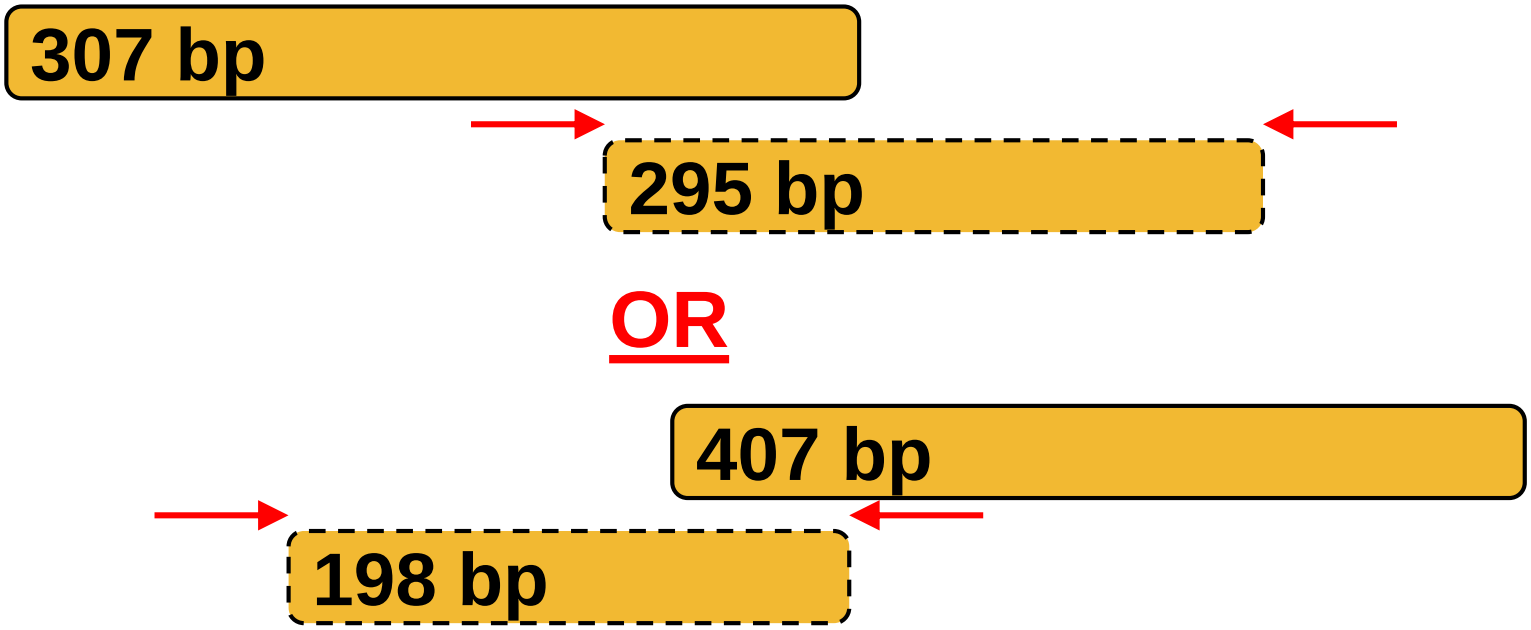


Step 2: If fails, proceed to “rescue protocol” depending on nature of failure

PCR

Age: 15-60 Years

- “Rescuing” incomplete barcode sequences by extending the existing fragment beyond 500bp via amplification of a fragment smaller than the one which failed

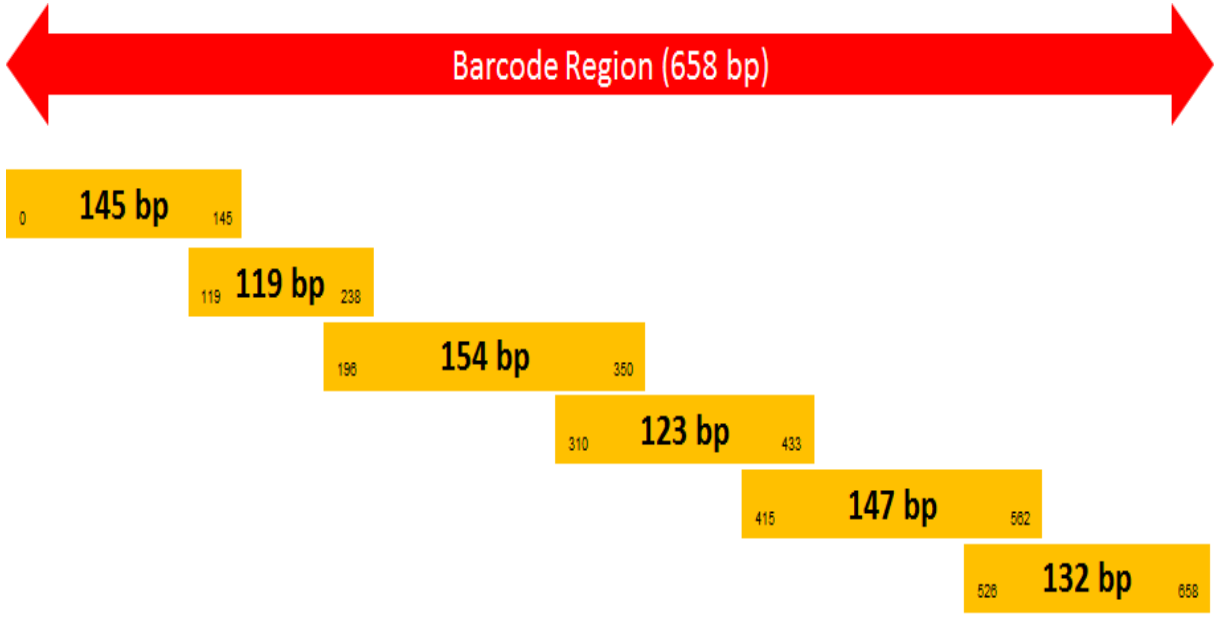


PCR

Age: 60-240+ Years

- Amplifying fragments >200bp is highly unlikely, but several short, overlapping fragments to get full-length barcodes can be recovered using Sanger or NGS sequencing...

PCR: 6rxns
SEQ: 12 rxns



PCR & Sequencing

Summary of Museum Methods

Protocols are in place for recovering full-length barcodes from any museum specimen regardless of age

Age	Target amplicons	Final sequence length	Method	No. reactions (PCR/SEQ)
0 - 15 yrs	658 bp	658 bp	**Sanger**	1/1
15 - 60 yrs	307 bp, 407 bp	658 bp	**Sanger**	2/4
60-240+ yrs	12 amplicons; range from 119 - 366 bp	658 bp	**Sanger or NGS**	6/12

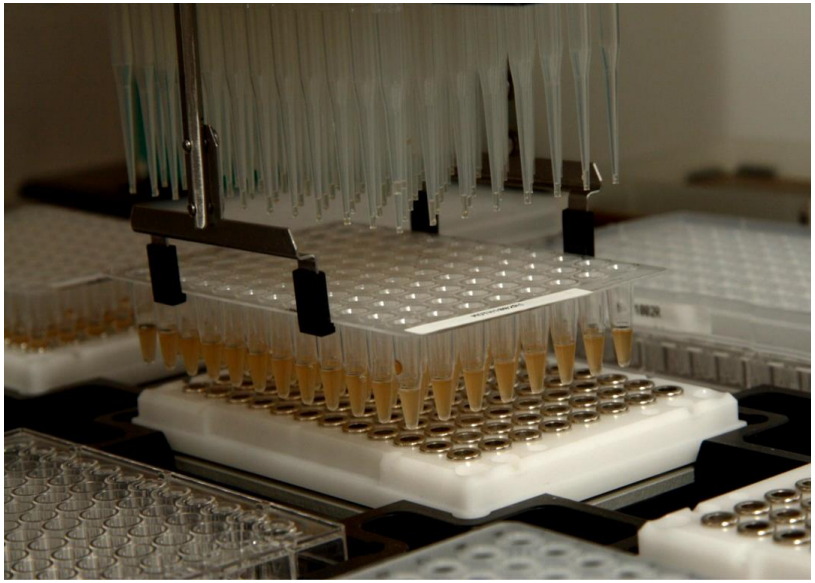
Taxon Specific Protocols

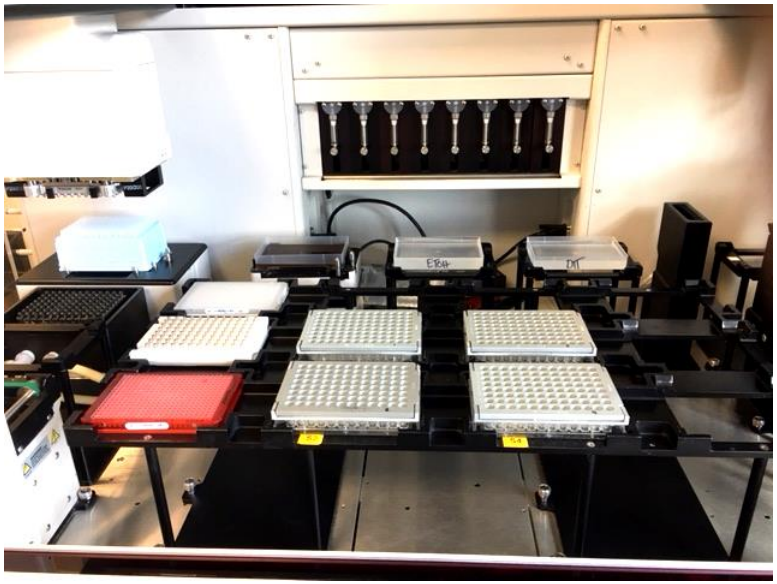
Taxon	Lysis	Marker	First Pass Primers	Failure Tracking Primer
Bivalvia	**CTAB**	**COI-5P**	BivF4_t1 + BivR1_t1	dgLCO-1490 + dgHCO-2198
Gastropoda	**CTAB**	**COI-5P**	C_GasF1_t1 + GasR1_t1	PCR1: [C_GasF1_t1 + MGasR1_t1] PCR2: [MGasF1_t1 + GasR1_t1]
Cnidaria	**CTAB**	**COI-5P**	C_LepFolF + C_LepFolR	R&D Primers
Mollusca	**CTAB**	**COI-5P**	dgLCO-1490 + dgHCO-2198	LCO1490_t1 + HCO2198_t1
Porifera	**CTAB**	**COI-5P**	dgLCO-1490 + dgHCO-2198	R&D Primers
Algae	**CTAB**	**COI-5P**	GHalF + GazR1	GWSFn + GWSRn
Cephalopoda	**CTAB**	**COI-5P**	LCO1490_t1 + HCO2198_t1	
Echinodermata	**CTAB**	**COI-5P**	LCOech1aF1 + HCO2198	EchinoF1 + HCO2198
Bryozoa, Ascidiacea	**CTAB**	**COI-5P**	R&D Primers	
Hemiptera	**invert**	**COI-5P**	LepF2_t1 + LepR1	PCR1:[LepF2_t1 + MHemR] PCR2:[MHemR + LepR1]
Nematoda	**invert**	**COI-5P**	C_NemF1_t1 + C_NemR1_t1	MNemF1_t1 + C_NemR1_t1
Odonata	**invert**	**COI-5P**	OdoF1_t1 + OdoR1_t1	C_LepFolF + C_LepFolR
Platyhelminthes	**invert**	**COI-5P**	plat-diploCOX1F + plat-diploCOX1R	MplatCOX1dF + MplatCOX1dR
Polychaeta	**invert**	**COI-5P**	polyLCO + polyHCO	C_VF1LFt1 + C_VR1LRt1
Rotifera, Tardigrada	**invert**	**COI-5P**	LCO1490_t1 + HCO2198_t1	
Zooplankton (+ small crustaceans)	**invert**	**COI-5P**	ZplankF1_t1 + ZplankR1_t1	LCO1490_t1 + HCO2198_t1
Hymenoptera	**invert**	**COI-5P**	LepF1 + LepR1	PCR1:[LepF1 + C_ANTMR1D] PCR2:[RonMWASPdeg_t1 + LepR1]
Lepidoptera	**invert**	**COI-5P**	LepF1 + LepR1	PCR1:[LepF1 + MLepR2] PCR2:[MLepF1 + LepR1]
Other invertebrates	**invert**	**COI-5P**	C_LepFolF + C_LepFolR	PCR1: [C_LepFolF + MLepR2] PCR2: [MLepF1 + C_LepFolR]
Amphibia + Reptilia	**vert**	**COI-5P**	AmphF2_t1 + AmphR3_t1	C_VF1LFt1 + C_VR1LRt1
Aves	**vert**	**COI-5P**	BirdF1_t1 + COIbirdR2_t1	LTyr + COI908aH2
Fish	**vert**	**COI-5P**	C_FishF1t1 + C_FishR1t1	C_VF1LFt1 + C_VR1LRt1
Mammalia	**vert**	**COI-5P**	C_VF1LFt1 + C_VR1LRt1	C_FishF1t1 + C_FishR1t1
Fungi: Ascomycetes, Lichen	**CTAB**	**ITS**	ITS5 + ITS4	
Fungi: Basidiomycetes	**CTAB**	**ITS**	ITS1-F + ITS4	
Plantae	**CTAB**	**rbcL, matK**	rbcLa-F + rbcLa-R	MatK-1RKIM-f + MatK-3FKIM-r

Sequencing

Sequencing Cleanup

- **Manual** – Sephadex (gel-filtration)
- **Robotic cleanup** – magnetic beads (Aline Biosystems, etc.)





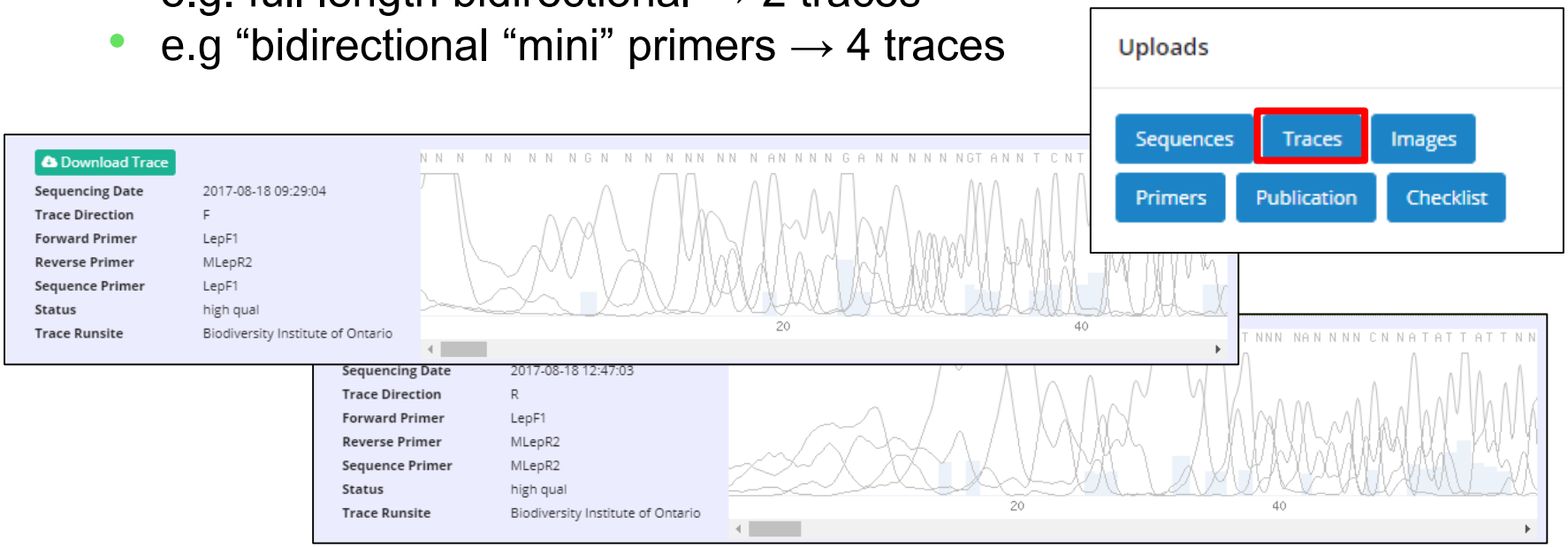
Sequencing

- Five 3730xl DNA sequencers
- Twenty-384 well plates sequenced each day

Sequencing

Trace Upload

- **Raw traces to be uploaded to BOLD for each sequence**
  - Done from BOLD Workbench
  - e.g. full length bidirectional → 2 traces
  - e.g “bidirectional “mini” primers → 4 traces



*** NOTE Helpful for quality control**

Sequencing

Sequence Editing

- Import raw traces into a sequence editing software (CodonCode)
- Align fragments, museum specimen may generate 4-6 fragments
- Remove primer sequences
- Form contigs
- Make base calls, avoid calling double peaks
- Due to lack of overlap, some fragments may not form a contig
- Contigs do not form due to chimeric sequences
- Longest fragment best matching the taxonomy is selected for upload

Failure Tracking

Positive Hit-Picking

Threshold Value is 75 Bands

E-gel Evaluation

>75 Bands

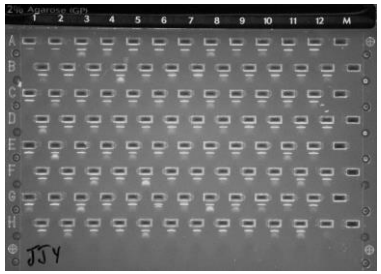
< 75 Bands

Sequencing

Positive Hit-Pick

Sequence Positives

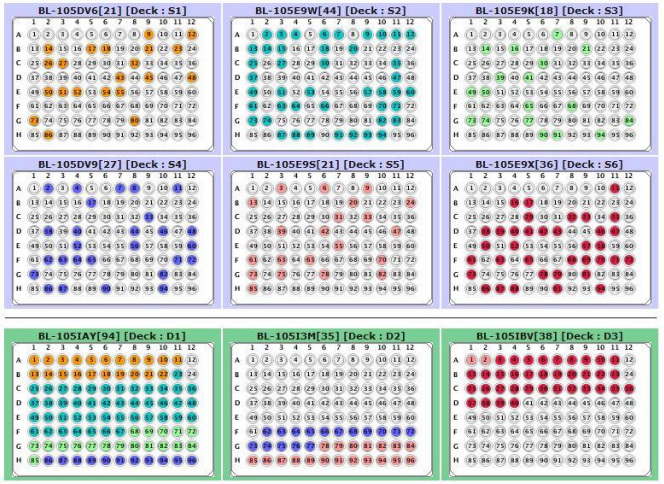
Failure Tracking



Failure Tracking

Hit-Picking – LIMS

C4		0	0	GREAR139-17	2.26 yrs
C5		0	0	GREAR140-17	2.22 yrs
C6		1	1	GREAR141-17	2.26 yrs
C7		1	1	GREAR142-17	2.22 yrs
C8		0	0	GREAR143-17	2.22 yrs
C9		1	1	GREAR144-17	2.22 yrs
C10		0	0	GREAR145-17	2.26 yrs
C11		0	0	GREAR146-17	2.26 yrs
C12		0	0	GREAR147-17	2.17 yrs
CM		0	0		
D1		0	0	GREAR148-17	2.26 yrs
D2		0	0	GREAR149-17	2.26 yrs
D3		1	1	GREAR150-17	2.26 yrs
D4		0	0	GREAR151-17	2.26 yrs
D5		0	0	GREAR152-17	2.26 yrs
D6		1	1	GREAR153-17	2.26 yrs
D7		1	1	GREAR154-17	2.17 yrs
D8		1	1	GREAR155-17	2.17 yrs
D9		0	0	GREAR156-17	2.26 yrs
D10		0	0	GREAR157-17	2.26 yrs
D11		1	1	GREAR158-17	2.26 yrs
					2.22



DNA Archiving

Storing DNA Plates in the Archive

Locate By Plate

Select Search By * Search by Name

Search Text * CCDB-02600 Submit Clear

Plate Name CCDB-02600 Update

Location : BIO-LAB > CBG > Room LL-002 > Deep Freezer 6 > Shelf 3 > R112 > Row 8 > Col D

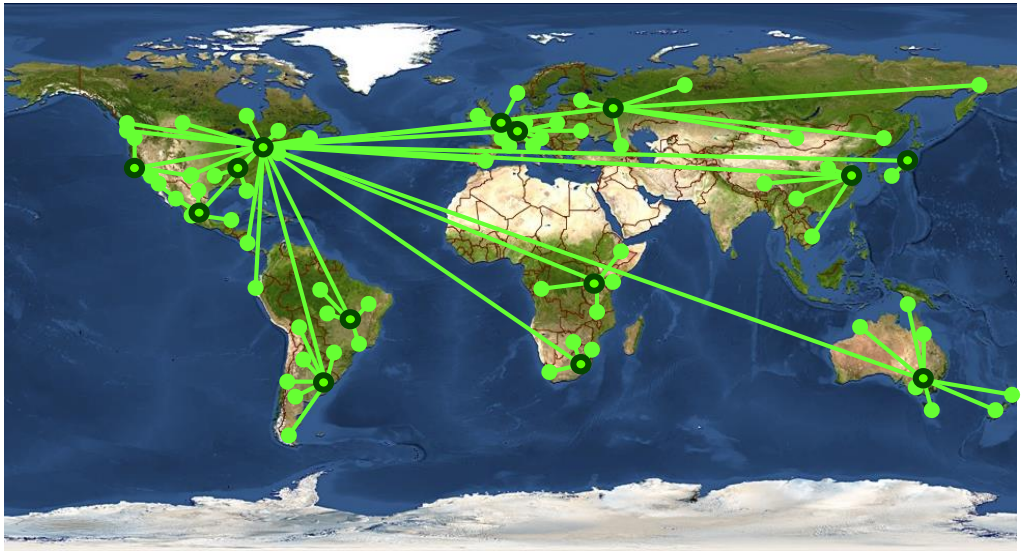
Plate Information

Sample Id	Coordinate
10-SRNP-100588	A01
10-SRNP-100589	A02
10-SRNP-100590	A03
10-SRNP-100591	A04
10-SRNP-100592	A05
10-SRNP-100593	A06
10-SRNP-100594	A07
10-SRNP-100595	A08
10-SRNP-100596	A09
10-SRNP-100597	A10
10-SRNP-100598	A11
10-SRNP-100599	A12
10-SRNP-100600	B01
10-SRNP-100601	B02
10-SRNP-100602	B03
10-SRNP-100603	B04
10-SRNP-100604	B05

Current capacity of DNA bank: ~5.5 million

DNA Archiving

Why do we Need Dry DNA Storage Systems?



- DNA & PCR products exchange between iBOL nodes
- Backup system for freezers

DNA Archiving

Trehalose & Preservation

The 15% solution for preservation

Analysis of biomolecules continues to revolutionize our understanding of the diversity of life and evolution. Unfortunately, for many taxa the only reliable method of preserving both their proteins and nucleic acids is to freeze samples below −80°C (Ref. 1). This makes collection difficult in remote and tropical locales where the biological knowledge gap is greatest. In a recent letter in *TREE*[2], and elsewhere[3], solvents have been suggested for *in situ* preservation of biomolecules. However, such treatments often result in low-quality DNA[4] as well as denaturation and leakage of proteins into the solvent[2]. Recently it has been shown that desiccation in trehalose (a disaccharide of glucose) dramatically increases the stability and heat durability of purified proteins and biological membranes[5–7]. Here, we report that air-drying of biological samples in 15% (w/v) trehalose is an effective and logistically simple alternative to freezing for allozyme and DNA field studies.

We modified the protocol of Colaço *et al.*[5] in an attempt to preserve biomolecules in tissues from *Daphnia pulex*, *Drosophila melanogaster* and goldfish (*Carassius auratus*). Samples were stored either frozen in water (at −80°C), air-dried in 15% trehalose (at 37°C for 12 hrs followed by storage at ambient temperature) or air-dried in water (controls). For *Daphnia* assays, two live adults were randomly assigned to microplate wells with 20 µl of either 15% trehalose or water for drying; or to 20 µl of water in microcentrifuge tubes for freezing. For assays with the other taxa, tissues were ground to facilitate exposure to the trehalose solution. Extracts of goldfish muscle (300 mg/ml) and *Drosophila* (30 mg/ml) were prepared by grinding and centrifugation (15 min @ 14 000 *g*) in either 15% trehalose or water. Trehalose-free extracts were halved into −80°C and air-dried treatments. Extract aliquots of 10 µl were used for each treatment.

After four weeks, samples were rehydrated to original volumes, standard cellulose acetate electrophoresis[8] was conducted, and staining intensities were quantified by scanning transmittance densitometry. We found that trehalose drying provided excellent preservation of both allozyme activity and structure. Among the 45 allozyme loci treated with trehalose, only one locus in one species (*Got* in *Drosophila*) showed a significant loss in activity compared to the −80°C treatments and only two loci showed enzymatic breakdown (*Got* and *Fum* in *Drosophila*). Furthermore, three *Drosophila* loci treated with trehalose actually showed significantly greater activity than the −80°C treatments. Drying without trehalose, however, resulted in significant breakdown or activity reduction at 71% of all loci. Although our study demonstrates enzyme stability for just a month, trehalose-dried restriction enzymes have been stored for 98 days and blood antibodies for several years without loss of original activities[9].

An added benefit of trehalose drying is the preservation of DNA, which is well-known to be protected by drying[4]. DNA extracted from trehalose-preserved *Daphnia* (one month) was of comparable quality and yield to DNA from fresh specimens. We also successfully PCR-amplified 600–1800 bp mtDNA products from trehalose-dried *Drosophila* and *Daphnia* tissue extracts and conclude that our preservation technique has broad applications.

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References

1 Dessauer, H.C., Cole, C.J. and Hafner, M.S. (1991) in *Molecular Systematics* (Hillis, D.M. and Moritz, C., eds), pp. 25–41, Sinauer Press
2 Oakenfull, E.A. (1994) *Trends Ecol. Evol.* 9, 26
3 Nakanishi, M., Wilson, A.C., Nolan, A., Gorman, G.C. and Bailey, G.S. (1969) *Science* 163, 681–683
4 Pyle, M.M. and Adams, R.P. (1989) *Taxon* 38, 576–581
5 Colaço, C., Sen, S., Thangavelu, M., Pinder, S. and Roser, B. (1992) *Bio/Technology* 10, 1007–1011
6 Carpenter, J.F. and Crowe, J.H. (1988) *Cryobiology* 25, 459–470
7 Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) *Science* 223, 701–703
8 Hebert, P.D.N. and Beaton, M.J. (1989) *Methodologies for Allozyme Analysis Using Cellulose Acetate Electrophoresis*, Helena Laboratories, Beaumont, TX, USA

1994. Trends Ecol. Evol. 9: 230

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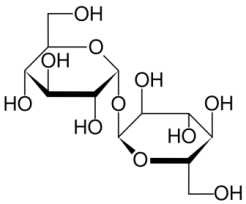
2007, *111*, 5542−5544
Published on Web 05/02/2007

Natural DNA Mixed with Trehalose Persists in B-Form Double-Stranding Even in the Dry State

Bo Zhu,† Takao Furuki,† Takashi Okuda,‡ and Minoru Sakurai*,†



Research Reports

Long-term storage of DNA-free RNA for use in vaccine studies

Kathryn L. Jones[1,2], Debbie Drane[3], and Eric J. Gowans[1,2]

BioTechniques 43:675-681 (November 2007)
doi 10.2144/000112593

JOURNAL OF CLINICAL MICROBIOLOGY, June 1998, p. 1798–1800 Vol. 36, No. 6
0095-1137/98/$04.00+0

Stabilized, Freeze-Dried PCR Mix for Detection of Mycobacteria

PAUL R. KLATSER,[1]* SJOUKJE KUIJPER,[1] COR W. VAN INGEN,[2] AND AREND H. J. KOLK[1]

J Forensic Sci, Sept. 2005, Vol. 50, No. 5
Paper ID JFS2004411
Available online at: www.astm.org

Steve Smith, B.Sc., Hons.[1,2] *and Phillip A. Morin, Ph.D.*[1,3]

Optimal Storage Conditions for Highly Dilute DNA Samples: A Role for Trehalose as a Preserving Agent

DNA Archiving

Biomatrica, PVA and Trehalose

Curr. Issues Mol. Biol. 12: 135-142. Online journal at http://www.cimb.org

Green Technologies for Room Temperature Nucleic Acid Storage

Eunice Wan[1], Matthew Akana[1], Jennifer Pons[1], Justin Chen[1], Stacy Musone[1], Pui-Yan Kwok[1,2,3], Wilson Liao[2].

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Abstract

Maintaining the long-term integrity of nucleic acids in the laboratory has traditionally required the use of freezers. However, novel nucleic acid stabilization technologies may allow for the storage of DNA and RNA at room temperature in a cost-effective, environmentally friendly manner. In this study, we evaluated two novel products for room temperature DNA storage: Biomatrica's DNA SampleMatrix technology and GenVault's GenTegra DNA technology. We compared the integrity and quality of DNA stored using these products against DNA stored in a -20°C freezer by performing downstream testing with short range PCR, long range PCR, DNA sequencing, and SNP microarrays. In addition, we tested Biomatrica's RNAstable product for its ability to preserve RNA at room temperature for use in a quantitative reverse transcription PCR assay.

Introduction

The rapid growth of genomics research has led to an unprecedented need for the storage of large numbers of biological specimens, including DNA, RNA, proteins, cells, and tissues. The traditional method for long-term storage of such specimens has been through laboratory freezers at -20°C, -80°C, or in liquid nitrogen. However, emerging technologies may offer the opportunity to store these samples at room temperature, thus reducing the carbon footprint associated with freezers. Two companies, Biomatrica (San Diego, CA) and GenVault (Carlsbad, CA), have developed products to store nucleic acids at room temperature.

Biomatrica's DNA SampleMatrix is based on a glass polymer that "shrink-wraps" and protects DNA from heat and UV light through a mechanism similar to that used by extremophiles, small organisms that can survive in dry environments for up to 120 years (Crowe et al., 1998).. The polymer is dispensed as a dissolvable coating on the inner surface of individual tubes or 96- or 384-well plates. After the addition of DNA in solution, the sample is dried down for room temperature storage. When ready for use, DNA is recovered by the addition of water or buffer. Similarly, Biomatrica's RNAstable product relies on a synthetic matrix that forms a thermostable barrier around RNA during the drying process. Characteristics of Biomatrica's DNA SampleMatrix and RNAstable are presented in Table 1.

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GenVault's GenTegra DNA is an inorganic mineral matrix with oxidation protection and antimicrobial activity for storage of purified DNA at room temperature. GenTegra DNA is supplied as a transparent coating at the bottom of each GenTegra DNA tube. Purified DNA is added to the GenTegra tube and dried down in a laminar flow hood or GenVault's FastDryer, a boxed enclosure with built-in fans. Recovery of DNA occurs with the addition of water. GenVault also offers GenPlates, a product consisting of FTA paper elements placed into a multi-well plate. Whole blood or buffy coat is aliquoted onto the paper elements, allowing for room temperature storage and downstream DNA recovery. Characteristics of GenTegra DNA are listed in Table 1. As the cellulose FTA paper in GenPlates does not represent a new technology (Hsaio et al., 1999; Smith and Burgoyne, 2004), additional information on this technology is not shown in Table 1.

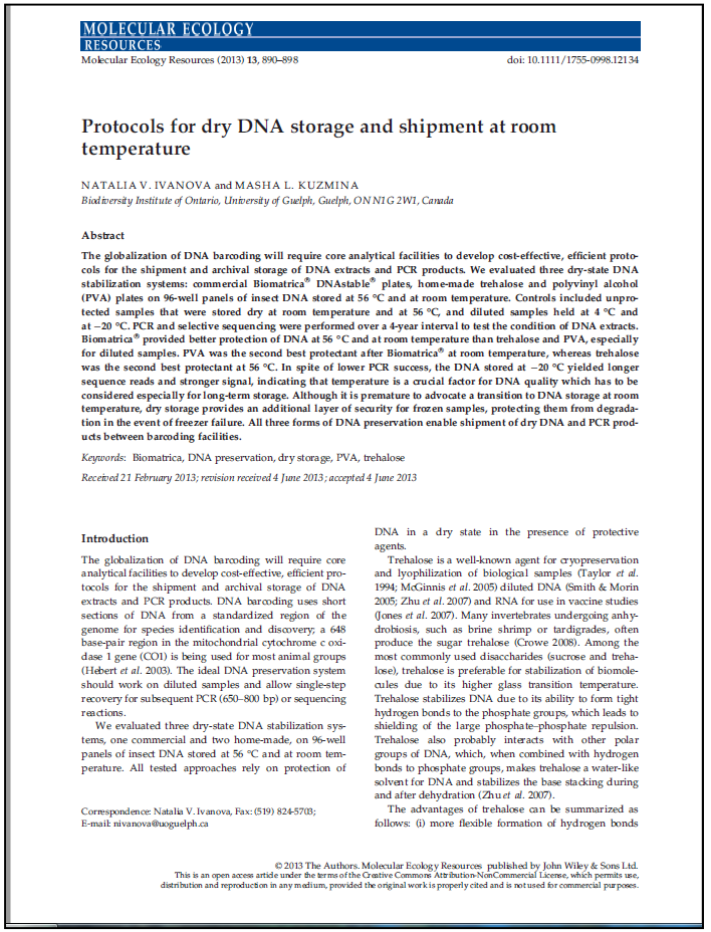
In this study, we evaluated the integrity and quality of DNA stored for 3 weeks at room temperature using Biomatrica and GenVault products against DNA stored at -20°C. Our downstream testing included short range PCR, long range PCR, DNA sequencing, and SNP microarrays. In addition, we tested Biomatrica's RNAstable product for its ability to preserve RNA at room temperature for use in a quantitative reverse transcription PCR assay. Finally, we tested the ability of Biomatrica's GenPlates to store whole blood samples at room temperature.

Materials and Methods

Human genomic DNA from 8 different whole blood samples was extracted using Machery-Nagel Nucleospin Blood XL Kits according to the manufacturer's instructions. The concentration of the DNA was determined using a NanoDrop 1000. 1-5 µg of DNA in 20 µl water was aliquoted into Biomatrica SampleMatrix wells or GenTegra tubes, with the remaining DNA kept frozen at -20°C. For SampleMatrix, drying was performed overnight in a laminar flow hood. After drying, the DNA plate was sealed in aluminum foil and stored at room temperature in a desiccant dry box. DNA was stored at room temperature 1 week for GeneChip analysis and 3 weeks for all other assays. For GenTegra, drying was performed overnight in the GenVault FastDryer unit. GenTegra tubes were capped and stored at room temperature in a drawer for 3 weeks. DNA was recovered by the addition of 20 µl water. The concentration of rehydrated DNA was measured using a NanoDrop 1000.

DNA recovered from room temperature storage and -20°C frozen storage was run on a 2% agarose gel. Both types of DNA were subjected to four downstream applications:

1) Short Range PCR, 531 bp product
from *ZNF750* gene, forward primer
5'-AATACTGTGCCTCCCAGGGTAT-3', reverse primer
5'-GTACTTACCAGAGGTGGGCAGTG-3', PCR
conditions: 95°C for 5 min; 34 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min; 72°C for 10 min



MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2013) 13, 890–898 doi: 10.1111/1755-0998.12134

Protocols for dry DNA storage and shipment at room temperature

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Abstract

The globalization of DNA barcoding will require core analytical facilities to develop cost-effective, efficient protocols for the shipment and archival storage of DNA extracts and PCR products. We evaluated three dry-state DNA stabilization systems: commercial Biomatrica® DNAstable® plates, home-made trehalose and polyvinyl alcohol (PVA) plates on 96-well panels of insect DNA stored at 56 °C and at room temperature. Controls included unprotected samples that were stored dry at room temperature and at 56 °C, and diluted samples held at 4 °C and at −20 °C. PCR and selective sequencing were performed over a 4-year interval to test the condition of DNA extracts. Biomatrica® provided better protection of DNA at 56 °C and at room temperature than trehalose and PVA, especially for diluted samples. PVA was the second best protectant after Biomatrica® at room temperature, whereas trehalose was the second best protectant at 56 °C. In spite of lower PCR success, the DNA stored at −20 °C yielded longer sequence reads and stronger signal, indicating that temperature is a crucial factor for DNA quality which has to be considered especially for long-term storage. Although it is premature to advocate a transition to DNA storage at room temperature, dry storage provides an additional layer of security for frozen samples, protecting them from degradation in the event of freezer failure. All three forms of DNA preservation enable shipment of dry DNA and PCR products between barcoding facilities.

Keywords: Biomatrica, DNA preservation, dry storage, PVA, trehalose

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Introduction

The globalization of DNA barcoding will require core analytical facilities to develop cost-effective, efficient protocols for the shipment and archival storage of DNA extracts and PCR products. DNA barcoding uses short sections of DNA from a standardized region of the genome for species identification and discovery; a 648 base-pair region in the mitochondrial cytochrome c oxidase 1 gene (CO1) is being used for most animal groups (Hebert *et al.* 2003). The ideal DNA preservation system should work on diluted samples and allow single-step recovery for subsequent PCR (650–800 bp) or sequencing reactions.

We evaluated three dry-state DNA stabilization systems, one commercial and two home-made, on 96-well panels of insect DNA stored at 56 °C and at room temperature. All tested approaches rely on protection of DNA in a dry state in the presence of protective agents.

Trehalose is a well-known agent for cryopreservation and lyophilization of biological samples (Taylor *et al.* 1994; McGinnis *et al.* 2005) diluted DNA (Smith & Morin 2005; Zhu *et al.* 2007) and RNA for use in vaccine studies (Jones *et al.* 2007). Many invertebrates undergoing anhydrobiosis, such as brine shrimp or tardigrades, often produce the sugar trehalose (Crowe 2008). Among the most commonly used disaccharides (sucrose and trehalose), trehalose is preferable for stabilization of biomolecules due to its higher glass transition temperature. Trehalose stabilizes DNA due to its ability to form tight hydrogen bonds to the phosphate groups, which leads to shielding of the large phosphate–phosphate repulsion. Trehalose also probably interacts with other polar groups of DNA, which, when combined with hydrogen bonds to phosphate groups, makes trehalose a water-like solvent for DNA and stabilizes the base stacking during and after dehydration (Zhu *et al.* 2007).

The advantages of trehalose can be summarized as follows: (i) more flexible formation of hydrogen bonds

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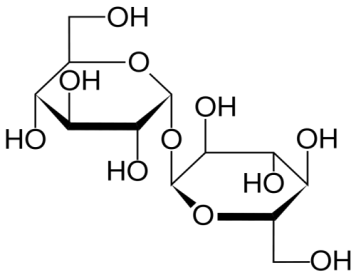
Quality Control

DNA & PCR Products Recommendations

- Convenient shipment of DNA and PCR products at room temperature
- Backup options for -20 or -80°C archiving
- Trehalose and PVA are sufficient for shipment
- Biomatrica is a better choice for forensic, degraded or diluted samples
- More data is needed to advocate transition to dry storage at room temperature

Biomātrica®
THE BIOSTABILITY COMPANY



Quality Control

Shipping of Insect Abdomen Lysates

Follow GLP

Commercial kits available (ATL buffer, Qiagen)

1 Prepare plate/tubes

- Clean bench area with dilute bleach.
- Add 180 µl ATL buffer to each tube/well.
- Add 20 µl Proteinase K to each tube/well.

2 Harvesting abdomens

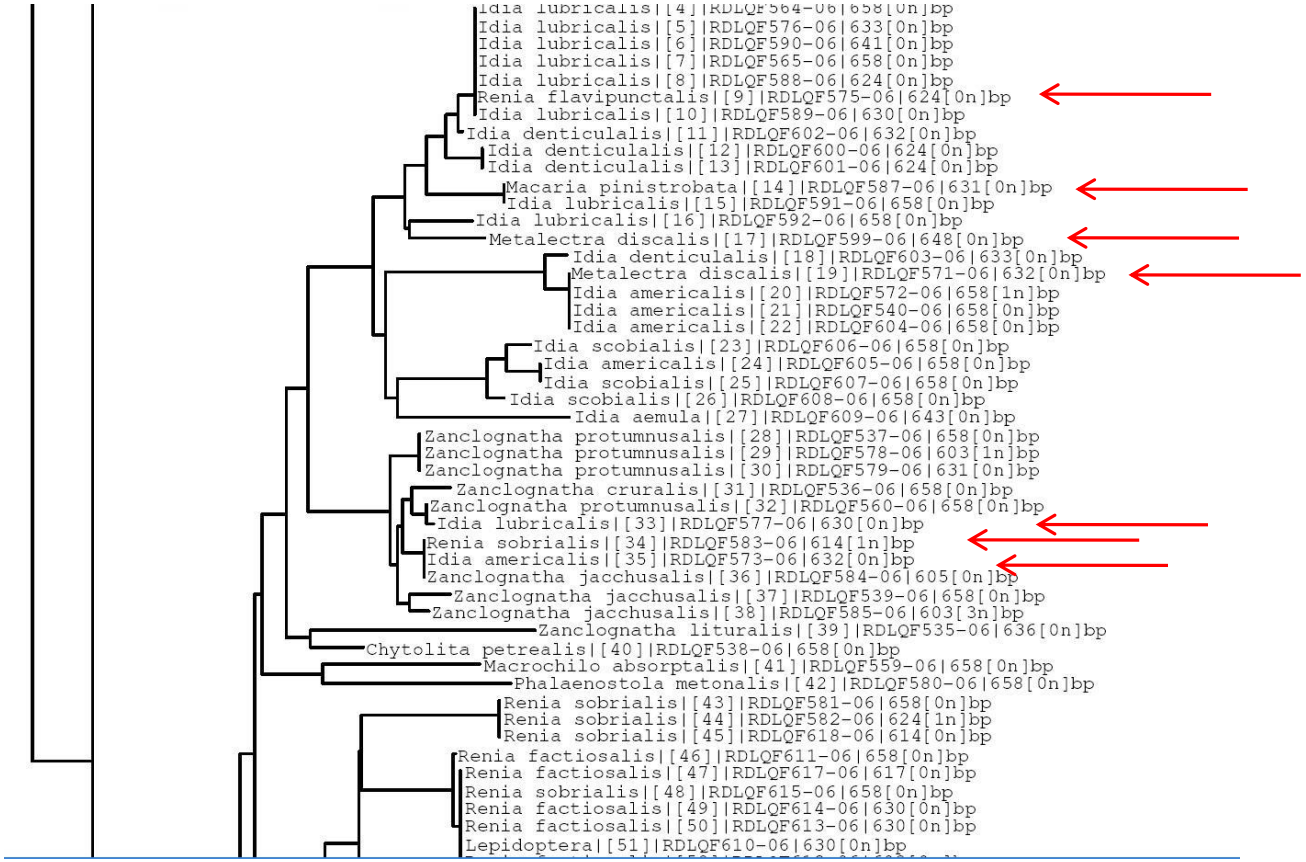
- Treat forceps in bleach and ethanol.
- Remove abdomen to tube/plate
- Incubate over night at 55°C

3 Collecting lysate

- Remove lysate from around abdomens to a lysis plate.
- Seal lysis plate and store in freezer
- Ship frozen

Quality Control

Failure to Match Identification



Quality Control

Failure to Match Identification

Inability to provide species identification due to:

- Failure to yield barcode sequence
- Loss of data integrity
- Failure to resolve species

1 Methodological factors

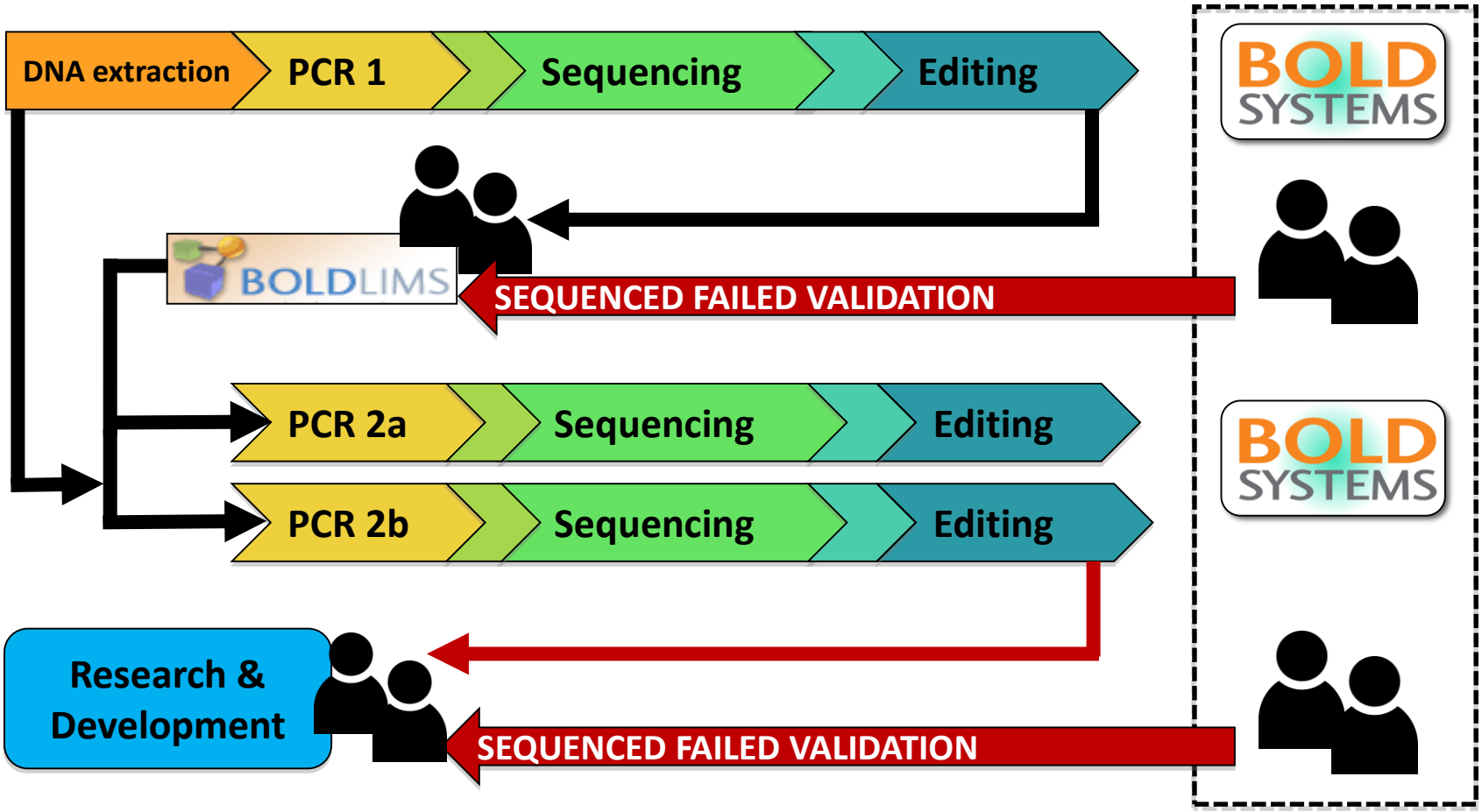
- Data recording errors
- Incorrect identification
- Sequence quality
- Contamination
- Reverse sequence orientation

2 Biological factors

- Incomplete lineage sorting
- Introgressive hybridization
- Pseudogenes (NUMT)
- Symbionts

Quality Control

QA/QC Procedures & Work Flow



CCDB Protocols

http://ccdb.ca/resources/

CCDB
Canadian Centre for DNA Barcoding
Protocols
Glass Fiber Plate DNA Extraction

Supplementary material for the paper:
Ivanova NV, deWaard J, Hebert PDN (2006) An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes*, 6, 998-1002.

CCDB - DNA Extraction

Simple methods of DNA extraction, such as a proteinase K digestion and/or Chelex extraction are usually sufficient especially while dealing with fresh specimens, but give poor quality DNA extracts.

In 2006 CCDB directed its efforts towards the development of an automation-friendly inexpensive method yielding high-quality DNA extracts. This resulted in a major advance – the elaboration of a membrane-based protocol that matches the performance of the best commercial kits, but is 75% less expensive ($0.50 versus >$2.00 per sample). Currently this protocol replaced previously used Chelex extraction for fresh specimens and commercial kits – GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) and Genomic DNA Nucleo-Spin® 96 Tissue Kit (Machery-Nagel) used for archival specimens.

We have now employed both manual and robotic versions of our DNA extraction protocol on more than 50000 vertebrate and invertebrate specimens (>5000 species) with highly positive results.

In September 2006 CCDB purchased two Biomek® FXP liquid handling stations both equipped with 96 multichannel head and Thermo Cytomat hotel. Currently our Biomek® FXP is able to process 20 96-well plates in ~5 hours of unattended operation.

Manual Protocol: Centrifugation Method

AcroPrep™ PALL Glass Fiber plate

Bind & wash on a square-well block.

Position GF plate for elution step with a PALL collar.

Glass fiber (GF) plate DNA isolation for recent and archived specimens:

Note: Use Insect Lysis Buffer and PALL2 plate for DNA extraction from Arthropods and centrifuge PALL2 plates at 6000 g.

1. For 1 plate mix 5 ml of Vertebrate Lysis Buffer and 0.5 ml of Proteinase K, 20 mg/ml in a sterile container. Add 50 µl of Lysis Mix to each well of 96-well Eppendorf plate.
2. Add a small amount of tissue (e.g. 2-4 mm of insect leg or 2-3 mm³ of ethanol preserved tissue) to each well of 96-well solid skirted microplate (flame sterilize instruments between samples), cover plate with caps.
3. Incubate at 56°C for a minimum of 6 hours or overnight to allow digestion.
4. Centrifuge at 1500 g for 15 sec to remove any condensate from the cap strips.
5. Add 100 µl of Binding Mix to each sample using multichannel pipette. Cover plate with cap strips. Shake vigorously for 10 – 15 sec and centrifuge at 1000 g for 20 sec to remove any sample from the cap strips.
6. Remove cap strips and transfer the lysate (about 150 µl) from the wells of microplate into the wells of the GF plate (PALL1) placed on top of a square-well block using multichannel pipette. Seal the plate with self-adhering cover.

CCDB Protocols www.dnabarcoding.ca
By Natalia Ivanova, Jeremy deWaard & Paul Hebert — Canadian Centre for DNA Barcoding

CDB
Centre for DNA Barcoding
Protocols
COI Amplification

erase choice

ling production rates demand high success
f the barcode region. One particularly criti-
CR amplification is the polymerase enzyme.
of DNA barcoding protocols across a broad
mic groups, from insects to mammals, it
e higher-cost polymerase from Invitrogen™
NA Polymerase) delivered both greater in-
and amplification success in cases where
ed. Results indicated that Platinum® *Taq* of-
erformance, and it is now the standard PCR
the CCDB. Platinum® *Taq* also offers a num-
er standard *Taq* polymerases. It is a robust
ds less optimization compared to standard
® *Taq* requires a "hot start" for activation,
me breakdown and fewer non-specific PCR
m® *Taq* is also stable at room temperature,
nced preparation and storage of PCR plates

lose facilitates PCR and makes possible
oted master-mixes. Currently CCDB uses
r making PCR plates. Mixes are aliquoted
ell plates, using Biomek® FX robot, plates
PCR film and stored at -20°C for up to 3
tch is labeled, recorded in the system and
performance. The combination of a ther-
m® *Taq* with trehalose ensures high perfor-
multiple freeze-thaws. Results with regular
may be less satisfactory.

can be stored at -20°C for up to 3 months
cycles don't affect performance). The con-
uld be mixed by pipetting before use.

& Equipment for PCR amplification

: dissolve of 5 g D-(+)-trehalose dehydrate
0), in 50 ml of total volume of molecular
Store at –20°C.
buffer for Platinum *Taq* (Invitrogen™).
.
(Invitrogen™). Store at –20°C.
mix (New England Biolabs®). Store at
µl aliquots.
r stock: dissolve desiccated primer (Invitro-
umber of nmol x 10 µl ultrapure H_2O. Store

- 10 µM primer working solution: add 20 µl of 100 µM primer stock to 180 µl of molecular grade ddH_2O. Store at –20°C.
- Platinum *Taq* polymerase (Invitrogen™). Store at –20°C in 50 µl aliquots.
- Microplate (Eppendorf® plates).
- Cap strips (ABgene®) or Aluminum sealing film.
- Thermocycler (Mastercycler® ep gradient, Eppendorf®).

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	

Aliquot 1/8 of total mix volume in 8-tube PCR strip (if making more than one plate, pour mix into disposable container) and dispense desired volume (10.5 µl for 12.5 µl reactions) in 96-well plate and then add 1-2 µl of DNA extract. If you plan to fill several 96-well plates include extra volume to allow for pipetting mistakes and dead volume in the digital multichannel pipettor (e.g. for making 10 plates with 12.5 µl reactions each, include about 40 extra reactions).

General recommendations

- The use of filter tips is recommended for all PCR reagents to avoid contamination. Clean the bench top with alcohol before setting up reactions.
- Always use a sterile tip when removing *Taq* polymerase and the other reagents from their tubes.
- Keep DNA templates (i.e. other PCR products) away from the PCR reagents while you are setting up the reaction mixes. Add DNA after all of the reagents have been returned to the freezer.
- Always include a sample without template as a negative

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DB
re for DNA Barcoding
Protocols
Sequencing

equencing of COI for the
yzer (Applied Biosystems)

reaction recipe will depend on the
ethod. Recently, CCDB switched
utoDTR™ method from EdgeBio®.
p method is less sensitive to PCR
nd allows longer high quality reads
BigDye™ in a sequencing reaction,
vity. However, Sephadex® clean-up
ethod that could be used in any low
acility.

tion setup
p

action according to the table below.
urified PCR product per sequencing

1 Reaction	96-well Plate: 104 Reactions
0.25 µl	26 µl
1.875 µl	195 µl
5 µl	520 µl
1 µl	104 µl
0.875 µl	91 µl
9 µl	936 µl
0.5 - 1.2 µl	

r is: 400 mM Tris-HCl pH 9.0 + 10 mM
g buffer.

olume (11.5 µl) in 8-tube PCR strip (if
late, pour the mix into a disposable
desired volume (9 µl) in 96-well plate
l of PCR product.

kes possible the freezing of aliquoted
ently, CCDB uses a batch strategy
plates. Mixes are aliquoted directly
g the Biomek® FX robot; plates are
d stored at -20°C for up to 3 months.
ecorded in the system and tested to

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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Acknowledgements

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CBG

UNIVERSITY of GUELPH

CANADA FIRST RESEARCH EXCELLENCE FUND

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