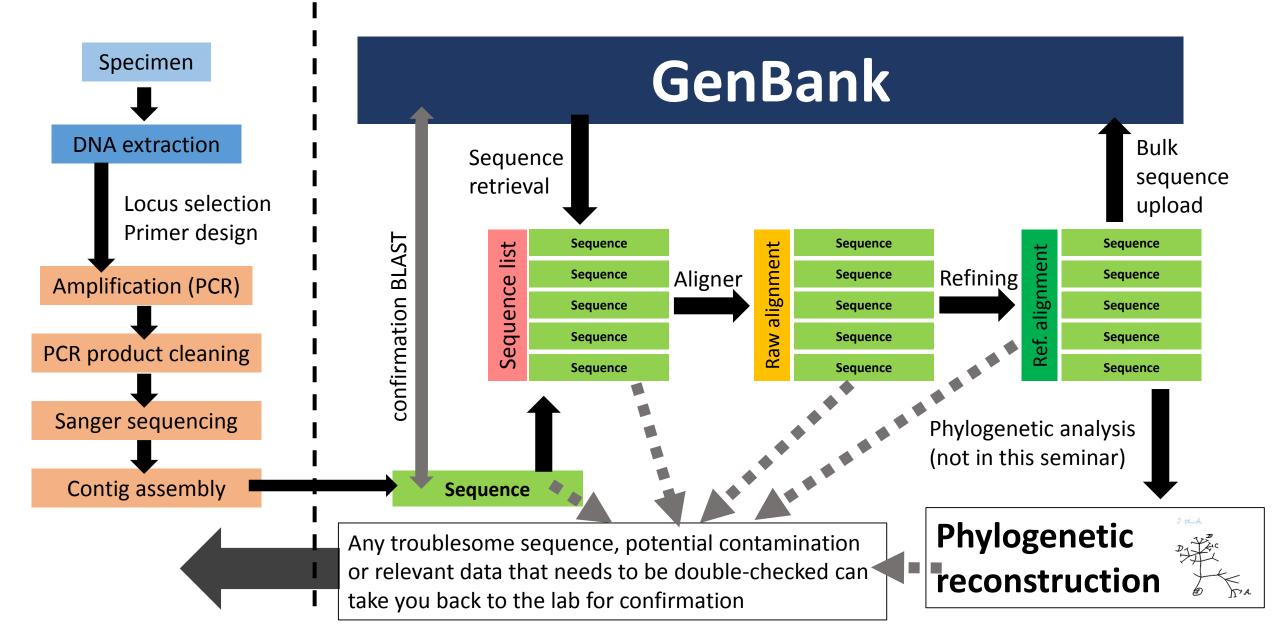
A primer for practical phylogenetic data gathering. Uconn EEB3899-007. Spring 2015 Session 1

# DNA extraction, amplification and sequencing



# A road map to phylogenetic data gathering



- DNA extraction
- DNA amplification (PCR)
- Validation
- PCR product cleaning
- Sequencing reaction
- Preparation for ABI sequencing
- Contig assemblage and sequence exportation

### • DNA extraction

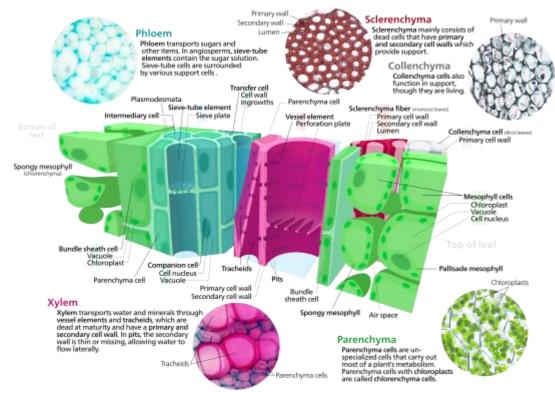
- DNA amplification (PCR)
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Typical steps, shared by different kits or protocols:

#### 1. Mechanical homogenization

- Grinding with mortar/pestle and sand/liquid N<sub>2</sub>
- Bead beater
- Heat shock

### Plant cells: tough nuts to crack!

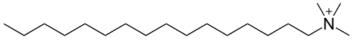


#### 2. Digestion

Relies on the presence of <u>chaotropic salts</u>, <u>denaturing agents</u> and <u>detergents</u> (often mixed in a "lysis buffer")



SDS (Sodium Dodecyl Sulfate)



Br⁻

CTAB (cetyltrimethylammonium bromide)

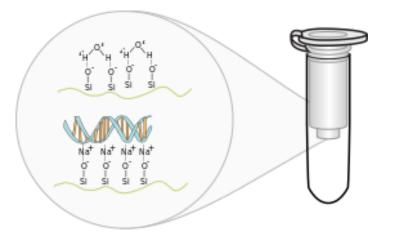
- Performed usually at 65°C
- Longer digestion times may improve results
- RNAse is usually recommended in many protocols

The clean lysate may be transferred after filtering or precipitation of a pellet

#### 3. Binding and cleaning

#### A. Silica membrane kit

- Binding buffer
- DNA stays in the membrane

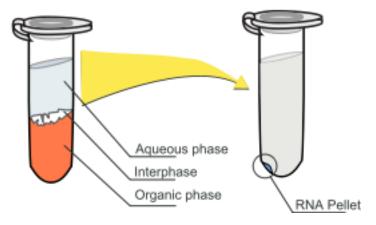


#### B. Phenol-chloroform extraction

- Lysate is divided in two phases
- DNA is precipitated with isopropanol

Phase separation

Isopropanol precipitation

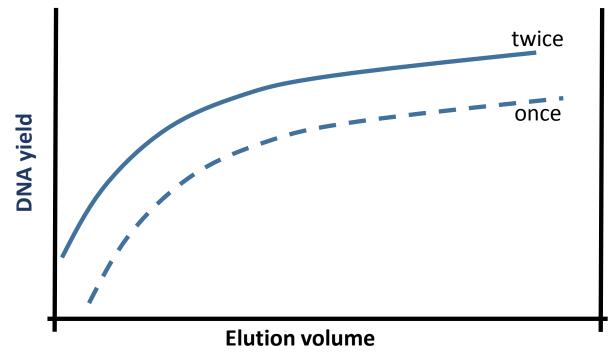


- In both cases, the DNA is washed with ethanol-based buffers
- The ethanol must be evaporated completely before elution

4. Elution

Usually performed with (warm) Tris-HCl buffer. EDTA based buffers are not recommended

Optimization of results may involve playing with number of elutions, volume and time

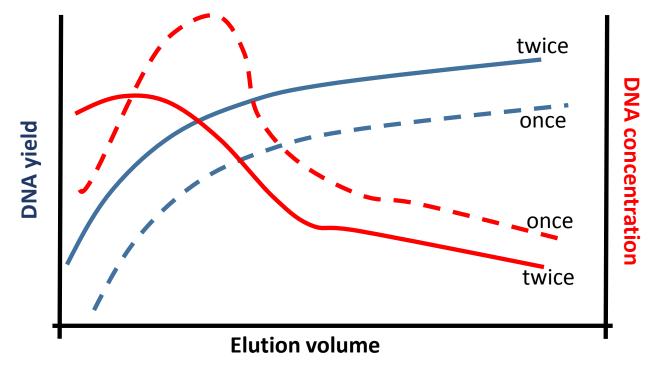


from Macherey-Nagel NucleoSpin

4. Elution

Usually performed with (warm) Tris-HCl buffer. EDTA based buffers are not recommended

Optimization of results may involve playing with number of elutions, volume and time



from Macherey-Nagel NucleoSpin

#### 5. Storage

Please, please, PLEASE: label and database your DNA sample so it can be used in the future

Double stranded DNA is very stable in Tris-HCl buffer or even in water

- 20°C for medium term
- 80°C long-term



#### **Quality control (optional)**

Nanodrop or Qubit

#### 5. Storage

Please, please, PLEASE: label and database your DNA sample so it can be used in the future

Double stranded DNA is very stable in Tris-HCl buffer or even in water

- 20°C for medium term
- 80°C long-term



#### **Quality control (optional)**

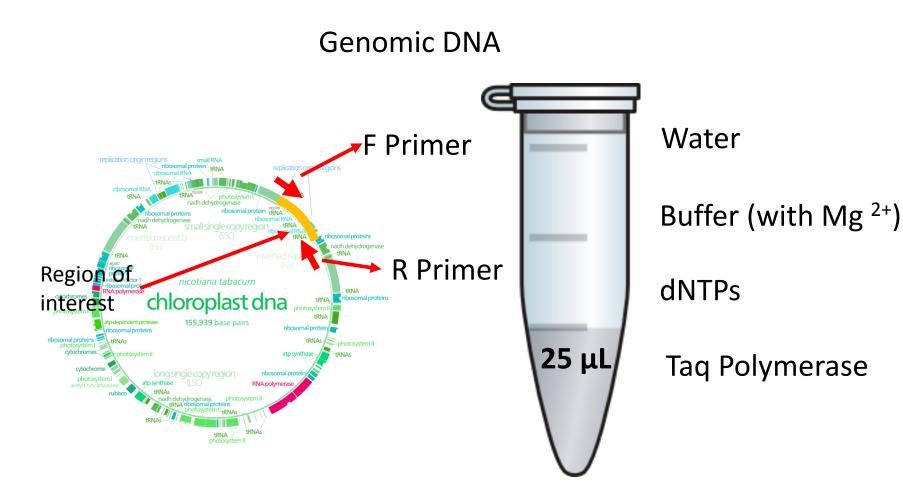
Nanodrop or Qubit

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# **PCR: Polymerase Chain Reaction**

(you probably know already about this)

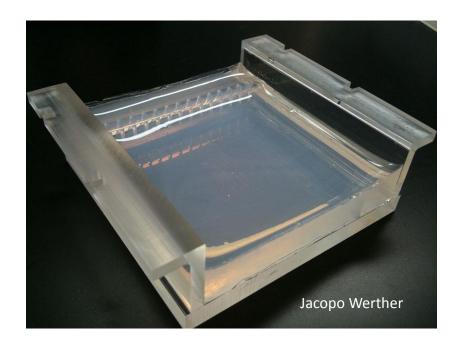
http://youtu.be/iQsu3Kz9NYo



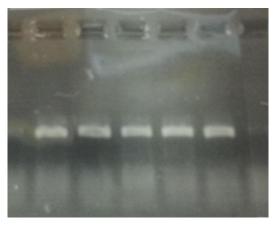
Kary Mullis

- DNA extraction
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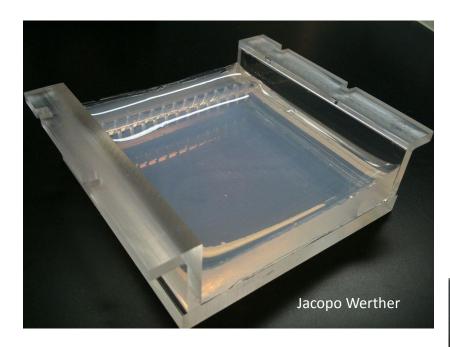
# Validation of a PCR reaction: agarose gel electrophoresis



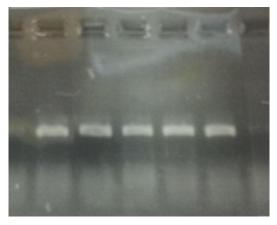
#### Good, sharp bands



# Validation of a PCR reaction: agarose gel electrophoresis



#### Good, sharp bands



#### Primer dimer



#### Double product

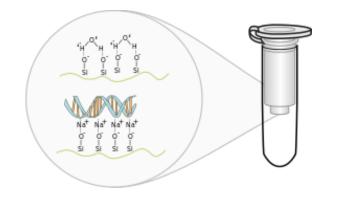


- DNA extraction
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# PCR product cleaning

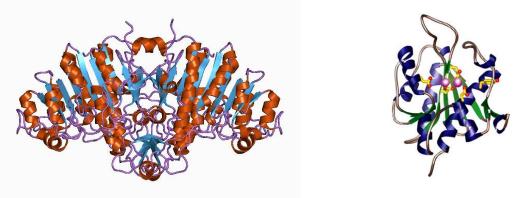
Basically, you need to get rid of primer residues, remaining nucleotides, enzymes, etc

**Option 1**: commercial kit (silicamembrane) method



Pro: gives a very clean product Cons: more expensive and time consuming. Lots of plastic waste **Option 2**: enzymatic (Exo-SAP) method

- Shrimp Alkaline Phosphatase (SAP) dephosphorylates remaining dNTPs
- Exonuclease I degrades single-stranded DNA



 Both enzymes are then denatured by heat

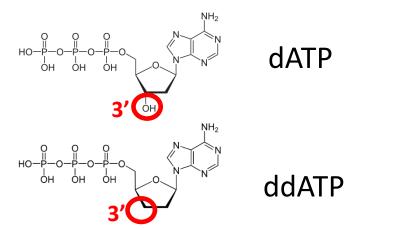
Pros: fast and cheap. Little waste involved Cons: you do not really "get rid" of the residues. Also: enzymes expire

- DNA extraction
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- PCR product cleaning
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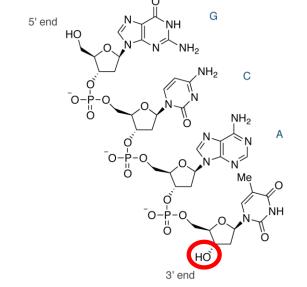
# **Sequencing reaction**

It resembles a normal PCR but

 Using a mix of normal dNTPs and fluorescent labeled ddNTPs



Frederick Sanger



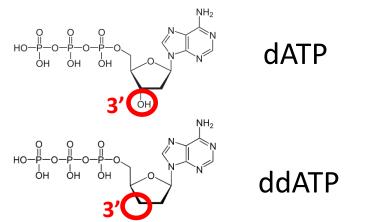
A DNA molecule that incorporates a ddnucleotide, cannot grow further

- Using only one primer

# **Sequencing reaction**

It resembles a normal PCR but

- Using a mix of normal dNTPs and fluorescent labeled ddNTPs



Fradariak Sangar

Frederick Sanger

⁻o-₽

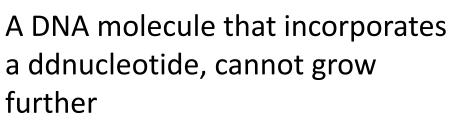
3' enc

5' end

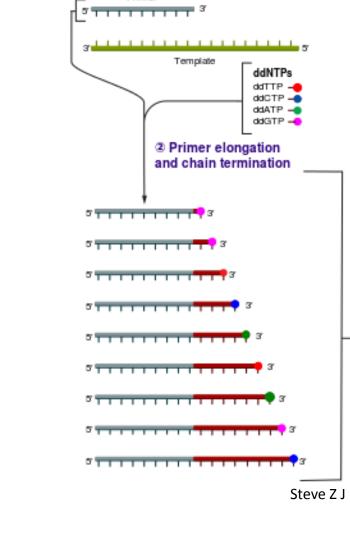
\_0-þ

⁻o−⊧

G



- Using only one primer



This results in a population of fragments with all the possible sizes and with a fluorescent 3' terminal nucleotide

- DNA extraction
- DNA amplification (PCR)
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# **Preparation for ABI sequencing**

Plan in advance the use of the ABI (bunches of 16 sequences), book a time slot on the whiteboard

Filter your samples through a Sephadex G-50 column into the sequencing plate



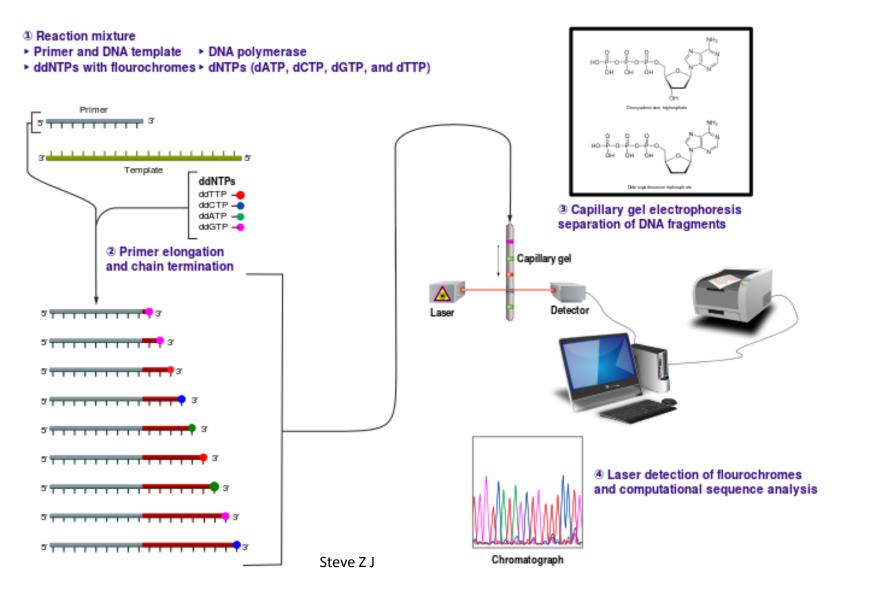
Have ready a spreadsheet with the information of each of your samples and the well it will take in the sequencing plate. All that information should also be in you lab book

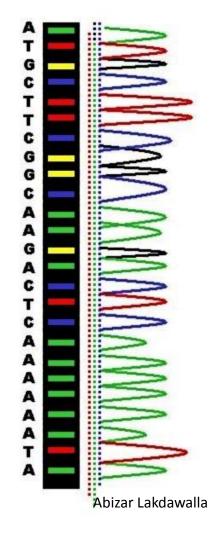
Container Name	Description	ContainerType	АррТуре	Owner	Operator	
Medinaseq19_23Jun2014		96-Well	Regular	Medina	Medina	
AppServer	AppInstance					
SequencingAnalysis						
Well	Sample Name	Comment	Priority	Results Group 1	Instrument Protocol 1	Analysis Protocol 1
A01	Physcomitrella_magdalenae_3828_atpbrbclF		100	GoffinetLab_sequencing	Seq50-POP7-E(BD1.1)	Seq_E
801	Physcomitrella_magdalenae_3828_psbAtrnHF		100	GoffinetLab_sequencing	Seq50-POP7-E(BD1.1)	Seq_E
C01	Physcomitrium_bukobense_3829_atpbrbclF		100	GoffinetLab_sequencing	Seq50-POP7-E(BD1.1)	Seq_E
1						

Taxon name, DNA ID, Region, primer

**Destination folder** 

### **ABI Sequencing**





Output of the ABI: a chromatogram in .ab1 format

- DNA extraction
- DNA amplification (PCR)
- Validation
- PCR product cleaning
- Sequencing reaction
- Preparation for ABI sequencing
- Contig assemblage and sequence exportation

Import your ab1 files into a Sequencher (Geneious, etc) project

Sequencher	File Edit Select Contig	Sequence	View Window Help
Assembly Pa Parameters: (D Name	New Project New Project From Template Open Project Open Recent Close Project	•	ied Project   ieractively Assemble to Reference   in Overlap = 20, Min Match = 85% (0 items selected out of 0)   Size Quality   Kind Label
-	Import Export Open Window	► ► ₩0	Sequences Folder Of Sequences From Template
_	Close Window Get Info	₩W ₩I	ACE Project CAF Project GCG Contig
	Save Project Save Project As Save Project As Template Revert To Saved Project	₩S	Sequencher Project Sequence From Vecbase
	Set Header & Footer Page Setup Print Print Trace in One Page	ЖР	
C			

#### Import your ab1 files into a Sequencher (Geneious, etc) project

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📲 Physcomitrium_bukobense_3829_trnlfR_Medinaseq19_23Jun2014_F02 737 BPs 81.1% AutoSeq Frag	🔞 Physcomitrium_buk	bense_3829		لinaseq19_23	lun2014_F02	737 BPs	81.1%3	AutoSeq Frag	-	Mo

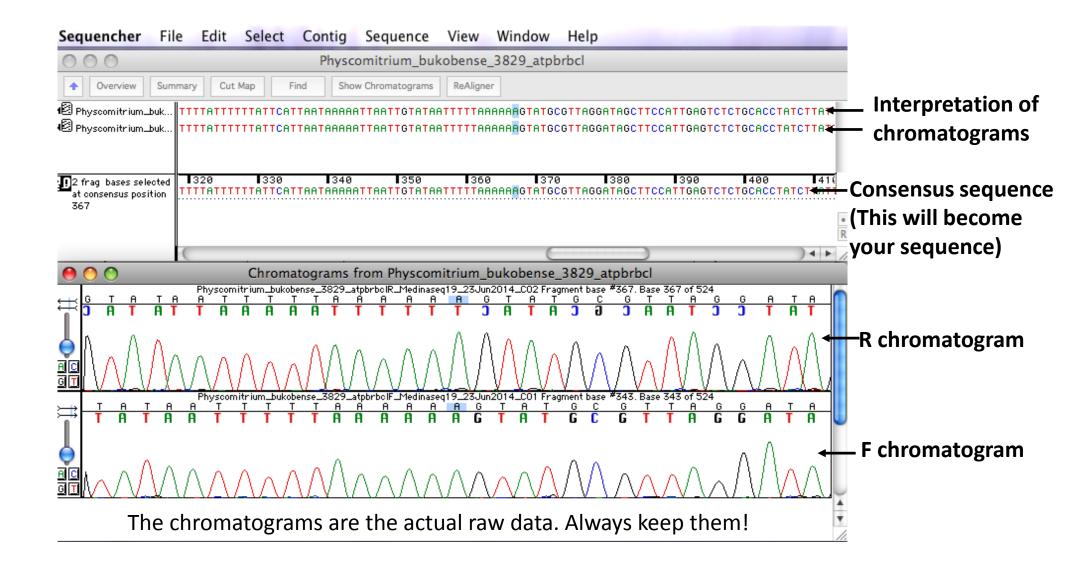
#### Assemble automatically your sequence pair

Sequencher File I	Edit Select Co	ntig Sequence	View Window	Help			
$\odot \odot \odot$							
Assembly Parameters							
Parameters: (Dirty Data	, With ReAligner, 3	ˈɡap placement): Mir	) Overlap = 20, Min	Match = 85%	(2 items se	elected out of	f 12)
Name			Size	Quality	Kind	Label	Mod
▶ 🗐 Contig[0002]			548 BPs		Contig of 2	-	We
🔞 Physcomitrium_bukobens	e_3829_ITS1F_Medinased	19_23Jun2014_A03	506 BPs	94.9%	AutoSeq Frag	-	Mo
🛛 🖉 Physcomitrium_bukobens	504 BPs	93.7%	AutoSeq Frag	-	Mo		
📲 Physcomitrium_bukobens	454 BPs	93.6%	AutoSeq Frag	-	Mo		
📲 Physcomitrium_bukobens	447 BPs	93.7%	AutoSeq Frag	-	Mo		
📲 Physcomitrium_bukobens	701 BPs	93.9%	AutoSeq Frag	-	Mo		
I Physicomitrium_bukobens	700 BPs	92.6%	AutoSeq Frag	-	Mo		
I Physicomitrium_bukobens	452 BPs	94.9%	AutoSeq Frag	-	Mo		
I Physcomitrium_bukobens	596 BPs	90.8%	AutoSeq Frag	-	Mo		
♥️ Physcomitrium_bukobense_3829_trn1fF_Medinaseq19_23Jun2014_F01			663 BPs	90.0%	AutoSeq Frag	-	Mo
🗟 Physcomitrium_bukobens	Physcomitrium_bukobense_3829_trnlfR_Medinaseq19_23Jun2014_F02			81.1%	AutoSeq Frag	-	Mo
	Assembly Completed						- 1
	Time Elapsed: 0 Items Selected: Comparisons Pe	2	Assemble by N Number of Con Number of Frag	itigs: 1			
Close							< > //

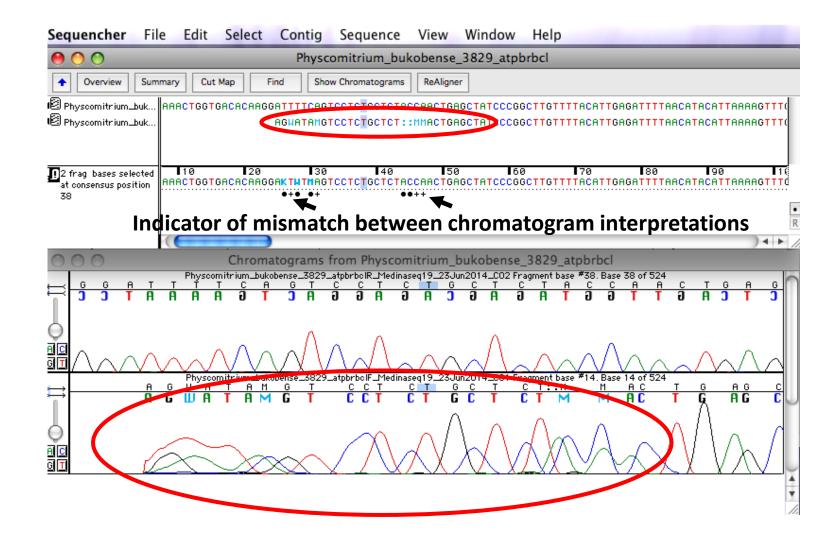
#### Rename the contig with the relevant information

Sequencher File Edit S	elect Contig Sequence V	/iew Window	Help		_	_
$\odot \odot \odot$	Untitled	Project				
Assembly Parameters AbN /	Assemble Automatically Assemble Interact	tively Assemble to	Reference			
Parameters: (Dirty Data, With R	eAligner, 3' gap placement): Min (	)verlap = 20, Min	Match = 85%	(1 items s	elected out o	of 11)
Name		Size	Quality	Kind	Label	Mod
Physicomitrium_bukobense_3829_a	tpbrbel	548 BPs		Contig of 2	-	We
Physcomitrium_bukobense_3829	_atpbrbclR_Medinaseq19_23Jun2014_C02	524 BPs	94.5 <b>B</b>	AutoSeq Frag	-	Mo
Ph: 😝 🔿 🥎	Physcomitrium_bukobense_38	29_atpbrbcl		Seq Frag	-	Mo
Physe				Seq Frag	-	Mo
Physe 🔶 Bases Summa	ry Sort Options Find	Get Info ReAlig	her	Seq Frag	-	Mo
Physe				Seq Frag	-	Mo
······································	m_bukobense_3829_atpbrbcIR_Medinas			Seq Frag		Mo
	itrium_bukobense_3829_atpbrbcIF_Me	dinaseq19_23Jun20	)14_C01	→ Seq Frag		Mo
Physe 200			1818	Seq Frag		Mo
			548			Mo
Physe		P		Seq Frag		Mo
Physe	· · · · · · · · · · · · · · · · · · ·			Seq Frag		Mo
Physe ++++++				Seq Frag	-	Mo
	Multiple fragments same direction Both strands Both strands plus CELECTOR ACTION Frame 1	Bumps on ragments show motifs, sollow rectangles show features		•		





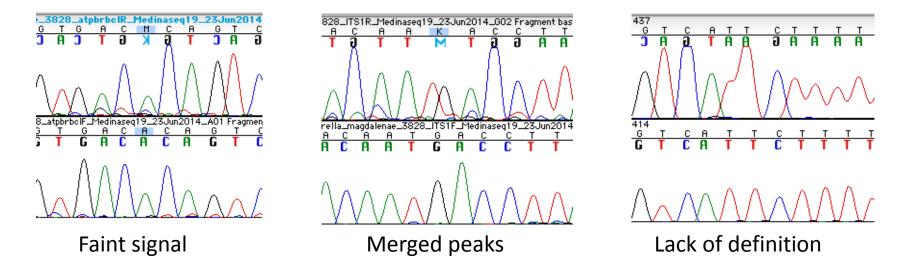
The chromatograms are usually "dirty" at the extremes



Export the consensus sequence as a FASTA (.fas) file when ready

Sequencher	File Edit Select Contig	Sequence	e View Window Help	
Image: Control of the second secon	New Project New Project From Template Open Project Open Recent Close Project	•	ukobense_3829_atpbrbcl ReAligner таатаааааттааттотатааттттааааааастатосоттаосатаосттосаттоа таатаааааттааттотатаатттттааааааастатосоттаосатаосттосаттоа	
2 frag bases sele at consensus posi	Import Export	Þ	Sequence(s)	
348	Open Window Close Window Get Info	жо жw жI	Consensus Contig Selection As Subproject	• R
	Save Project Save Project As Save Project As Template Revert To Saved Project	₩S	Overview As Text Selected Bases Selected Bases As Protein Summary	$\cap$
	Set Header & Footer Page Setup Print Print Trace in One Page	ЖP	seq19_23Jun2014_C01 Fragment base #324. Base 324 of 524 A T T G T A T A A T T T T A A A T T G T A T A A T T T T A A A T T G T A T A A T T T T A A	J

What to expect when editing sequences (best case scenarios)



Problematic, noisy sequences can (sometimes) be edited (as we will see in the next session)

Always keep a backup of the raw chromatogram files, the Sequencher/Geneious project and your fasta sequences