Mon June 20, 2011
Jennifer Weller

DNA extraction

Solutions and Dilutions

Sample list

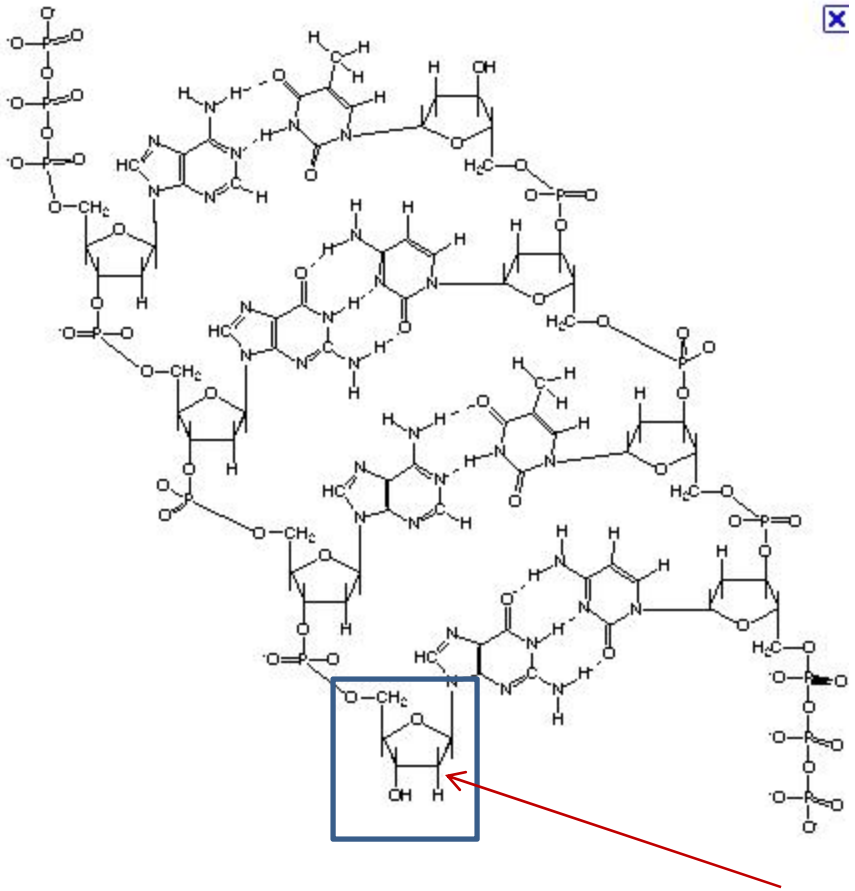
Statistics and Excel (we didn't get to this, but it is still useful to look at)

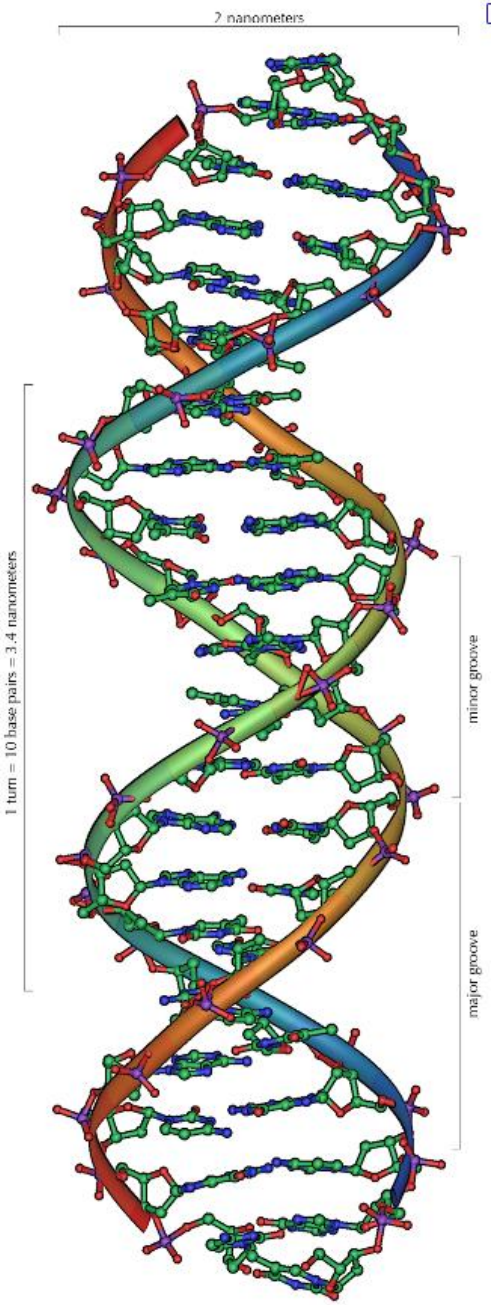
Living organisms have cells

- The directions for what a cell does are in the DNA, so ‘all’ cells have DNA.
  - Red blood cells in mammals are oxygen-carrying packages – no DNA is included.
  - Viruses get other cells to do the work, some have RNA instead of DNA
- Some cells have two sets of nested containers – the inner part is the nucleus with the DNA

Deoxy-ribonucleic acid

- Also ribonucleic acid….

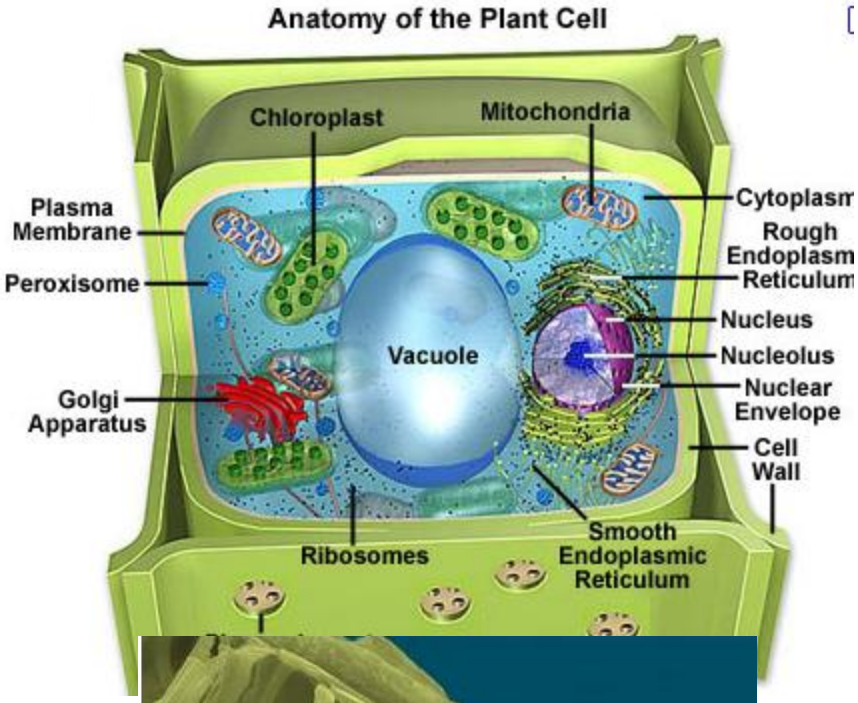


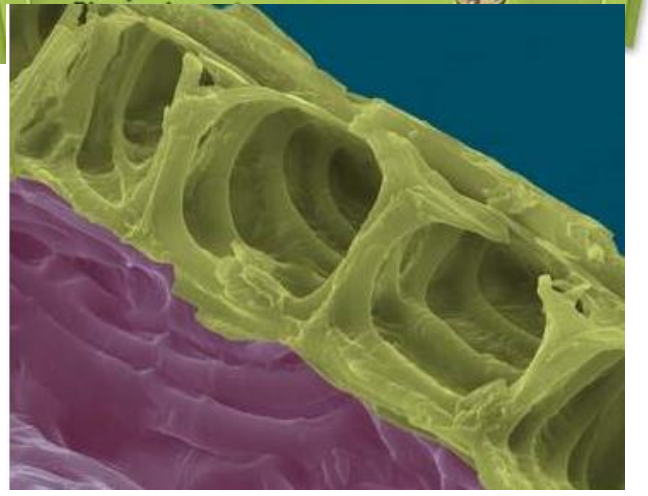


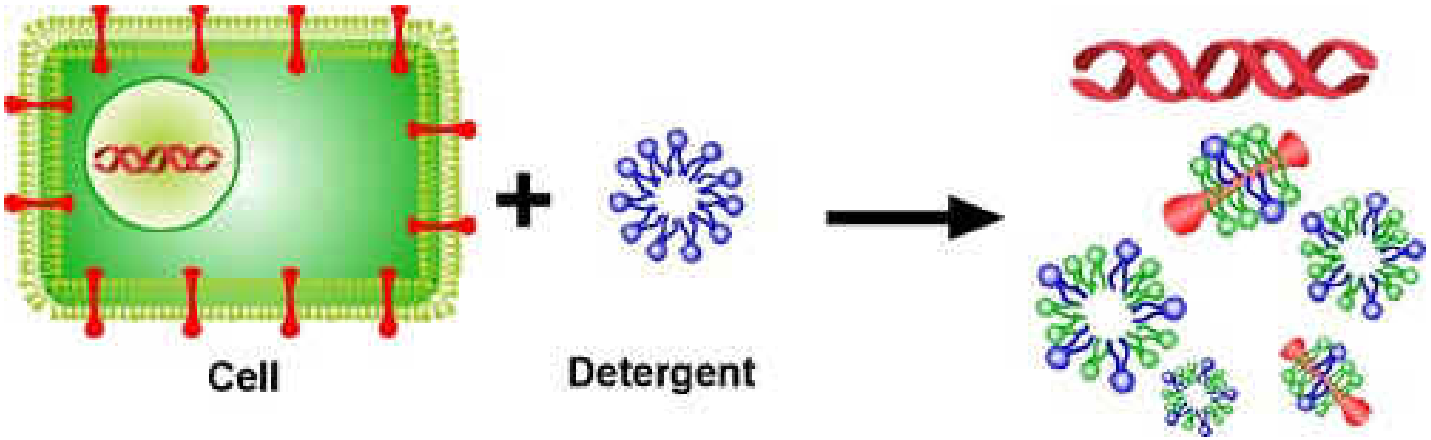
http://familypedia.wikia.com/wiki/DNA

Cell structure is based on separating the inside from the outside.

- Membranes and cell walls form the barriers for cells.
- To get out the DNA you have to break open the cell.
  - Soap will dissolve membranes
  - Cell walls have to be crushed
    - Things inside the cells tend to get crushed as well.

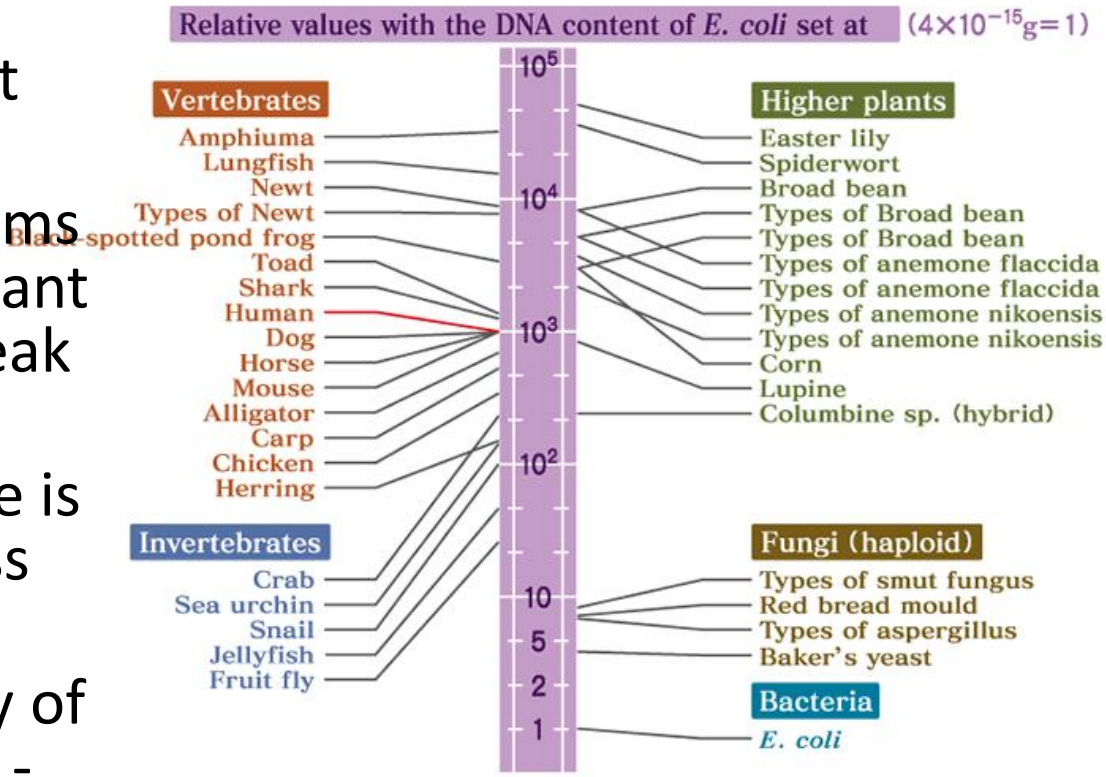





Cell
Detergent

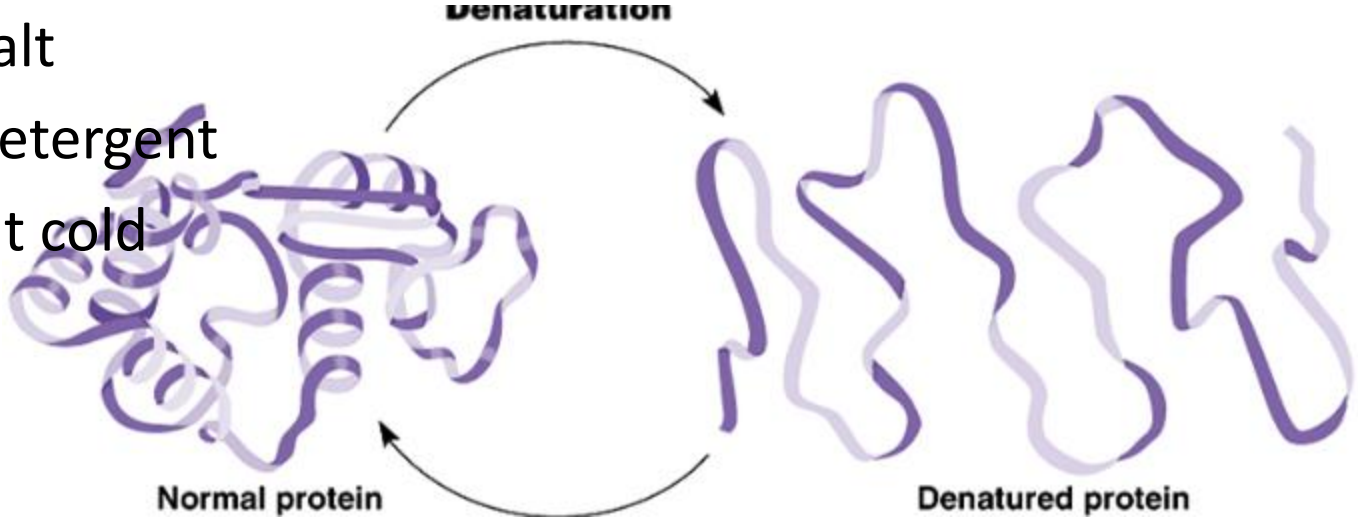
The amount of DNA per cell is very small, so you need some idea about your assay needs.

- How much DNA (the mass) does <u>one</u> plant cell have?
- If I want 10 micrograms of DNA how many plant cells do I need to break open?
- How much leaf tissue is this (what is the mass one leaf cell)?
- What if my efficiency of recovery is only 50% - how much more leaf tissue will I need?



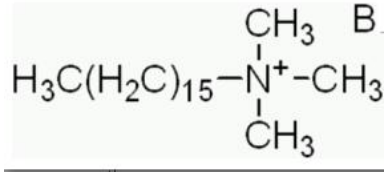
Lysis buffer contains chemical that protect the DNA while we extract it.

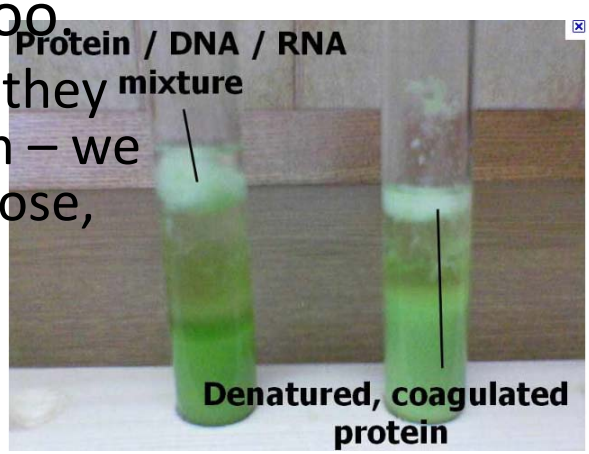
- Cells have active ingredients that can break down DNA – these are chemicals and enzymes.
  - We can stop the processes by keeping the sample cold and blocking or inactivating the enzymes
    - Add salt
    - Add detergent
    - Keep it cold



Plant cells have a lot of carbohydrate and a lot of small chemicals that can react with the DNA.

- You can change how carbohydrates behave by reacting them with other compounds.
  - We will use a detergent called CTAB that makes the carbohydrate mix with chloroform better than with water.
  - The reaction is slow so we use heat and mixing to make sure it is complete.
- A lot of the small chemicals will dissolve in phenol or chloroform, so we add a little phenol too.
  - This will cause proteins to denature so that they won't dissolve in either water or chloroform – we have to add another method to separate those, centrifugation.



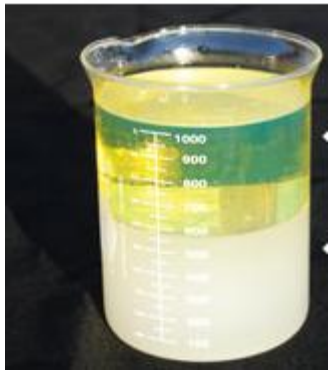


The DNA and RNA stay soluble in the buffer, the goal is to remove everything else.

- What is the difference between something suspended in a liquid and something dissolved in the liquid?
  - Does it sink or float?
  - Mashed up cells are suspended, with some of the inside stuff (cytosol) dissolving if the liquid is water-based (aqueous).
- What is the buffer?
  - Water with some salts and detergent, and also something that keeps the sample from becoming too acidic or basic when the cells break open.
    - TRIS is a very common biological buffer – it balances the acidity to what is common inside cells.

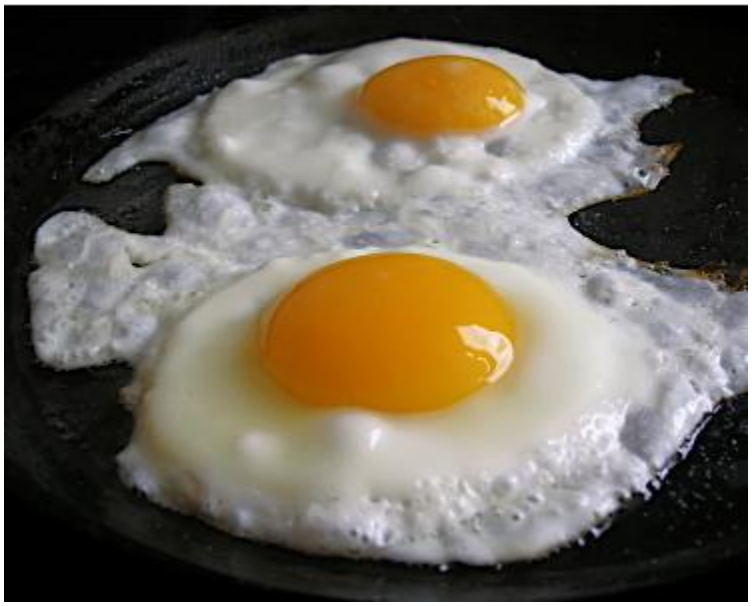
Separating samples using different solubility

- Most biological samples are handled as solutions – in liquids.
- Different parts are MORE soluble in different types of liquids
  - DNA dissolves in water-based liquids
  - Membranes dissolve in oily liquids
    - If you use a detergent you can break up the membrane and the bits + detergent will be soluble in water.
- You can play with differential solubility to separate the parts
  - Oil and water do not mix. If I have something that will dissolve in water and I shake it with oil, it will stay in the water layer.
  - If I put something in the water layer that is more soluble in oil, if I shake oil and water together it will transfer to the oil layer. I have extracted it from the water layer, or phase.



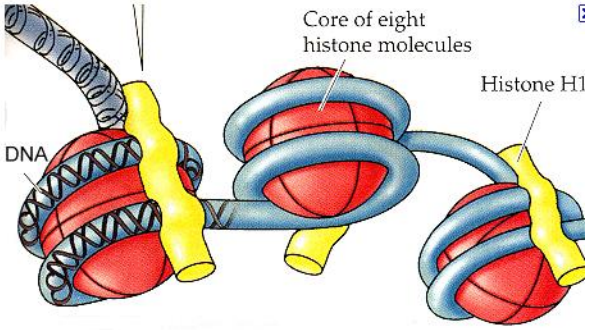
Changing specific properties can also be used in purification methods.

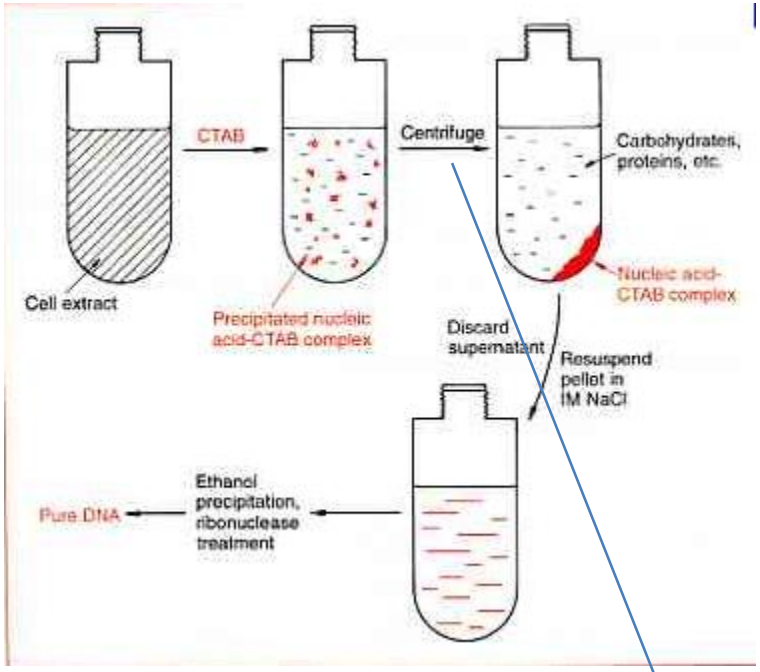
- Some materials change properties depending on their shape.
- Proteins (some) can dissolve in water-based buffers when they are folded up properly but not when they are unfolded
  - Egg white – uncooked it can be shaken up with water to make a solution. Cooked, not so much.
    - The heat ‘denatured’ the protein so it lost its folded up structure.

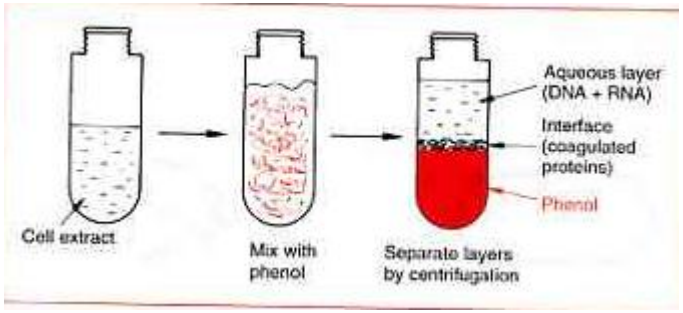


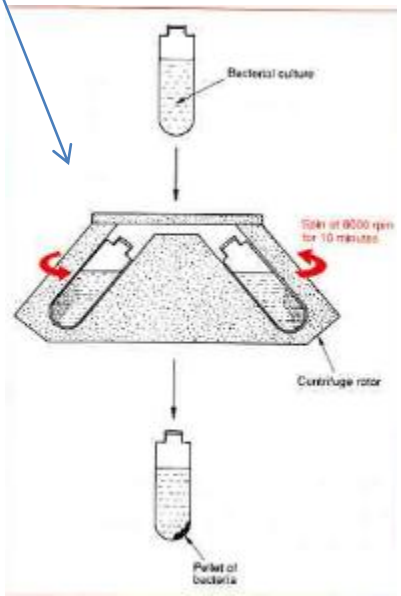
Chromosomes are packaged with proteins that can be hard to remove.

- The six feet of DNA length in your cells has to get wrapped up in order to fit.
  - It is looped around proteins (histones) and these can be hard to remove.
  - There are proteins that break down other proteins (Proteases) – if we add these to the sample the histones are much easier to remove from the DNA.
  - Then use detergent and phenol to denature the protein pieces and phenol and chloroform to extract them away from the DNA.




CTAB
Centrifuge
Carbohydrates, proteins, etc.
Nucleic acid-CTAB complex
Cell extract
Precipitated nucleic acid-CTAB complex
Discard supernatant
Resuspend pellet in 1M NaCl
Ethanol precipitation, ribonuclease treatment
Pure DNA

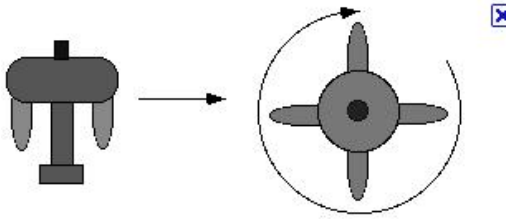

Aqueous layer (DNA + RNA)
Interface (coagulated proteins)
Phenol
Cell extract
Mix with phenol
Separate layers by centrifugation

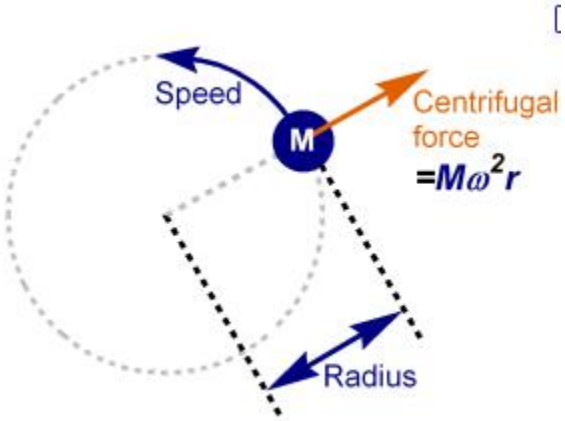

Bacterial culture
Centrifuge rotor
Pellet of bacteria

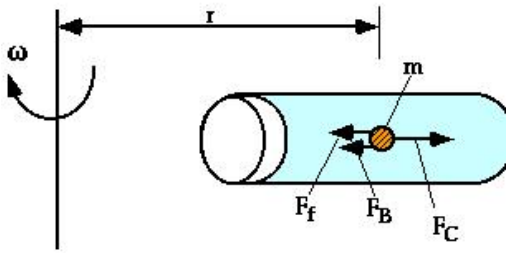
Concentrating the DNA

- The DNA is in solution, in the buffer, but it is very dilute – I want to concentrate it – how?
  - Evaporation
  - Precipitation – make it insoluble.
    - Add an alcohol – DNA does not stay in solution in 70% alcohol. Add 100% alcohol to the buffer until the final solution is 70% alcohol.
    - It will settle out of the solution but I can collect it more quickly if I centrifuge it.

Centrifugation

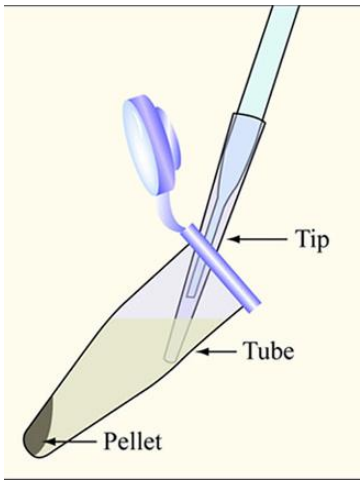


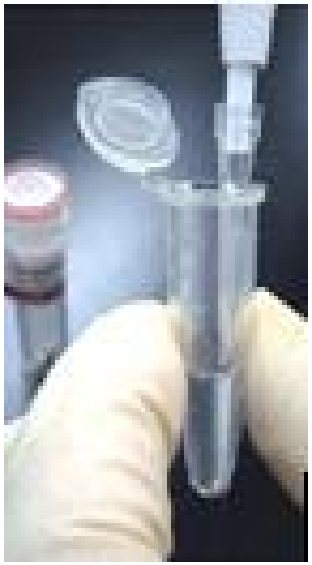




Balance tubes that are directly across from each other!!!

Handling small samples





Re-solubilizing the DNA

- Remove the alcohol from the tube, try not to touch the pellet of DNA.
  - Remove it slowly so the meniscus travels down the tube and not drops are left behind.
  - Let the pellet air dry
  - Add a small volume of buffer to the pellet and let it sit so the DNA goes back into solution, or shake it gently to speed this up.
    - I want to give this enough time that the DNA solution is homogeneous (the same everywhere) – why?

Now I have DNA for my genetic assay

- Actually, I still have to remove the RNA (They purify together). I will use an enzyme to do this, RNAase, and then remove the enzyme, use ethanol to precipitate the DNA one more time, and resuspend it.
- Then I will check the concentration of the DNA with a spectrophotometer, and double-check this using an agarose gel.
- THEN I will be ready to do a genetic assay.

Following a protocol

- Read through all of the directions **before** starting anything.
- Make sure you understand how to do all of the steps
- Make sure you have all of the required solutions, and enough of them'
- Make sure you have the right equipment and supplies (gloves, tips, tubes, etc)
- If something needs to be set to some temperature before use get it started – don't begin until the correct temperature is reached.
- If some of the steps need to be timed be sure you have a timer available.
- If some of the chemicals need to be stored on ice get an ice bucket and ice before taking them out of storage.
- If some material is to be discarded make sure you know if it can go down the sink or needs to be put in a special waste container – and make sure you know where that is.
- If you will not have time to complete the protocol in one day make sure you understand where the allowed stopping times are, and how the sample must be stored (at room temperature in alcohol? In a freezer?).

Lab activities

- Part 1 dilutions
  - You will have 5 concentrated stock solutions, each starts at 20X, darkly colored.
  - Make a 2-fold serial dilution
  - Make a 10-fold dilution. Can you tell where the 10-fold dilution would fall in the 2-fold series?

Making Solutions

- A lot of biology involves adding several reagents (in solution) together in the correct amounts, mixing them carefully and then adding an enzyme to start a chemical reaction.
- The order of addition is usually important.
- Careful mixing and making sure you end up with the correct final volume is also important.

Centrifuging samples

- When you are spinning small masses at very large force, small differences will cause unbalancing and possibly having the rotor come off the spindle of the centrifuge.
- Therefore you must always have two tubes that have the same mass that are put directly across from each other in the rotor.
  - Do not ‘eye balance’ by having the liquid levels seem the same, put them on a balance and either add or take away solution to make them weigh the same.
- Centrifuge to collect all of a solution in the bottom of a tube
- Centrifuge to collect material that is not dissolved in the bottom of the tube (‘pelleting’).

Skills I: Mixing solutions

- Add 500ul of Solution A to Solution B – observe carefully both the pipette tips and how the solutions ‘layer’ – which one is denser?
  - Can you get all of each solution out of the tip and into the tube?
  - Try mixing the two solutions
    - Finger mixing
    - Shaking
    - Pipetting up and down
  - Are droplets left on the sides of the tube?
- Add 500 ul of Solution C to Solution D – answer the same questions as above.
- Add 500 ul of Solution C to Solution E – answer the same questions as above.

Skills 2: Serial Dilutions

- Using Solution A, make 2-fold serial dilutions:
- In each of 5 tubes put 1ml of water
- Label the tube 1,2, 3, 4, 5
- Add 0.5 ml of Solution A (500ul) to tube 1 and mix thoroughly – make sure no droplets are on the sides (centrifuge if necessary).
- Remove 500ul from Tube 1 and add to Tube 2.
  - Mix thoroughly and collect
- Remove 500 ul from Tube 2 and add to Tube 3, mix and collect
- Remove 500 ul from Tube 3 and add to tube 4, mix and collect
- Remove 500 ul from Tube 4 and add to tube 5, mix and collect.
- Verify that you have the expected volume in each tube
  - Repeat the volume measurement
  - Weigh the sample on an appropriate scale

Skills 3: Diluting solutions

- In molecular biology most reagents come in pre-made solutions, at some excess concentration from what you need – usually a fold-change like 5X or 10X. Often they are frozen, sometimes they have splashed around
  - Thaw the solution, mix it, then spin it. Them put it on ice if the directions say to do this.
- Label the tube you are measuring into.
- To make 200ul of a 'reaction mix'. (Do this 3 times to check your reproducibility)
  - Add 20 ul of Solution A (which is 10X
  - Add 40ul of Solution B (which is 5X)
  - Add 2ul of Solution E (which is 10%)
  - Mix these together, spin in the centrifuge and make sure you have the correct volume.
  - If the volume is correct add 133 ul of water, mix carefully and centrifuge.
  - What is the total volume supposed to be?
    - The difference is the amount left for adding your sample.
  - What is the actual volume?
  - What is the final concentration of each reagent when you have brought the volume to 200ul with your sample?

Skills 4: Recovering pellets and layers

- In many molecular biology applications you spin a sample to separate solutions that won't mix into layers, or to collect something that is not dissolved at the bottom of a tube.
- You remove the top layer of a solution to a new tube – one or the other of the layers has the sample you want
  - Try to get as much of the top layer off as possible without contaminating it with the bottom layer
- You remove all of the solution from the tube, leaving the pellet behind
  - Try not to touch the tip used to remove the liquid to the pellet
  - Try to remove as much liquid as possible, without disturbing the pellet.
- Mix 500 ul of Solution A with 200ul of Solution C.
  - Centrifuge
  - Remove the top layer to a new tube
    - Did you get all of the top layer off?
    - Measure the volume of each layer and record it
    - Measure the mass of each layer and record it (you should weigh 1ml of each solution in order to have a reference)
- Add 500 ul of Solution D to the tube the says Sample A.
  - Mix thoroughly, then centrifuge for 30 sec.
  - Remove the liquid to a new tube
    - Were you able to remove all of the liquid without disturbing the pellet?
    - How much liquid did you remove?

Samples in Collection

Purple ink writing (no initials or date, carried at ambient temperature until end of day

- TG5 (leaves and one soil vial)
- Sight 1 Stats on leaf
- Sight 2 (sprout) and leaf and vial
- TS4 (infected_)
- TS3 (infected) and leaf and vial

Black ink writing - leaves in Baggies

- TS4 MV & AS
- JH and SA Site 3 12:36
- PNR Sample 4 6-15-11 12:30 Resistant tree
- AG SS 12:21 Resistant Tree
- SA & AH TS3
- MR SP Sample 5 Near Bottom 05/16/11 12:30
- M? and CR Sample 4 No blight 6/15/11
- MR & SP Sample 4 6/15/11 12:27
- Bassam Resistance 12:30
- Sample 2 6/15/11 Sprout of American Chestnet Blight presence MJ CR
- AA 6/15 Chestnut hybrid Disease Resistant
- Sample #3 Chestnut BC Castenaea dentata sprout
- MV and AS Tree 1
- Tree 2 AS and MV – leaves and vial
- Tree #1 Sample #1 SA and JA
- SA and AH Sample 1 ? leaf from sprout
- MR and SP Sample 2 Near 1 and base 6/15/11 10:17
- PNR sample 3 6/15/11/10:47 Infected Tree
- Site 3 near boulder leaf JH and SA
-

Samples in collection – pg 2

- SA and AH sample 2 Leaves
- BZ sample 2 chestnut 6/15/11
- MR SP Sample 1 6/15/11 10:12
- AG SS 10:12 Sprout 1
- PNR Sample 1 6/15/11 10:14am
- Sample 1 American Chestnut BA(?) June 15, 2911 10:13
- PNR Sample 2 6/15/11 10:19am upper hill chestnut leaf
- MT CR Sample 1 6/15/11 Sprout of American Chestnut contained fungus – Rocky area
- BS MH Nearby Tree of Sample 3
- Sample 4 BC infected 10:45 ?
- MR SP Sample 3 6/15/11 11:12
- Tree 3 MV and AS leaf and vial
- BC 11:47 Sample 6? Infected
- Sample 7 BC 10:51
- MT CR Sample 3 6/15/11 sprout blight rocky area leaf and vial

Vials - 15

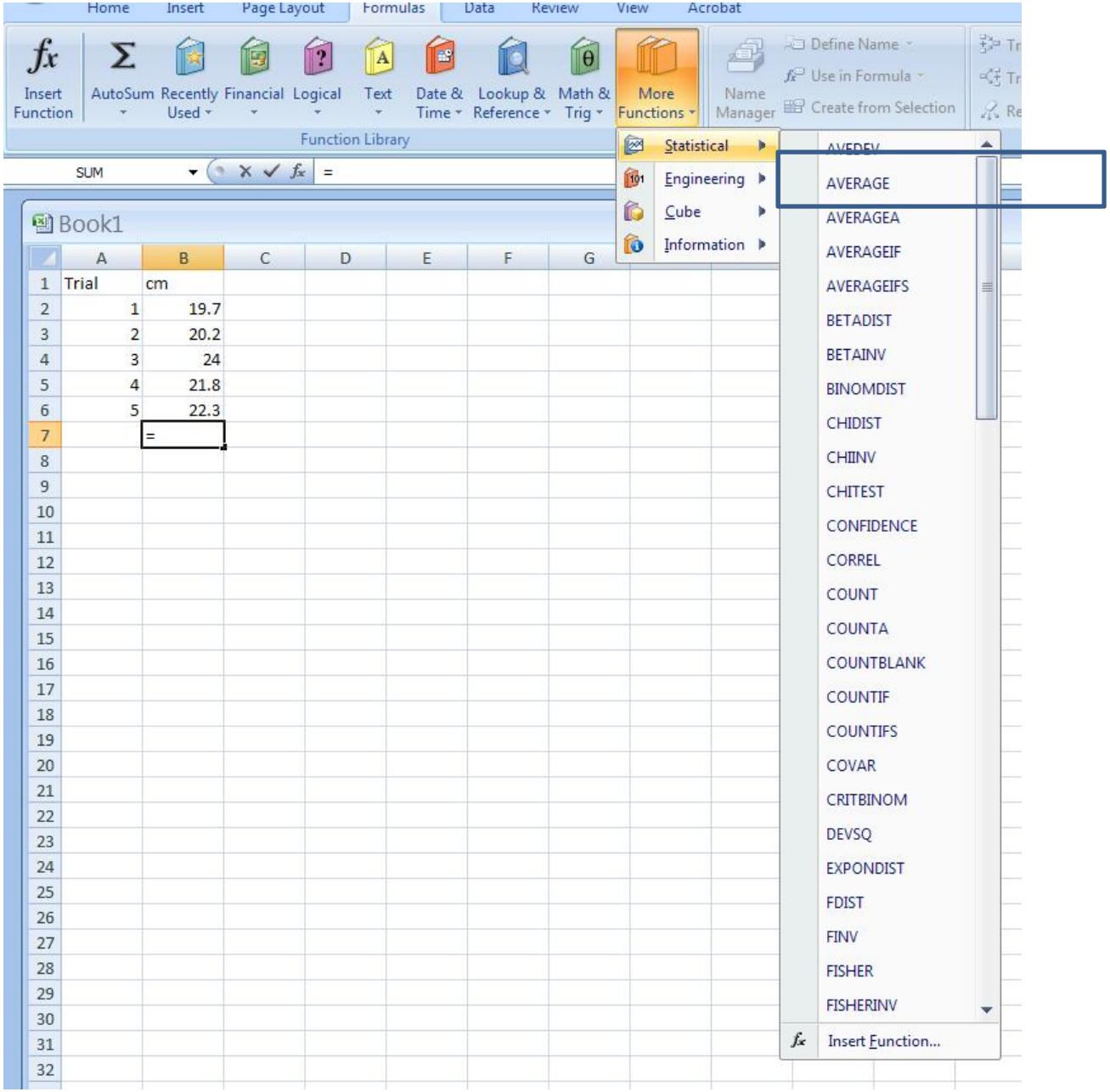
- Marquel17 unleaf seeds
- BC bark resistant soil 1 upper ? 10:??
- ?? Marks may have come off
- SA3 AH SS2
- SA & AH SS?
- Site 1 MH
- AG SS Sprout 2 10:20
- SA AH TS3
- Site 1 MH Blight
- MV AS Tree 1
- PNR (K?)6/15/11 10:30soil & resistant2:4
- MH soil from resistanttree
- MH far (?) 3
- BC soil (?) close? 8-3?

Statistics and Excel

- We made multiple measurements to see how good our precision was.
- Usually you take the mean or median in order to get the best estimate of accuracy.
- The mean: add the replicates together and divide by the number of measurements
- The median: take the middle value (separating the top half and the bottom half)

Finding the range of your measurements

- The smallest interval that contains all your data is the range (the highest to lowest value)
  - The variance shows how far the numbers are from the average (mean).
  - You calculate it by subtracting the mean from each measurement and taking the square , summing these values and dividing by the number of values.
- The standard deviation is often used – it is the square root of the variance, so it has the same units as the measurements.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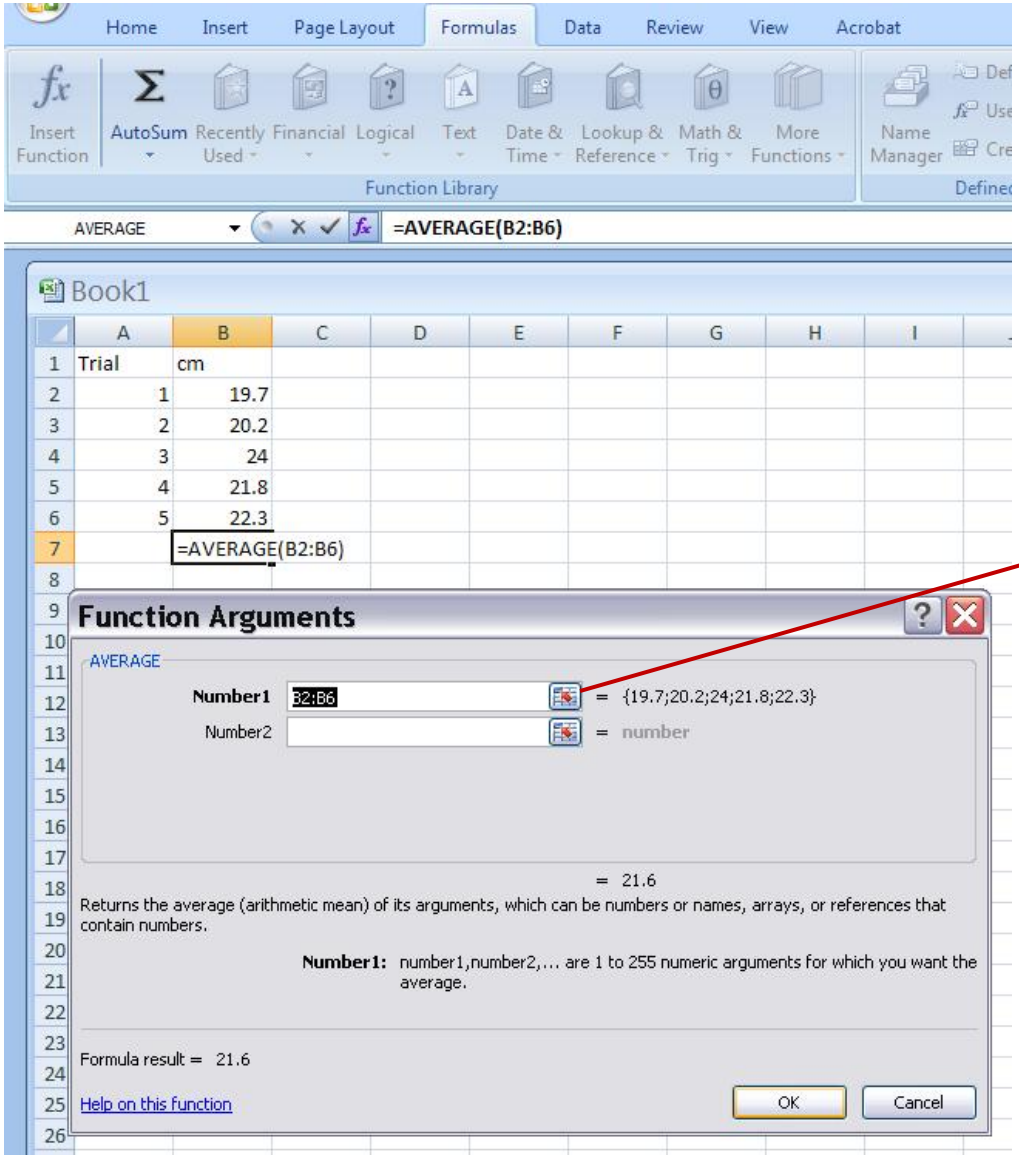
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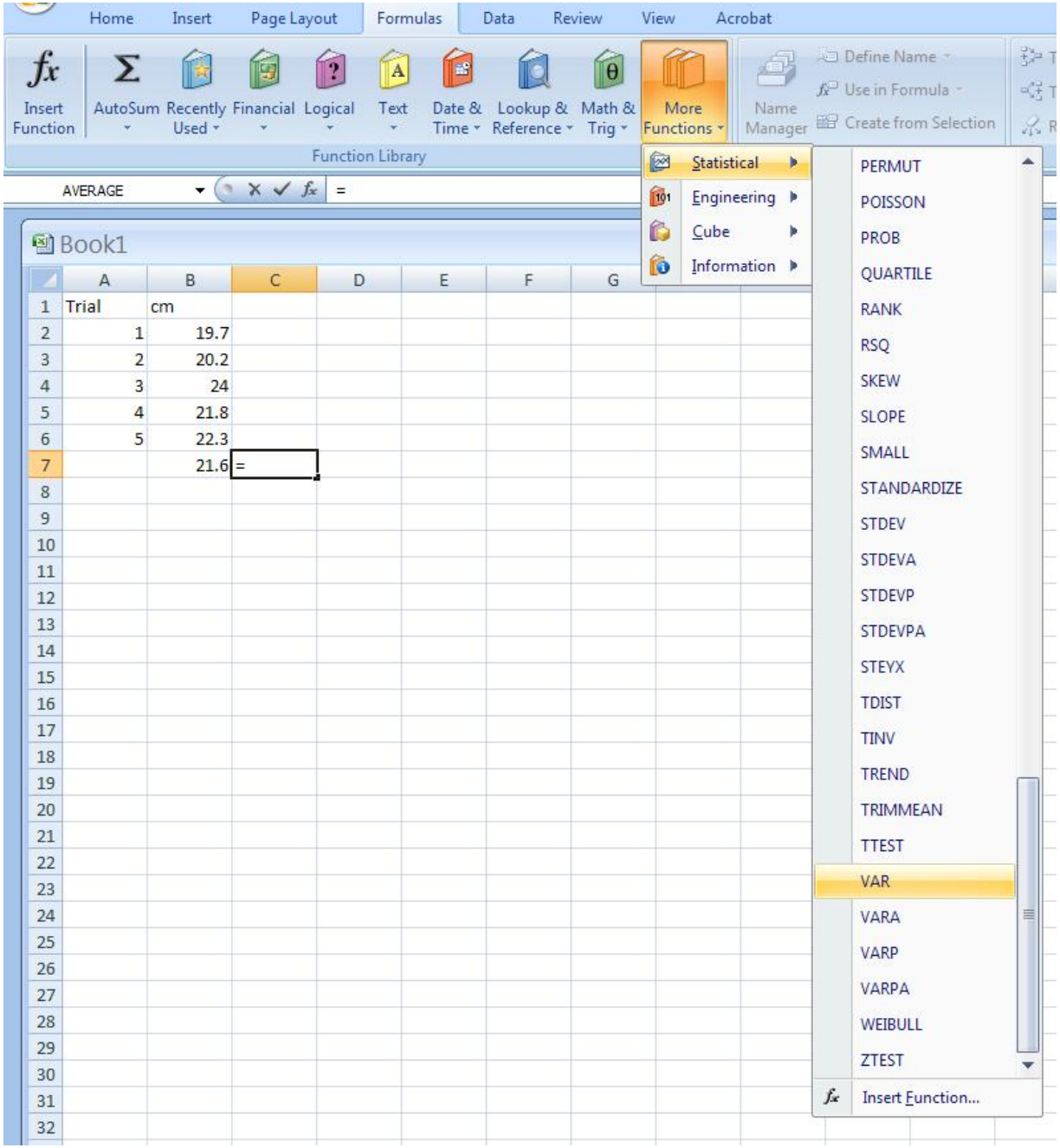
AVERAGE
=AVERAGE(B2:B6)
Book1
Trial
cm
1 19.7
2 20.2
3 24
4 21.8
5 22.3
=AVERAGE(B2:B6)
Function Arguments
AVERAGE
Number1
B2:B6
= {19.7;20.2;24;21.8;22.3}
Number2
= number
= 21.6
Returns the average (arithmetic mean) of its arguments, which can be numbers or names, arrays, or references that contain numbers.
Number1: number1,number2,... are 1 to 255 numeric arguments for which you want the average.
Formula result = 21.6
Help on this function
OK
Cancel

B2:B6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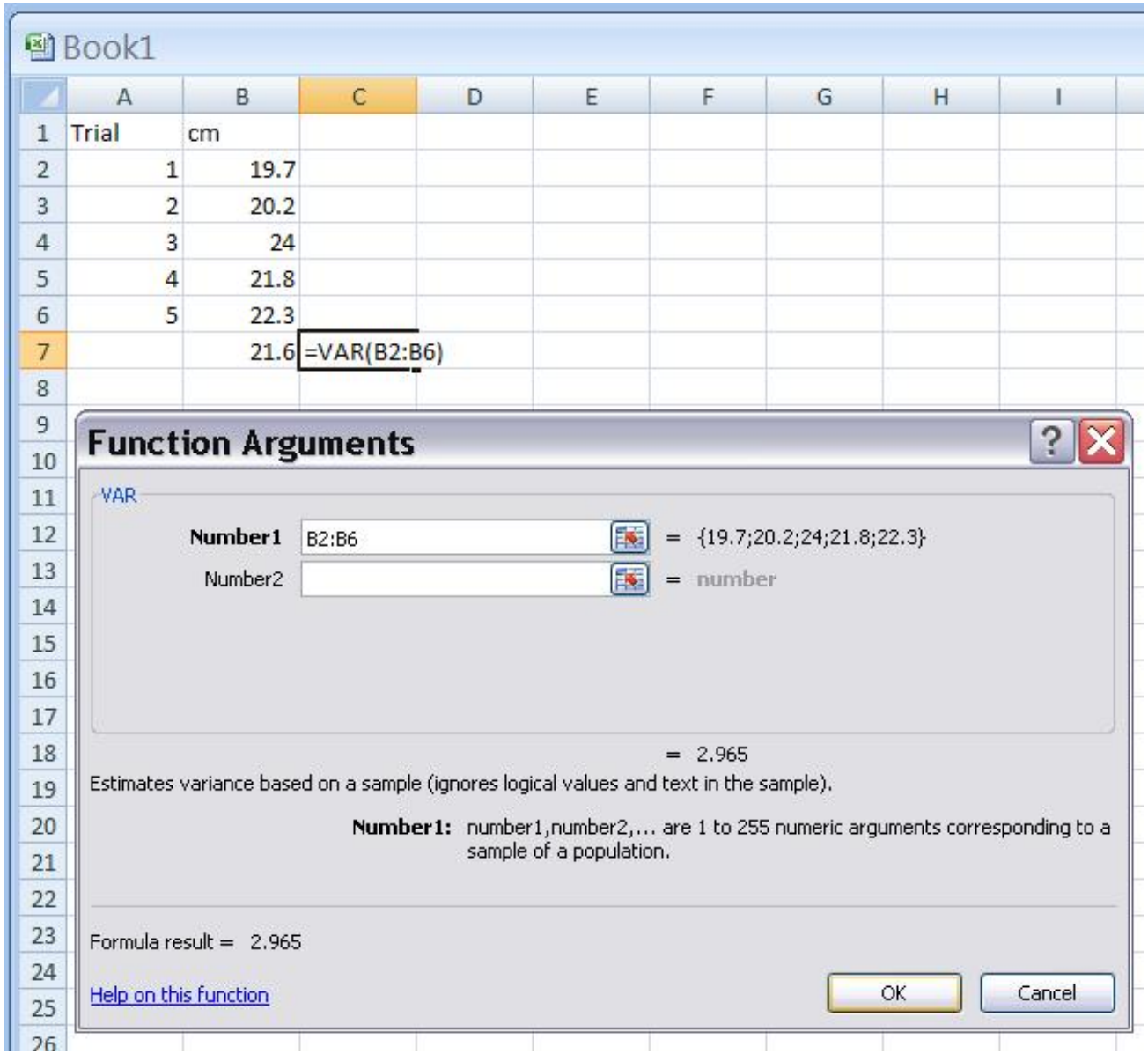
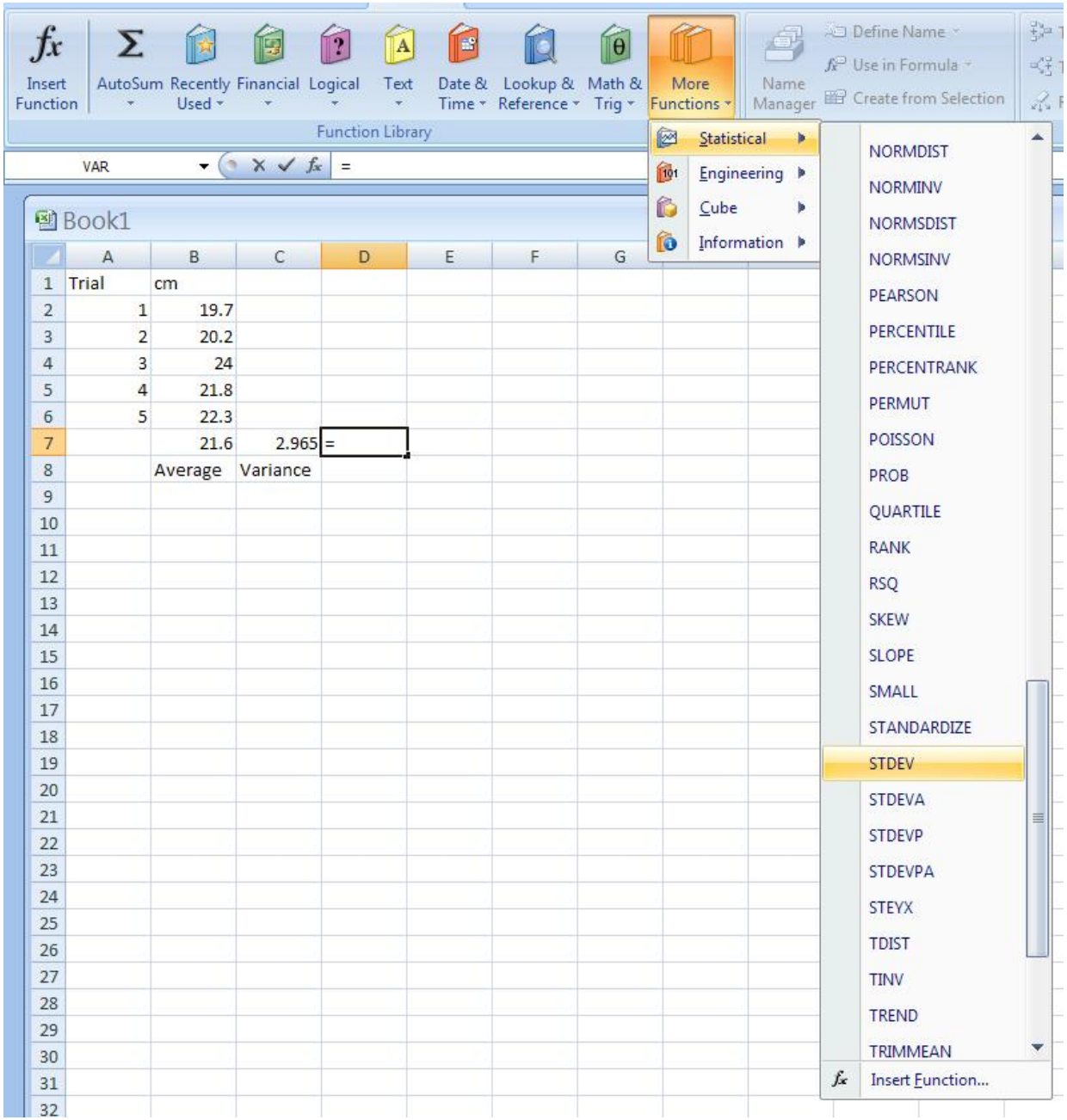
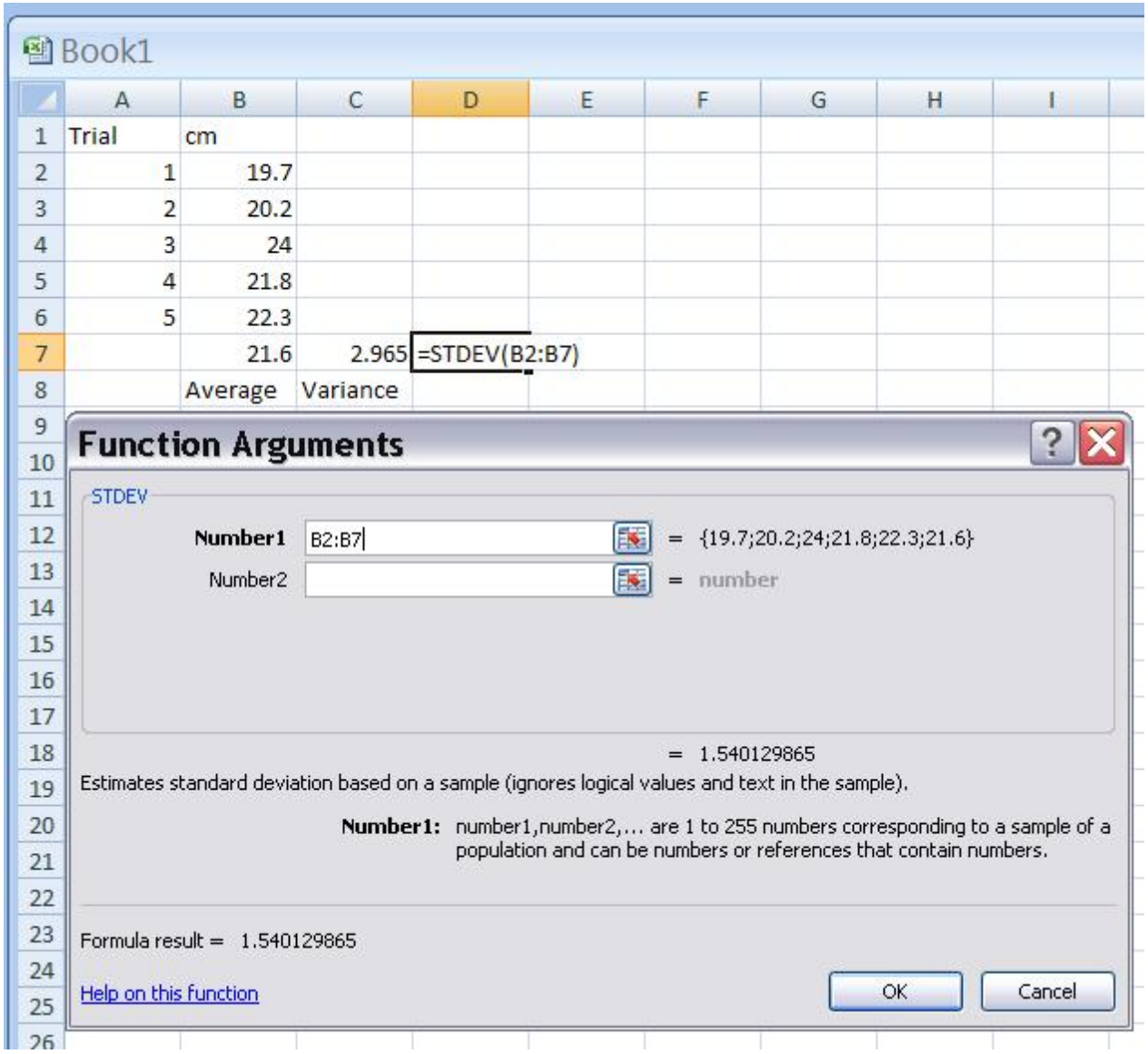
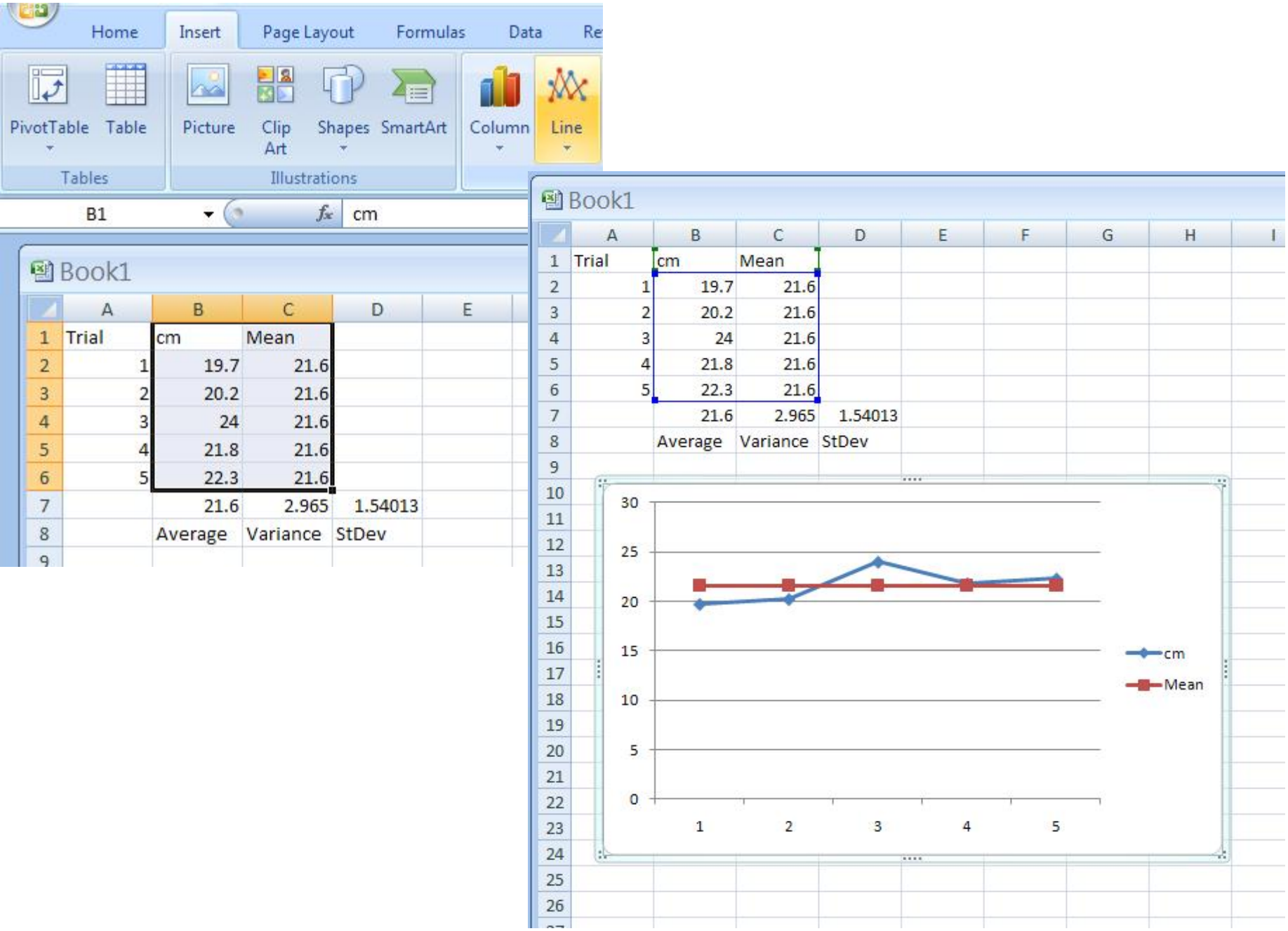
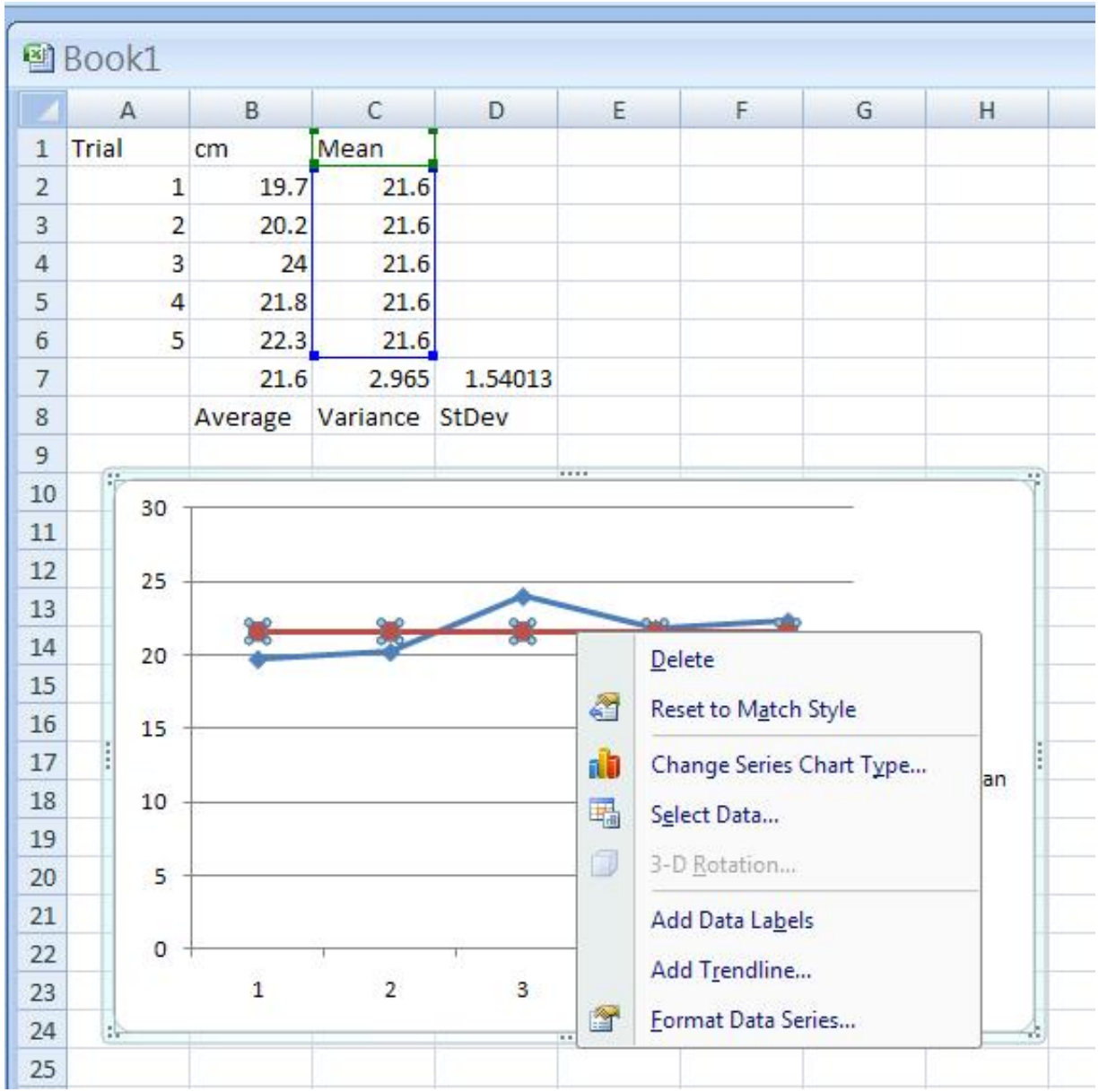
cm
1 19.7
2 20.2
3 24
4 21.8
5 22.3
21.6 =


Book1
A B C D E F G H I
1 Trial cm
2 1 19.7
3 2 20.2
4 3 24
5 4 21.8
6 5 22.3
7 21.6 =VAR(B2:B6)
Function Arguments
VAR
Number1 B2:B6 = {19.7;20.2;24;21.8;22.3}
Number2 = number
= 2.965
Estimates variance based on a sample (ignores logical values and text in the sample).
Number1: number1,number2,... are 1 to 255 numeric arguments corresponding to a sample of a population.
Formula result = 2.965
Help on this function
OK
Cancel


Insert Function
AutoSum
Recently Used
Financial
Logical
Text
Date & Time
Lookup & Reference
Math & Trig
More Functions
Name Manager
Define Name
Use in Formula
Create from Selection
Function Library
Statistical
Engineering
Cube
Information
VAR
=
Book1
Trial
cm
1 19.7
2 20.2
3 24
4 21.8
5 22.3
21.6
2.965
=
Average
Variance
NORMDIST
NORMINV
NORMSDIST
NORMSINV
PEARSON
PERCENTILE
PERCENTRANK
PERMUT
POISSON
PROB
QUARTILE
RANK
RSQ
SKEW
SLOPE
SMALL
STANDARDIZE
STDEV
STDEVA
STDEVP
STDEVPA
STEYX
TDIST
TINV
TREND
TRIMMEAN
Insert Function...

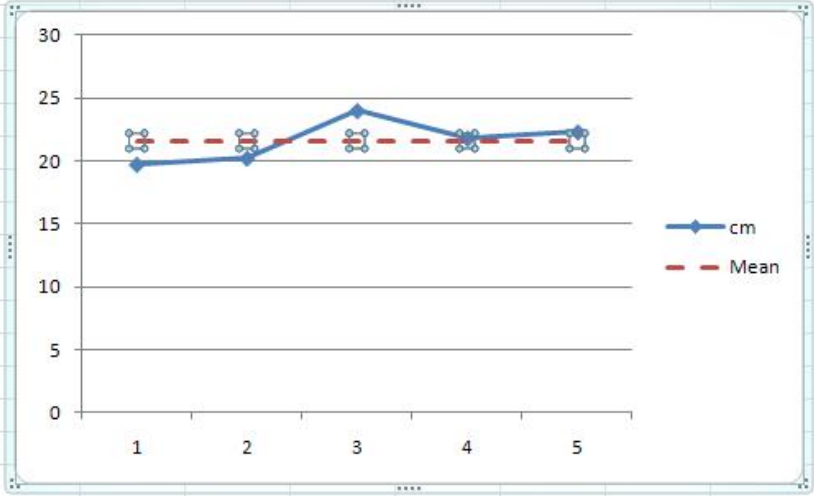

Book1
A B C D E F G H I
1 Trial cm
2 1 19.7
3 2 20.2
4 3 24
5 4 21.8
6 5 22.3
7 21.6 2.965 =STDEV(B2:B7)
8 Average Variance
Function Arguments
STDEV
Number1 B2:B7 = {19.7;20.2;24;21.8;22.3;21.6}
Number2 = number
= 1.540129865
Estimates standard deviation based on a sample (ignores logical values and text in the sample).
Number1: number1,number2,... are 1 to 255 numbers corresponding to a sample of a population and can be numbers or references that contain numbers.
Formula result = 1.540129865
Help on this function
OK
Cancel

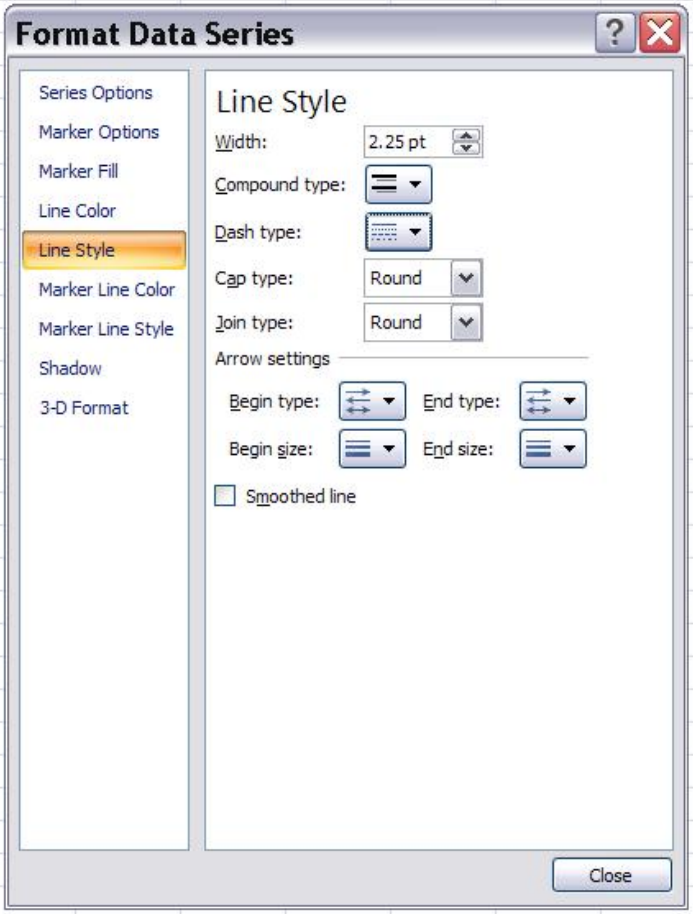

Home
Insert
Page Layout
Formulas
Data
PivotTable
Table
Picture
Clip Art
Shapes
SmartArt
Column
Line
Tables
Illustrations
B1
cm
Book1
Trial
cm
Mean
Average
Variance
StDev
30
25
20
15
10
5
0
1
2
3
4
5
cm
Mean


Book1
Trial
cm
Mean
1 19.7 21.6
2 20.2 21.6
3 24 21.6
4 21.8 21.6
5 22.3 21.6
21.6 2.965 1.54013
Average Variance StDev
30
25
20
15
10
5
0
1
2
3
Delete
Reset to Match Style
Change Series Chart Type...
Select Data...
3-D Rotation...
Add Data Labels
Add Trendline...
Format Data Series...

Book1

	A	B	C	D
1	Trial	cm	Mean	
2	1	19.7	21.6	
3	2	20.2	21.6	
4	3	24	21.6	
5	4	21.8	21.6	
6	5	22.3	21.6	
7		21.6	2.965	1.54013
8		Average	Variance	StDev





Homework is to do this with your own data. If you need help here is a site with simple tutorials using Excel.

- http://www.ncsu.edu/labwrite/res/gt/graphtut-home.html
- Use Excel to find the mean, variance and standard deviation of the measurements you made on Thursday and graph them.