ALLANTOIN

Updated Sept 2017

This Procedure is accompanied by a Video demonstration that can be downloaded at:
http://animalscience.ucdavis.edu/faculty/robinson/videos.html

Procedure:

- ✓ This is a colorimetric method described by Young and Conway (1942). In this procedure, allantoin is:
  - Hydrolyzed under a weak alkaline (0.5 M NaOH) condition at 100ºC (**boiling bath**) to allantoic acid (C4H6N4O3)
    - *Need a pH = 12 for 100% Allantoin recovery*
    - *Completely converted to allantoate @ pH 12 in ~ 6 min boiling. Longer heating (<20 min) does not affect results*
    - *But @ 90°C it takes 20 min, @ 100°C it takes 8-12 min.*

Has to BOIL

  - Degraded to urea and glyoxylic acid in a weak acid (0.5 M HCL) solution.
    - Everything should be degraded – thus not sensitive to time
    - *Hydrolysis complete at 2 min, but not affected up to 10 min @ 100°C. If 90°C it takes 3-5 min.*
  - The glyoxylic acid reacts with phenylhydrazine hydrochloride (Phen) to produce a phenylhydrazone of the acid.
    - *Need to be cooled immediately to freeze point to stop reaction. Longer than 3 min in ice bath has no affect.*
    - *MAIN thing is to cool RAPIDLY.*
    - *If cannot get bath to -10°C with salt, use alcohol*

Has to FREEZE (-10°C)

  - This product forms an unstable chromophore with potassium ferricyanide (Pot Fer) in acid conditions (HCL).
    - Unstable, therefore time sensitive
    - *Once acid is added,*
- ✓ This procedure requires critical timing of the reactions.
  - If solution is not cooled completely in ice bath, or acidified properly, product forms unevenly (temp sensitive) after Pot Fer is added
    - Warmer samples give faster reaction with Pot Fer – thus conversion to chromophore occurs faster in warmer water. Ice bath slows reaction to give time to add Pot Fer to all samples before timer starts
    - This results in uneven color distribution through the glass tube
  - Therefore ensure ice bath < 0ºC & cooling bath < 10ºC
  - Ensure HCL mixed in with thumb action
- ✓ The spec reading must be done within the shortest possible time-span, as OD decreases with time.
  - Generally decreases with ~ 0.01 over 15 min
- ✓ Therefore, no more than 15 samples should be processed in each run. Two sets of allantoin standards, two blanks (no allantoin standard in) with distilled (DI) water are also processed in duplicate in each run.

- *To test max number of samples that can be run:*
  - *Do test run with 5 test samples and standards*
  - *Do samples in triplicate. Thus 5 samples, 3 times = 15*
  - *If calculated concentrations between the 3 triplicates differ, then too much time elapse between sample prep and less samples need to be done in a run*
  - *If concentrations do not differ, 15 samples can be done in each run*

✓ Have 1 inter-run (STD) sample (duplicate = STD1 & 2) in each run – same sample type as is being analyzed (e.g., urine)

- Concentration of inter-run samples should not deviate more than 5% from average after all runs, otherwise run should be excluded and samples re-run

✓ Concentrations for standards at the beginning and end of run should be the same

- For standard curve, both sets of standards are averaged on a curve to get equation
- X-axis = λ and Y-axis = concentration, THEN equation can be used to calculate concentration from read OD
  - Record all λ into spreadsheet
  - Graphs are set up to show standards & duplicated samples
  - Check that standard curves look correct
    - 1st and 2nd set should not deviate a lot
    - 2nd set will lose some concentration
    - However, since average of both standard sets are used, it corrects for time lag between samples
  - Once all samples have been recorded, input equation values from standard curve into relevant cells to calculate concentrations (mg/dl)
    - Check that standard concentrations equal 20, 40, 60, 80 & 100 mg/dl
    - Check that duplicate samples do not differ by > 5 mg, if so, mark sample for rerun
    - Transfer STD 1 & 2 concentrations from previous run and check it doesn't differ by > 5 mg
  - If all looks in order, continue with next run

✓ Using blanks

- Make 2 blanks containing only DI water to calibrate the spectrophotometer for each run
- Calibrate when switching on as well as prior to each run if it shifted
- Note that used and new cuvettes have different OD readings, therefore do not mix new with used within a run

✓ Cleaning cuvettes:

- Place cuvette into cuvette holders with ▼ pointing to the infrared light in the spec-meter when read
  - Use disposable pipettes to add sample to cuvette, new pipette for each sample

- Note down reading
- Use central pressure vacuum to remove liquid from cuvette
    - Attach pipe to vacuum tap (White LV)
    - Have 2 volumetric flasks with outlet ("traps") attached with a tube
        - It is necessary to use 2 to ensure that moisture does not move into the vacuum system
        - Large flask: Have 1st trap's inside tube high up to allow buildup of liquid in flask
        - Small flask: Have 2nd trap's inside tube low down to prevent moisture being sucked into tube if it comes in
    - Attach long steel thin syringe to end of flexible pipe attached to large flask to suck out liquid from crucible
        - Hang tube with syringe high to prevent moisture from flowing back out
- Add next sample
- Cuvette can be discarded after each run and a new one used for next run to avoid permanent discoloration of cuvette

Tips:

- Place ice bath on cold packs in freezer for additional cooling
- Tape around glass tubes important for grip and identification

- **Repeating samples:**
    - If 2 replicates within run differ by more than 5, repeat with diluted urine.
    - If 3 out of 4 values are the same, remove incorrect value.
    - If no comparison, redo using centrifuged urine.
    - If these 2 replicates look the same as before, again differ more than 5, redo from scratch (defrosted original urine).

- **Test samples:**
    - Use samples from the same group as the main samples
    - Take them from the whole range of possible samples to ensure all concentrations are seen
        - Eg. One test sample from each dairy/treatment group
- **Repeatability:**
    - However the samples are treated, keep it the same between all samples
    - If the duplicate tubes are taken from the *same* location in the centrifuged tube, do this for all samples. If it is taken from *different* locations, also repeat (this may cause more variability)

1) Preparation:

◊ Check spectrophotometer
- ➢ Cuvettes to use inside = # of runs in TOTAL
  - ❖ Do not mix NEW and USED cuvettes, since λ differs
- ➢ How to operate Spectrophotometer

◊ Collect all required equipment/instruments

◊ Find/purchase all required chemicals

◊ Check urine sample dilution
- ➢ Allantoin require 17 – 68 times
  - ❖ If urine = 7 ml in total sample of 35 ml, then 35/7 = 5.
    = 7 in 35 ml *(35 ml should include 7 ml urine to give total volume)*
    = 1:5
  - ❖ To get to 1:60 dilution
    = 1:5 x 12
  - ❖ Since tube to dilute samples in = 5 ml (only 2 ml required/run)
    - Add 0.2 ml urine into 2 #2 tubes (duplicate)
    - Add 2.2 ml DI water into each tube

    = 2.4 ml of diluted sample
    = 0.2/2.4
    = 1:12

◊ Sort out urine samples
- ➢ Select urine samples to be analyzed and place in urine racks
- ➢ For 1st time analysis, use selected urine samples as test samples (~14)
- ➢ Select 1 test sample to use as inter-run STD – keep with urine samples

◊ Make lists to write down urine control #

◊ Have spreadsheet ready to process results and run SAS

◊ Create required tube labels
- ➢ Blue round labels with control # for urine samples to be analyzed
- ➢ Colored labels for Standards
  - Label with color stickers on LID and place in Lincoln rack
- ➢ Lab bench labels
- ➢ Chemicals in flasks

◊ Set up tube racks according to Section 3 (Apparatus)

◊ Prepare 3 sets of 3# glass tubes according to diagram
- ➢ Blank: B
- ➢ Standards: 20 – 100 mg/dl: Yellow
- ➢ STD 1 & 2: Blue
- ➢ Sample 1 – 14: Purple

1	2	3	4	5	6	7	8	9	10
B	20	40	60	80	100	STD 1	1	2	3
11	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
4	5	6	7	8	9	10	11	12	13
21	**22**	**23**	**24**	**25**	**26**	**27**	**28**		
14	STD 2	B	20	40	60	80	100		

◊ Mark tubes as in Section 3 (Apparatus)

◊ Place tubes in corresponding racks
- ➢ 26 Inter-run standard sample tubed in STD dilute rack
  - ▪ Add 26 tops
- ➢ 42 Dilute urine tubes in diluted rack
  - ▪ Add container of red tops
- ➢ First set of centrifuge tubes in centrifuge rack
  - ▪ Add container of tops
- ➢ 2 Inter-run STD to dilute

◊ Set up lab bench

◊ Go through procedure to check if sequence of bench works out

◊ Have table @ door of fridge & freezer to put ice/cold baths on

◊ Place extra stuff to restock on bottom of cart

◊ Prepare Standards and Reagents

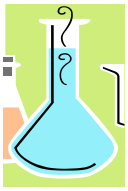
➢ *Do test runs until happy with results !!!!*

Timing:
- ❖ Morning setup = 90 min (prepare STD at start = 20 min)
- ❖ Complete 1st run = ~2h10
- ❖ Every additional run = ~1h
- ❖ Clean up = 25 min
- ❖ Prep for next day (marking) = 30 min
- ❖ TOTAL/day = 9h30min
- ❖ **6 RUNS = 8:00 – 17:30**

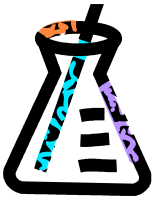
2) Apparatus:

- ◊ Spectrophotometer (1)
- ◊ Centrifuge (1)
  - ➢ Can 15 ml tubes fit?
  - ➢ Glass/Plastic -Tubes must go in smoothly, no pushing, otherwise it gets stuck or break
- ◊ Small scale (measure in grams)
- ◊ Kettle (1)
- ◊ Vortex (1)
- ◊ Boiling water bath – 100^{o}C (1)
  - ➢ Pot to boil water in
  - ➢ Heating plate to put pot on
  - ➢ Thermometer to measure temp
  - ➢ Warning/Beware sign to show plate is hot
  - ➢ Metal tubes on bottom of pot to keep racks from touching bottom – avoid excessive bubbling
  - ➢ Weight to keep plastic trays in water
- ◊ Ice bath (2) – plastic freezer containers (-10^{o}C)
  - ➢ Fill to cover ¾ of the tubes in tube rack (all the fluid that is in the tube should be cooled
- ◊ Cooling bath (2) – deep plastic container filled chilled water in fridge (< 20^{o}C)
  - ➢ Fill to cover ¾ of the tubes in tube rack (all the fluid that is in the tube should be cooled
- ◊ Thermometer
- ◊ Glass oven bowl to work in – laid with cheese cloth (1)
- ◊ pH meter and standards (1)
- ◊ Disposable droppers plastic – box (500)
- ◊ Large plastic cup to keep droppers on bench – marked pipettes
- ◊ Timer & battery
  - ➢ 4 different time settings (7, 4, 20, 30 min)
- ◊ Safety Goggles
- ◊ Lab coat
- ◊ Funnels (Small & Large plastic)
- ◊ Gloves:
  - ➢ Thermo, to work with hot/cold
  - ➢ Box of medium latex gloves
- ◊ Trolley to keep laptop & restock equipment on

- ◊ Bucket/Drum to discard liquid waste into (10 Gal)
- ◊ Paper towels (5 rolls)
  - ➢ Place in paper towel dispenser on bench
- ◊ Blue towels (stack)
- ◊ Trash can large (or container with trash bags) – on floor
- ◊ Ziplock bags - quart
- ◊ Weighing spoon
- ◊ Marbles & 3 x aluminum pie cups to hold them
- ◊ Stationary etc:
  - ➢ Pens & markers (small, large + x-large sharpie)
  - ➢ General writing pad
  - ➢ Stickers to mark glass flasks
  - ➢ Duct tape: Yellow, Blue, Pink & White
  - ➢ Alligator clips to hold procedure
- ◊ FLASKS – Mark all with stickers
- ◊ Glass volumetric flasks



  - ➢ 250 ml x 5 = Mixed standards (mark 20 – 100)
  - ➢ 250 ml x 2 = Pot Fer & Phen (mark name with weight to add)
  - ➢ 1000 ml x 3 = 0.5M HCL (1) & 0.5M NaOH (2)
  - ➢ 2000 ml x 1 = 0.5 M HCL
- ◊ Conical volumetric flasks



  - ➢ 1000 ml x 1 = Allantoin stock solution
  - ➢ 500 ml x 1 = 0.01M NaOH
  - ➢ 100 ml x 1 = 0.01M NaOH – to measure into
  - ➢ 500 ml x 1 = Add Alcohol for ice bath
  - ➢ 1000 ml x 1 = Add Alcohol for ice bath
- ◊ Glass flasks

  - ➢ 100 ml x 1 = DI H2O – Blank spectrophotometer
  - ➢ 250 ml x 2 = To keep for HCL and Pot Fer in fume hood (drip)
  - ➢ 300 ml x 1 = Phen – on bench
  - ➢ 500 ml x 1 = For pH rinsing & other rinsing water
  - ➢ 500 ml x 2 = To keep 0.5M HCL & 0.5M NaOH on bench
  - ➢ 1000 ml x 1 = Clean DI water
- ◊ Repipette bottles x 3 & pipette attachments
  - ➢ Set @ 3 ml = [HCL] in fume hood (10 ml)
  - ➢ Set @ 1 ml = Pot Fer in fume hood (5 ml)
  - ➢ Set @ 5 ml = Water (25ml)
  - ➢ Set @ 2.2 ml = Water (10ml)

- ◊ Eppendorf pipettes
  - 10 ml (1) - Set @ 1.5 ml (7.5 x 0.2 ml dispersals) (0.5 M HCL)
  - 50 ml (1) – Set @ 1 ml (1 x 1 ml dispersals) (0.5 M NaOH)
- ◊ Pipettes:
  - 1 ml (1) - Set @ 1.00 (samples, standards, blank) – use new tip
  - 1 ml (1) - Set @ 0.20 (urine to dilute)
- ◊ Pipette tips:
  - Blue, 1 ml (3 boxes (300)/day)
- ◊ Cuvettes, 10 mm light path, disposable (1/run/day) – for spectrophotometer
  - Do NOT use in boiling bath: Polymethylmethacrylate = methylmethacrylate = acrylic & polystyrene
    - Glass transition temperature < 100ºC (the critical temp at which it changes behavior from "glassy" to "rubbery")
- ◊ Tubes
  - #1 Plastic tube (15 ml)
    - 24 = Inter-run sample dilute
      - Mark as SDT 1-12 in duplicate (1 new tube/day)
      - Must be plastic = expand when freeze
    - 24 **Tops** to freeze
  - #2 Plastic tube (5 ml)
    - 342 = Diluted urine (171 x 2)
      - Only rack marked – use red tops to hold place in row
    - 14 **Tops** (red stoppers) placeholders
  - #3 Tall Glass tubes (15 ml)
    - 84 = Allantoin procedure (28 x 3 sets)
      - Mark according to procedure instructions with tape
      - Use quart/glass test tubes in boiling water bath
  - #4 Plastic tubes (40 ml)
    - 70 = Allantoin standards (12 x 5) + 10 stock solution
      - Mark with color labels (20 – 100)
  - #5 Plastic tubes (15 ml)
    - 171 = Centrifuge original urine samples
      - Transfer control # from original urine sample
      - Will be frozen at end of each day
    - ✓ 2 = Inter-run sample centrifuge
      - Mark as SDT (in duplicate since need ~13 ml)

- ◊ Tube racks
  - ➢ 3 x Plastic with handles = hold glass tubes in baths
    - Marked 1 - 28
  - ➢ 2 x Small metal = hold diluted urine samples
    - 1x Marked 1 – 7, in duplicate (8 – 14), for number of runs
  - ➢ 1 x Metal green = hold diluted Inter-run standards
    - Use to hold frozen STD
    - Marked Inter run STD
  - ➢ 1 x Metal green = for plastic tubes in & out of centrifuge
    - Marked centrifuge
  - ➢ 1 x steel square = drying glass tubes
  - ➢ 1 x Lincoln rack = Thawing original urine
  - ➢ 1 x Lincoln rack = Standard samples
  - ➢ 3 x Lincoln rack = All samples to be analyzed (171)
  - ➢ 1x Lincoln rack = Test urine samples

3) Chemicals:

✓ Calculations:

- 28 tubes/run
  - (7 samples x 2) + (5 standards x 2) + 2 blanks + 2 inter-run samples
- 6 Runs/day (4 New + 2 repeat) – *More than 6 runs/d can lead to fatigue*
- 28 NEW Urine samples/day
- Do 5 runs of testing to get technique right (or 2 full days of practice)

	PER TUBE	PER RUN	PER DAY	TOTAL	Round up	Weight/ volume	Volume	Total	Unit
Standards (each of 5)	1	2	12	120	150				
Inter-run STD	1	2	12	120	150				
DI Water	5	140	840	8400					
0.5 M NaOH	1	28	168	1680	2000	25.75	1000	52	g
0.5 M HCL	1.5	42	252	2520	3000	41.25	1000	124	ml
[HCL]	3	84	504	5040	5500	1	1	5500	ml
Phen	1	28	168	2500	2500	3.5	1000	9	g
Pot Fer	1	28	168	2500	2500	16	1000	40	g
Allantoin				750	1000	0.1	1000	0.1	g
0.01 M NaOH				500	500	0.2575	500	0.3	g

◊ 52 g Sodium Hydroxide (**NaOH**) *CORROSIVE*

- 25.75 g to make 1000 ml 0.5 M
- 0.2575 g to make 500 ml 0.01 M

◊ 6 L 12 M (37%) Conc Hydrochloric acid **[HCL]** *CORROSIVE / TOXIC*

- 5500 ml concentrated *(SG = 1.19)*
- 41.25 ml to make 1L 0.5M HCL

◊ 9 g Phenylhydrazine Hydrochloride ($C_6H_9N_2Cl$) **(Phen)** *POISONOUS*

- 3.5 g to make 1000 ml

◊ 40 g Potassium Ferricyanide ($C_6FeK_3N_6$) (**Pot Fer**) *HARMFUL*

- 16 g to make 1000 ml

◊ 11 L 90% Isopropyl **alcohol** – *normal rubbing alcohol*

- 5360 ml to make 8000 ml 70% Alcohol (start with 8 L)
- 1000 ml 90%/2 days to fill up (go for 10 days = 5 refills)

◊ > 0.1 g **Allantoin** (from Sigma or BDH)

- 0.1 g to make 1000 ml stock solution (need 750 ml)

4) Standards:

1) Prepare (500 ml) **0.01 NaOH**
   - Dissolve 0.2575 g NaOH in 500 mL DI H_2O (0.0515 g in 100 ml).
2) Prepare 1000 ml **Allantoin** stock solution (100 mg/L):
   - Weigh 0.1 g of allantoin and transfer it to a 1000 ml volumetric flask.
   - Dissolve in approximately 100 ml of **0.01 NaOH**, and q.s. with DI H_2O.
     - The addition of NaOH is only to help dissolve allantoin.
     - If doesn't dissolve, add more NaOH
3) Working standards of 20, 40, 60, 80 & 100 mg/L
   - Pipette into 250 ml volumetric flasks the reagents indicated below and
   - make up the 250 ml volume with DI H2O *(see ROUND UP total):*

Standards [mg/L]	Allantoin stock solution (@ 100mg/L)
20	50
40	100
60	150
80	200
100	250
Total Stock (ml)*	**750**

- 5ml stock, filled to 50 ml with DI H_2O = 5ml x 100mg/L = 0.5mg Allantoin
  - Thus 0.5mg Allantoin in 50 ml of DI H_2O = 0.5mg/50ml x 1000ml/L = 10mg/L

- 10ml stock, filled to 100 ml with DI H_2O = 10ml x 100mg/L = 1mg Allantoin
  - Thus 1mg Allantoin in 100 ml of DI H_2O = 1mg/100ml x 1000 = 10mg/L

- 15ml stock, filled to 150 ml with DI H_2O = 15ml x 100mg/L = 1.5mg Allantoin
  - Thus 1.5mg Allantoin in 150 ml of H_2O = 1.5mg/150ml x 1000 = 10mg/L

- 30ml stock, filled to 150 ml with DI H_2O = 30ml x 100mg/L = 3mg Allantoin
  - Thus 3mg Allantoin in 150 ml of DI H_2O = 3mg/150ml x 1000ml/L = 20mg/L

- Store standards in small quantities in the freezer
  - Divide into plastic container (milk sample tubes) of ~20 ml each
    - It expands a lot when frozen
    - Require ~ 20 ml/day = 13 x 19 ml tubes per standard
  - Only required quantity is thawed and rest discarded, thus calculate the amount required before each run
    - Allantoin is less stable in solution. If not freeze, prepare fresh every day

5) Reagents:

1) 0.5 M Sodium Hydroxide **(0.5 NaOH)** - *(can be kept)*
   - Dissolve 25.75 g NaOH in DI H_2O
   - Fill to 1L
   - Make in TRIPLICATE (*Total = 3L)*
2) 0.5 M Hydrochloric Acid **(HCL)** - *(can be kept)*
   - Add 41.25 ml *(82.5 ml)* of 12M HCL to ~250 mL DI H_2O
   - Fill to 1L *(2L)*
   - Make 2x 1L, 1x 2L *(Total = 4L)*
3) 12 M Concentrated Hydrochloric acid **[HCL]** at -20^oC
   - Put in Repipette bottles for easy measuring
   - Set pipette to 3 ml

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4) 0.023 M **Phen**ylhydrazine Hydrochloride - *(made fresh daily – Approx 224 ml/day)*

- Dissolve 0.1663g in 50 ml DI H_2O

Phen	*base*	*/day*
ml	50	250
g	0.1663	0.8315

5) 0.05 M **Pot**assium **Fer**ricyanide - *(made fresh daily – Approx 224 ml/day)*

- Dissolve 0.835g in 50 ml DI H_2O

Pot Fer	*base*	*/day*
ml	50	250
g	0.835	4.175

- Put in brown pipetting bottles for easy measuring

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6) Set up for analysis (day before):

1) Prepare 70% (v/v) **Alcohol** ice bath & place in freezer overnight
   - 1.5 L = add 1000 ml 90% Isopropyl alcohol to 500 ml water
   - Pour into ice containers x 2 (4 L each)
     - If water is frozen next morning, add more alcohol to lower the freezing temperature
     - Alternate ice baths after each run to keep it cold < -10^{o}C
     - Refill baths with alcohol when needed (500ml 90% + 250ml H20)
2) Prepare plastic cooling baths and keep in fridge overnight
   - Pour cold water into plastic container x 2
     - Alternate cooling baths after each run to keep it cold < 10^{o}C
3) Set timer (mark on timer to know which is which):
   - 1 = 7 min
   - 2 = 4 min
   - 3 = 20 min
   - 4 = 30 min
4) Defrost for the next day:
   - Allantoin standards x 5
   - Inter-run STD x 2
   - Urine samples to analyze (pick randomly from selected group)
     - Only defrost the samples to do in one day
       - ✓ 4 runs = 28 urine samples (can increase to 5/6 runs if needed)
   - Samples to be thawed for next day should be removed from freezer around mid-day and kept @ room temperature to ensure proper thawing. Afterwards it can be placed in fridge – ***set alarm to remember to remove next set of samples***
   - Mark samples with control #
     - Pick next set to thaw & put in rack *(pick randomly from main group but keep in sequence to make marking easier)*
     - Select sample, mark with sticker and note down control # on sample set sheet (keep in sequence)
     - Mark Centrifuge tubes with new control # (keep in sequence)
     - *Random design will come in when remove from centrifuge*
5) Stock equipment (see checklist in Section 10)
6) Fill all water containers
7) Put [HCL] in fridge
8) Prepare small pre-weighed sets of Pot Fer & Phen
9) Print Absorbance Run sheets

7) Morning Checklist:

- *Total = 90 min (prep Inter-run STD = 20 min)*

◊ Centrifuge urine samples *(Centrifuge take 9 x 4 tubes = 36)*
- Use #3 plastic tubes
  - Number according to urine sample control # (write with sharpie on tube)
- Mix urine samples **(& standard**) 3 times slowly by tilting it
- Pour > 5 ml urine into glass tubes (0.2 + 0.2)
  - See level on example tube – level with top of rack
  - Create duplicate tube for standard (10 x 2 = 20 ml)
  - Make sure tubes fit easily in centrifuge holes
- Centrifuge - 15 min @ 1200 x g (RCF)
- Get subsample/dilution tubes ready (#2 tubes)
- Put leftover urine back in fridge (toss at end of day)

◊ Dilute urine samples to run *(7 urine samples/run @ **0.2** + 2.2 DI H_2O (1:12))*
- Add urine (0.2 ml)
  - Write urine control # from centrifuge tube onto run sheet
  - Pipette urine into #2 tubes (duplicate), from **same** spot in all tubes
    - ✓ Use red tops to indicate which tubes have been filled
    - ✓ Use new pipette tip for each sample
    - ✓ ***ONLY 1st time:***
      - 1 urine STD (inter-run) sample @ **1** in 11 DI H_2O (1:12) into 13 *duplicate* #1 tubes (need 12 ml/day)
      - = 26 tubes of 12 ml diluted urine each
      - = need 13 ml of centrifuged urine for STD
      - = centrifuge duplicate tubes for STD
- Add DI H_2O (2.2 ml) to each urine tube

◊ Put leftover centrifuged samples in fridge (freeze at end of day)

◊ Set up laptop and work sheets

◊ Switch on Spectrophotometer (at back near power cord)
- Set Timer T4 = 30 min to warm up

◊ Prepare the fresh solutions for Pot Fer & Phen
- Calculate required amount for nr of runs to do
- Make extra Pot Fer (yellow) to enable using Repipet

◊ Switch on the water bath
- Fill with hot water
- Water needs to boil at 100^{o}C (takes ~30 min depending on size)
- Boil kettle

- ◊ Remove ice bath from freezer
- ◊ Set glass tubes in racks (3 sets) according to chart
- ◊ Put marbles in plastic container with paper towel
- ◊ Mix and open all chemicals, standards and samples
  - DI H2O
  - 0.5 M NaOH
  - 0.5 M HCL
  - Phen
- ◊ Place inter-run STD and AL standards on ice on bench
- ◊ Calibrate pH meter (keep standards in fridge with samples)
- ◊ Place Pot Fer (in Repipette bottle) in fume hood
- ◊ Prepare fume hood
  - Place empty marble container and catch cups for Pot Fer and HCL in hood on blue towels
- ◊ Check & refill boiling water bath
  - Boil water in kettle
- ◊ Set Spec wavelength and measure blank

8) Method *(put up over bench):*

1) Add **5 mL** of **DI H_2O** to each tube

2) Pipette **1 ml** of standard, sample and blank into test tubes – *check liquid level*

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3) Add **1 mL** of **0.5 NaOH** to each tube, place marble on and MIX by vortex (set on 3.5)

4) Place the rack in **boiling water** bath for ***7 min*** – *Time NB*

- Set timer 1

5) Add H2O to next run of tubes

6) Add Blanks & Standards to next run of tubes

7) Fetch New cooling bath from fridge, put HCL in freezer

8) Remove rack from boiling water, cool the tubes in ***cold water*** for 4 min

- Set timer 2, Refill boiling bath

9) Add urine samples to next run of tubes

10) Go to STEP 30

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11) Add **1.5 ml** of **0.5 M HCL** to each tube, MIX by thumb

12) Test pH – after adding the HCL, pH must be in the range of 2-3 (~1.9 is ok).

- Start each test run with 1 ml acid to determine if 1.5 is required
- If more HCL is necessary, adjust volume
- 1 ml (pH=12), 1.2 ml (pH=10), 1.4 ml (pH=1.7)
- pH of 0.5M HCL ~ 0.8

13) Rinse marbles & transfer to drying cup

- ***Next steps should NOT be interrupted***

14) Add **1 ml** of **Phen** to each tube, put marble on and MIX by vortex (set on 3.5)

15) Place the rack with tubes in **boiling water** bath for ***7 min*** – *Time NB*

- Boil kettle
- Input next set of control # into run sheet on laptop

16) Return cooling bath to fridge (put in freezer if not cold enough- - check temp)

17) Bring new ice bath and **[HCL]** from freezer

- Set [HCL] in fume hood

18) Toss pipettes

19) Rinse glass tubes

- Pour liquid into chemical waste container
- Rinse thoroughly with DI H2O, inside and out (remove any alcohol outside)

20) Transfer from the boiling bath to the ***ice bath*** for 4 min - Timer 2

- Refill boiling bath

21) Rinse glass tubes

- Set tubes upside down in tube rack
- Rinse bottom with DI H2O and set out to dry

22) In fume hood:

a. Pipette **3 ml** of *cold* **[HCl]** to all samples - don't mix yet

b. Pipette **1 ml** of the **Pot Fer** solution

23) Mix thoroughly by placing thumb on opening and tipping over

- Color must be uniform throughout the tube
- Don't touch bottom of tubes with mixing thumb (contaminated with alcohol)

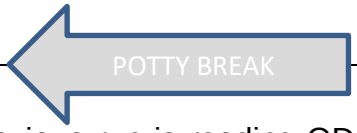
24) Start the timer for 20 min (Set timer 3)

25) Bring marbles back from fume hood

- Rinse & transfer to drying cup

26) Take ice bath back to freezer

27) Set up next set of glass tubes



28) Start next run @ step 3

- Next run will be in cooling bath by time previous run is reading OD. Stop next run at this point since it can wait here before continuing

29) Set up next set of glass tubes

30) Sort samples for next day

- Mark with stickers, transfer control # to dairy sheet, mark centrifuge tubes etc.

31) With **2 of 20 min** to go, organize spec

- Turn vacuum on
- Insert NEW cuvette into spec-meter, run BLANK
- Fill disposable pipette container

32) After 20 min, use disposable dropper to transfer required amount of sample into cuvette

- Leave dropper in tube until end of run, use new dropper for every sample
- Record absorbance @ 522 nm in spreadsheet on laptop
- Use suction to remove liquid from cuvette

33) Start next run @ 11

9) Evening Checklist: *Total = 25 min*

- ◊ Check if next set of samples are thawed. Place in fridge overnight
  - ➢ Samples should be taken out of freezer around mid-day to provide time for it to thaw properly
  - ➢ See **Section 7** for marking **(30 min)**
- ◊ Switch off Spectrophotometer
- ◊ Close all chemicals on bench
- ◊ Dump water out of boiling bath
- ◊ Put ice baths in freezer
  - ➢ Refill with 500 ml Alcohol + 250 ml water
- ◊ Put cooling baths in fridge
- ◊ Refill [HCL] and close > 100 ml
  - ➢ Put in fridge
- ◊ Switch of Fridge & Freezer lights
- ◊ Dump small dilution and glass tubes
- ◊ Leftover standards can be dumped
- ◊ Unused centrifuged urine can be dumped
- ◊ Empty vacuum trap if half full
- ◊ Put pH electrode away (in electrode fluid)
- ◊ Refill stock:
  - ➢ Disposable pipettes next to spec-meter - fill large plastic cup
  - ➢ Blue tips – 3 boxes/day
  - ➢ Red tops – fill glass bowl
  - ➢ White tops – fill plastic bucket
  - ➢ Gloves
  - ➢ Check chemicals according to previous calculation per day
  - ➢ Fill DI water repipette bottles
  - ➢ Absorbance run sheets – print

Check samples that were repeated in final run of day

- ✓ If 3 out of 4 values are the same, do not rerun sample
- ✓ If no comparison between 4 values, add sample to list to rerun next day
- ✓ If still no comparison in values – use average of all 8 values