Pulmo BioMed

Your Breath: Your Health™

Understanding SARS-CoV-2 Airborne Transmission.

The Role of Exhaled Breath

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Overview

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- Coronavirus in Breath.
  - Scientific evidence.
  - Key unknowns.
- Exhaled Breath Diagnostics.
  - Opportunities.
  - Challenge.
- The PBM-xHALE™ approach.
  - Platform IP.
  - Supporting key data.
- Clinical progress update.

Coronaviruses

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- Coronaviridae family:
  - HCoV: SARS, MERS-CoV, SARS-CoV-2.
  - Torovirus (animal diarrhea)

- Three groups:
  - 229E: 1960s – common cold to bronchilolitis (NL63)
  - OC43, HKU-1, SARS, MERS: mild to severe.
  - Group 3: avian.

- Tropism
  - Respiratory
  - Enteric
  - Central Nervous System
  - Ocular

- Animal reservoirs:
  - Cattle, Pigs, Turkeys, Camels, Mice
  - Dogs, Cats, Ferrets, Mink
  - Bats
  - Pangolins (?)
  - Snakes (?)

Phylogenetic study and more reservoirs: Anthony SJ *et al.* 2017 *Virus Evolution* **3(1):** vex012

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Coronaviruses

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  - Respiratory

ANIMAL RESERVOIRS AND GENETIC RECOMBINATION

Colds: Pigs, Dogs, Bats.
Pneumonias: Pigs, Dogs, Cattle, Mice, Camels, Bats.

SARS-CoV-2: 96% identical to bat CoV

Phylogenetic study and more reservoirs: Anthony SJ *et al.* 2017 *Virus Evolution* **3(1):** vex012

COVID-19: SARS-CoV-2 Virology

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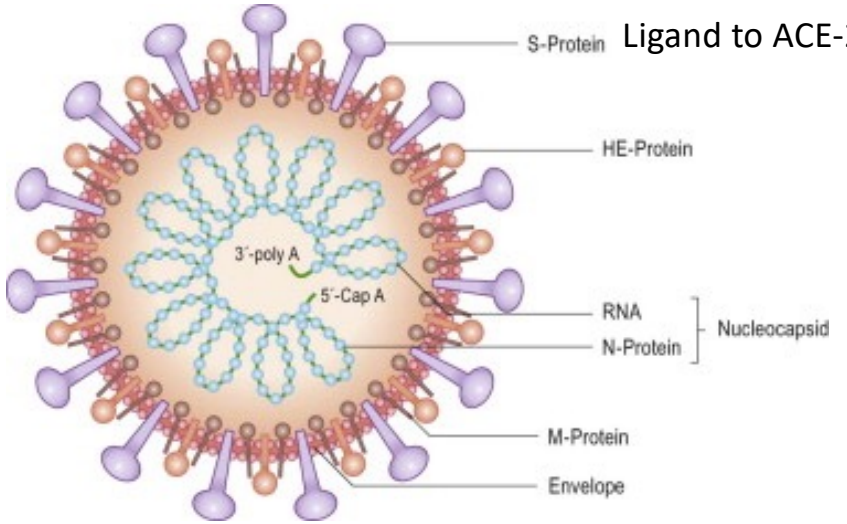
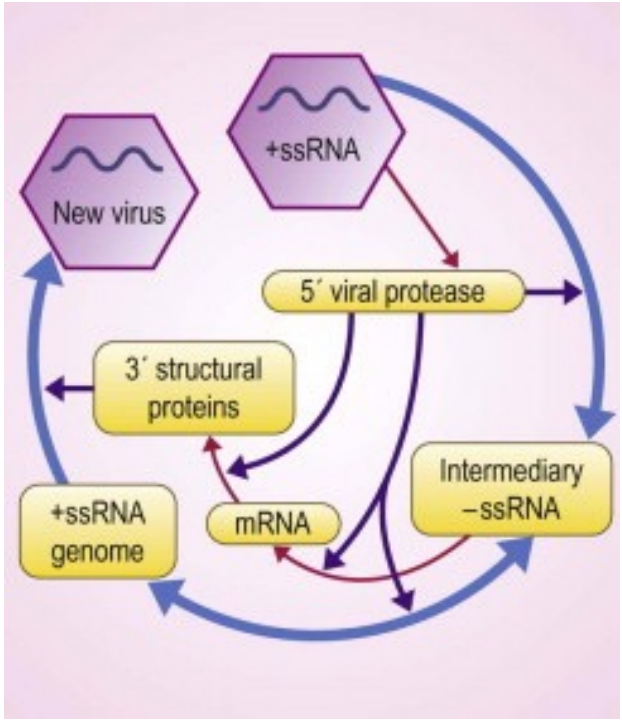


Ligand to ACE-2 (bat, civet, pig)[1]

80 × 160 nm diameter, 12–24 nm surface projections (spikes) that cause the corona



30,000 nt +RNA genome

1. Zhu, P. *et al*. Nature, DOI: 10.1038/s41586-020-2012-7.

COVID-19: SARS-CoV-2 Virology

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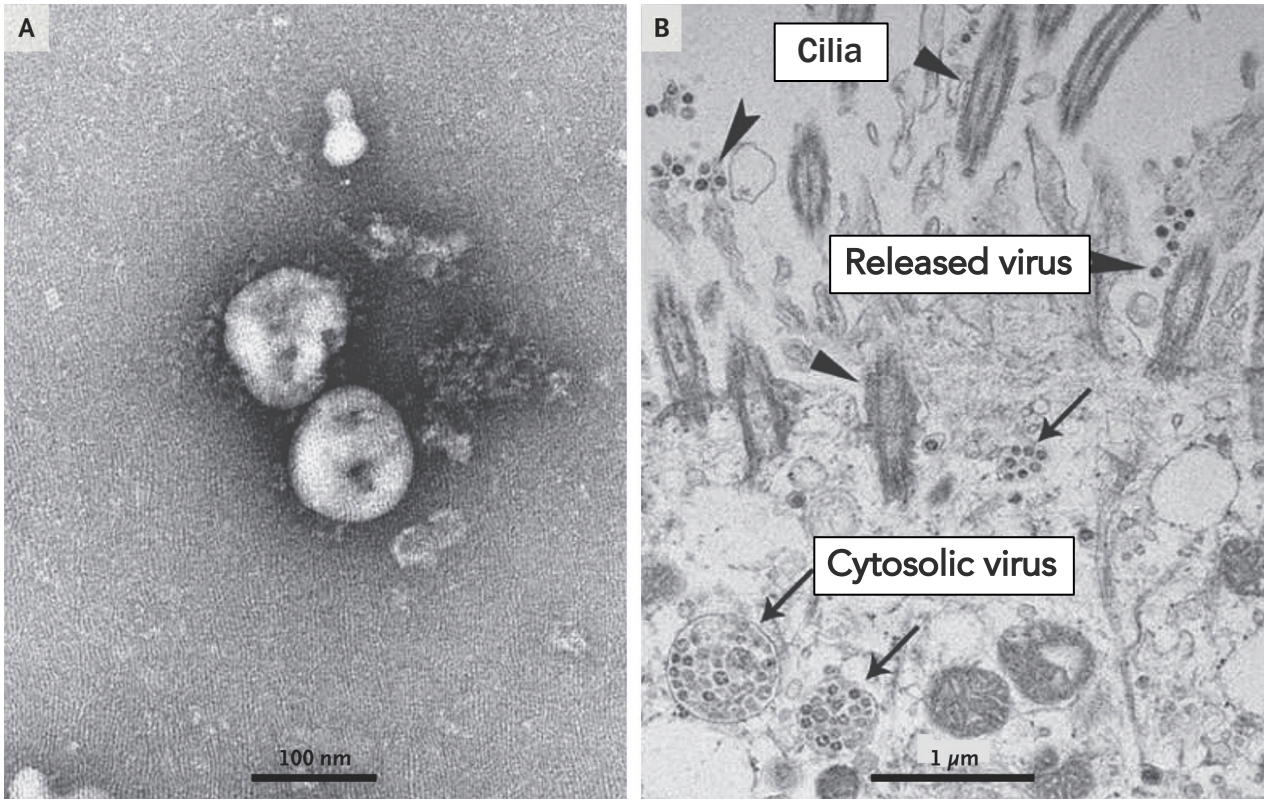

Zhu, N *et al.* 2020 *NEJM;* DOI 10.1056/NEJMoa2001017

SARS-CoV-2 cell entry

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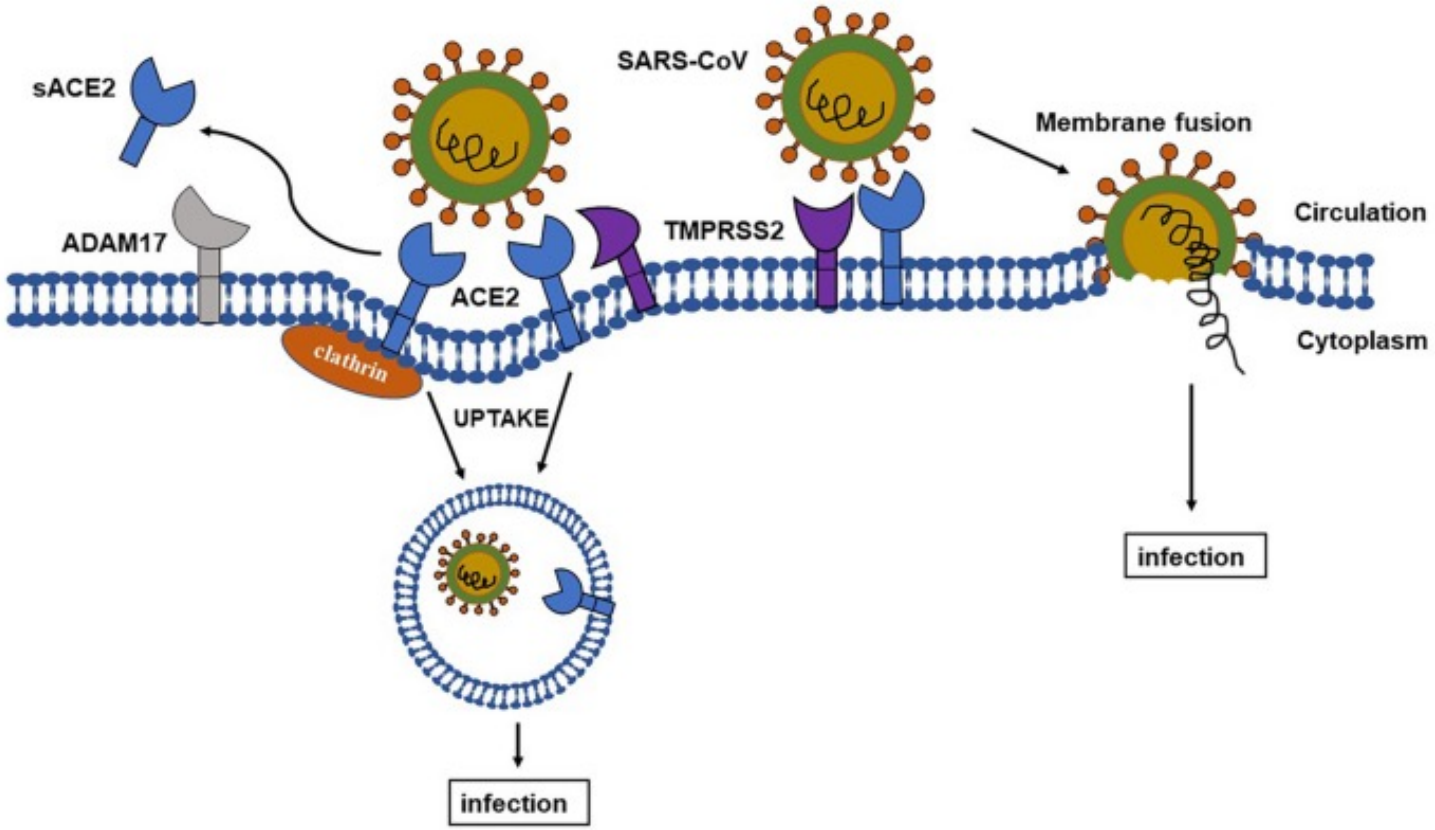

COVID-19: Clinical Presentation

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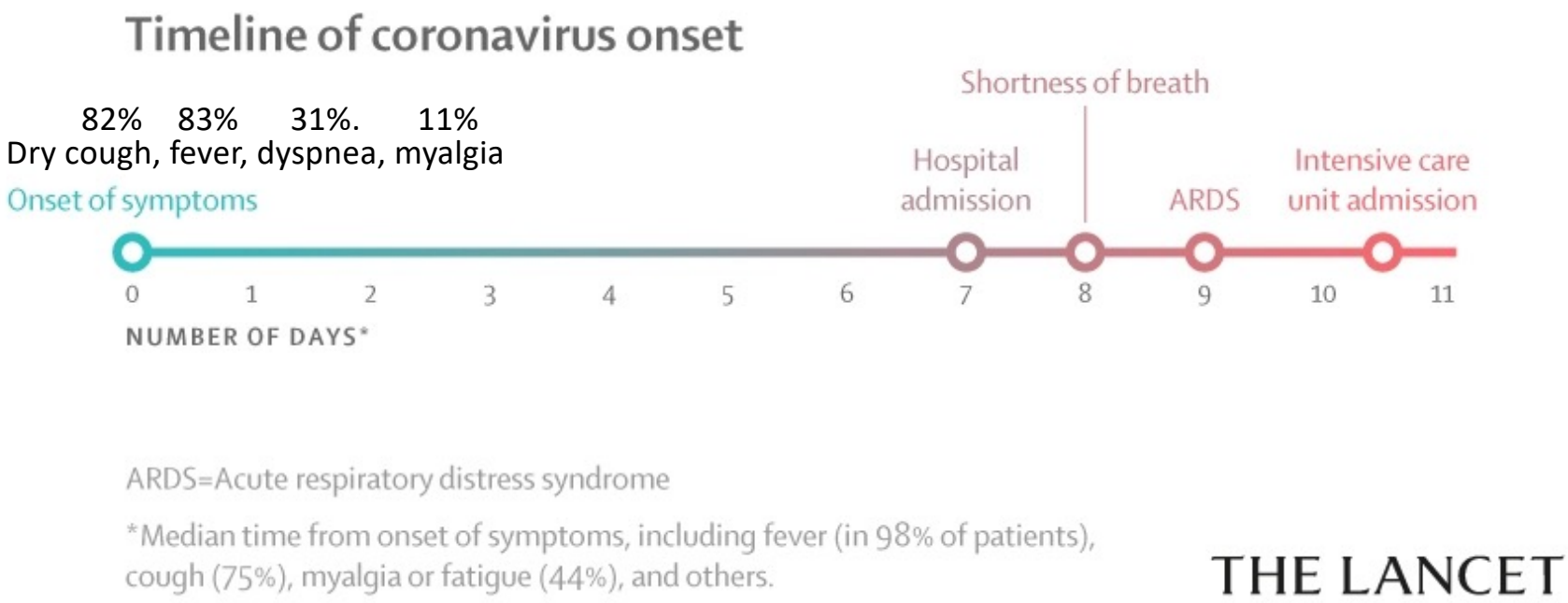

Day to day clinical progression, 1st US patient: Holshue, M. *et al.* 2020 *NEJM;* DOI*:* 10.1056/NEJMoa2001191

Exhaled breath: origin, content, COVID19.

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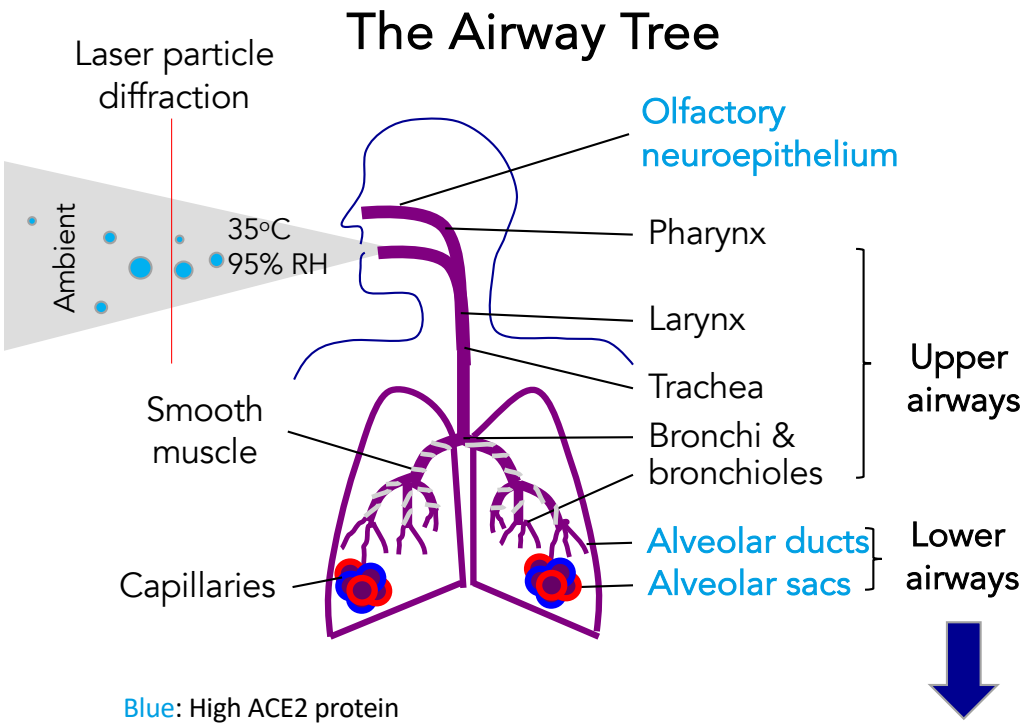

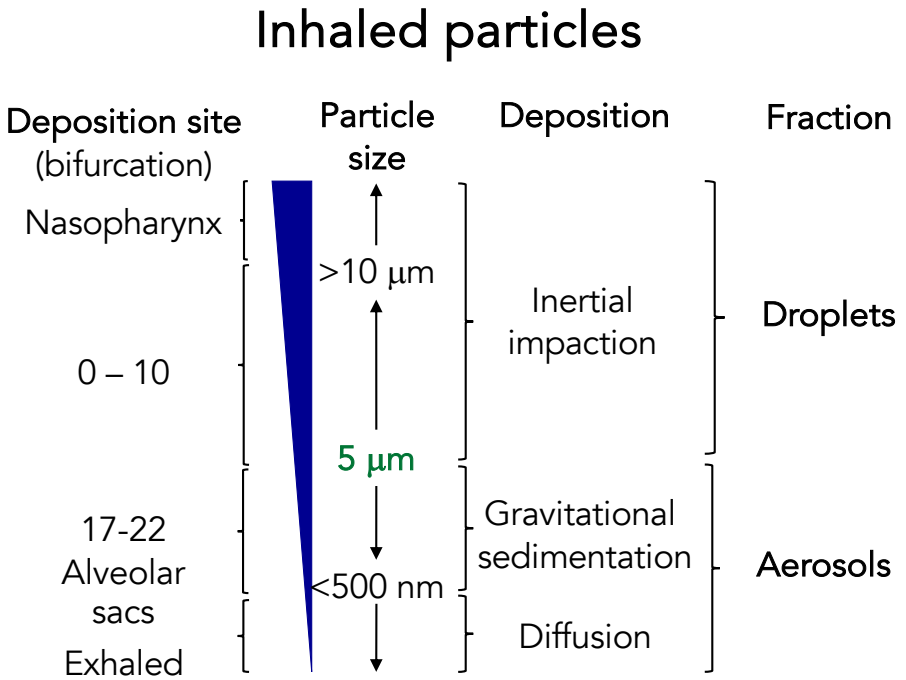

Airway lining fluid droplets
Molecular water

Moschos SA *et al. Pharm. Ther.* 2017

Coronavirus 19 (COVID-19) in breath: Confidence in rationale

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Biology

- SARS-CoV-2 binds ACE2 receptor.[1]
- ACE2 protein levels highest in lower lung.[2]
- Aerosols (<5 μm) best to reach lower lung.[3]

Pathology

- Disease of the lower lung: respirator need.
- Proposed transmission routes: fomites, droplets (cough, >5 μm), but:
  - Models & data[4] show transmission without symptoms (no cough!).
  - Aerosol science used in epidemiology out of date.[5]

1. Hoffman M. *et al. Cell* 2020; 2; Kuba *et al.* 3. Moschos SA *et al. Pharm. Thera.* 2017; 4: Tindale LC *et al. MedRxiv 2020;* 5. Bourouiba L. *JAMA* 2020.

Coronavirus 19 (COVID-19) in breath: Confidence in rationale

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Clinical evidence

- Transmission occurs up to 1 week before symptoms (peak @ -2.9 days).[1]
- Virus genome levels max in lower lung samples > nose > throat.[2-5]
  - <42% false negative oral swabs.
  - 10-25% false negative nasal swabs.
  - Viable virus levels low in nasal swabs.
  - Nasal detection ~70% days 0-5 from symptoms[6]

Experimental evidence:

- COVID-19 ward aerosol gel traps –ve, but ceiling air vents +ve:[7] Droplets pulled by gravity, aerosols pulled by air flow.[8]
- Aerosolised virus infectious for 16hrs after mechanical generation.[9, 10]
- *Other coronaviruses can naturally aerosolize (n=3000).*[11]

1. Tindale LC *et al. MedRxiv* 2020; 2: Winnichakoon P. *JClinMicro* 2020; 3: Wu *et al Clin Inf Dis* 2020; 4: Yang Y. *et al. MedRxiv* 2020; 5: Ai T. *et al. Radiology* 2020; 6: Zhao J. *et al.* Lancet 2020; 7*:* Ong SWX *et al.* 2020; 8: Bourouiba L. *JAMA* 2020. 9: Holbrook MG *et al. NEJM* 2020; 10. Fears AC *et al.* MedRxiv 2020; 11. Leung N.H. *Nature Med.* 2020.

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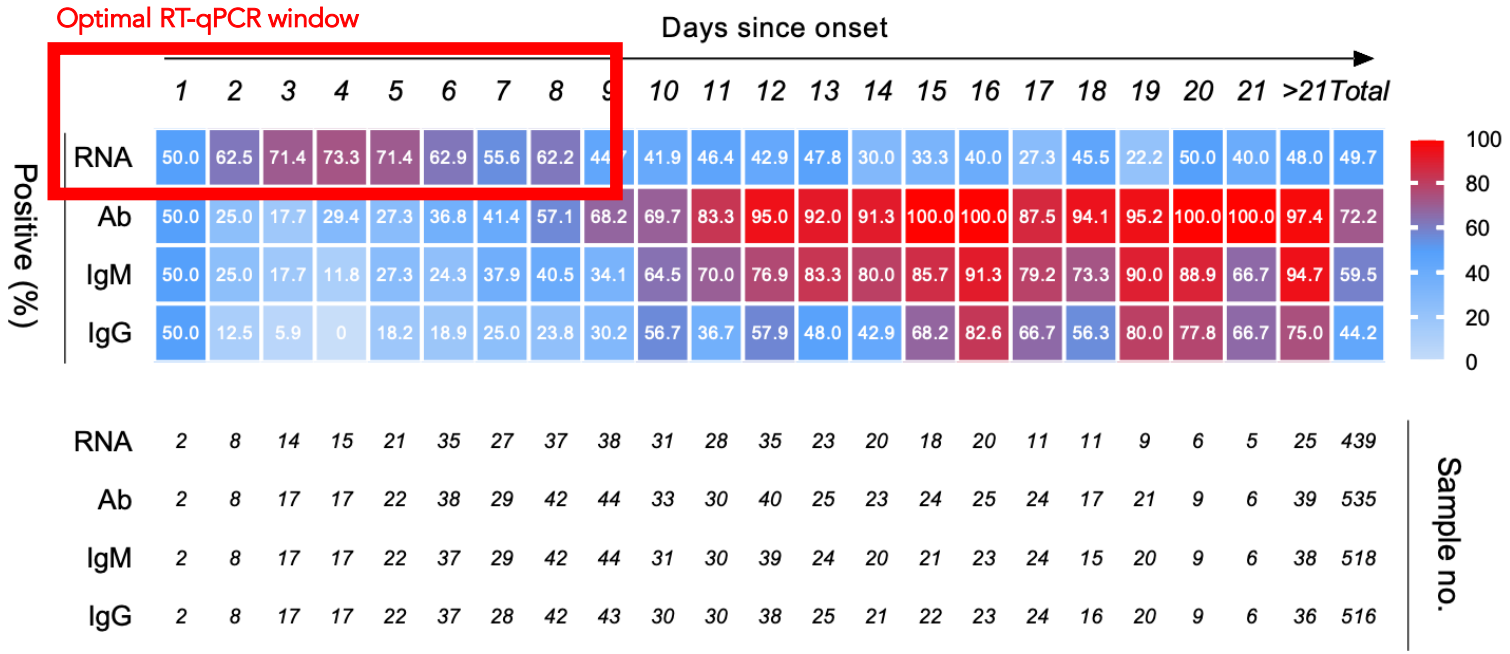

Detection of SARS-CoV-2 in nasal swabs (RNA) or blood (IgX) from symptom onset

Zhao J. *et al*. Lancet 2020.

Coronavirus 19 (COVID-19) in breath
Confidence in rationale

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Our hypothesis

- Disease is a function of amount of virus reaching the lower lung.
- Achieved mainly by breath aerosols or poor immune system.
- Explains close contact transmission chains.

We need to test breath aerosols for:

- The amount of virus present (genomes).

- Infectivity (viruses).

Diagnosing from Exhaled Breath Condensates (EBC)

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Breath is 95% hydrated:

- Volatile compounds (smells, eg garlic, alcohol).
- Vapour & aerosols.
- Biological molecules.

Health and Disease indicators:

- Lung infections.
- Liver diseases.
- Multiple cancers:
  - Blood.
  - Breast.
  - Brain.

Challenges to clinical use

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- Reproducibility.
- Contamination:
  - Saliva.
  - Ambient.
- Sample loss.
- Safety.
- Upper vs deep lung separation.

RTube™

Poor process control

EcoScreen™

Sample lost in black tube
17Kg + weight

Horváth, I., *et al*, 2017. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *European Respiratory Journal*, **49(4),** p.1600965.

Current competitors: volatiles

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Owlstone Medical (UK).

- Volatile compounds only:
  smells, not diseases/pathogens
- Sorbent tubes (510k FDA).
- No analytics.
- Unsafe for healthcare personnel.

Breath Biopsy™

eNose (NL)

Current competitors: volatiles

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Owlstone Medical (UK)

eNose (NL)

- Volatile compounds only (same issue).
- On-the-fly results.
- No sample.
- Unsafe for healthcare personnel.

eNose™

A fresh approach

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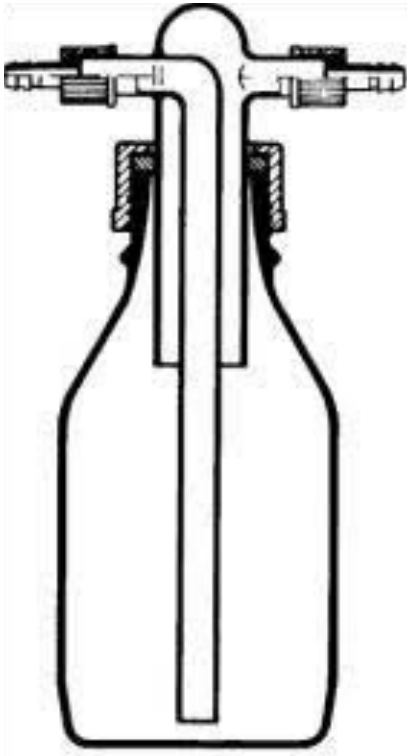
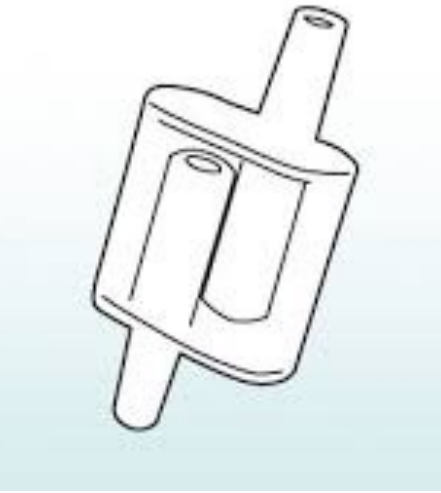
- Boyle's law 1662: PV = nRT.
- Dreschel bottle: late 1800's
- Focus on isolating the sample.
- Eliminate sample loss.
- Separate saliva.





Alco-pro saliva trap

Horváth, I., *et al*, 2017. A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *European Respiratory Journal*, **49(4),** p.1600965.

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EBC: condensation physics

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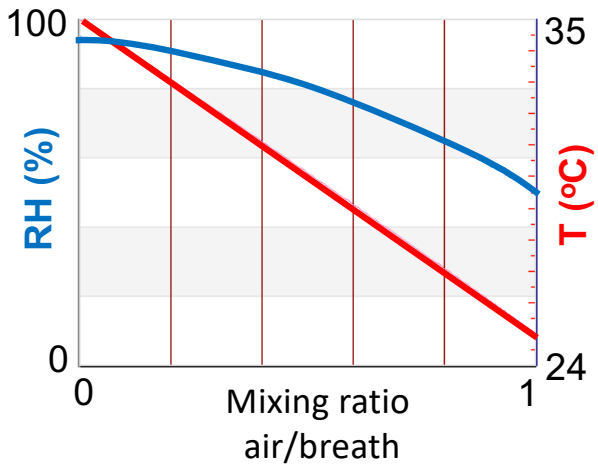
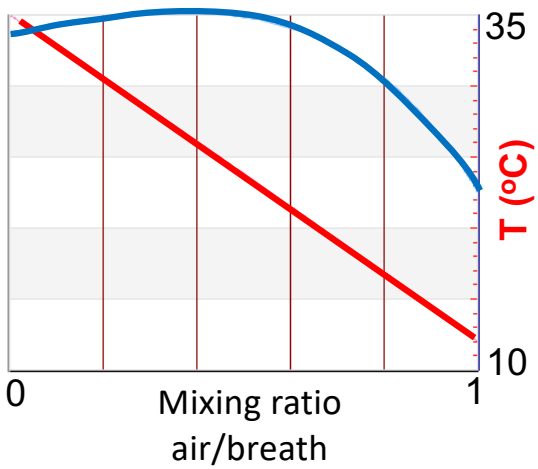
Ambient conditions

25°C, 50% RH



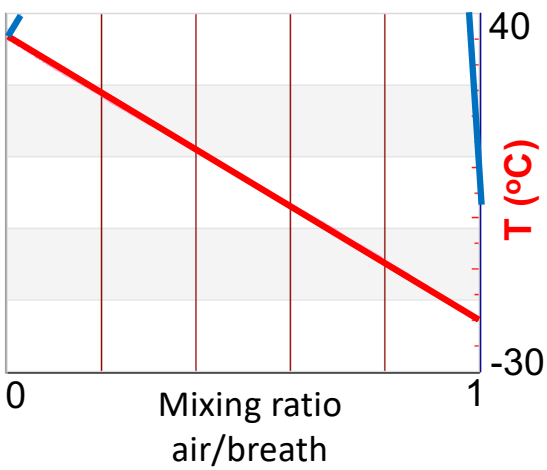
No condensation

12°C, 50% RH



0.19 g/kgr water/air

-20°C, 50% RH



11.1 g/kgr water/air

Dry ice: -100°C condensation yields 22.3g/kg @ 0-100% RH; tidal breath vol ~0.5 L; air density is ~0.13% of water; 1 kg air ~ 2hrs

Preliminary data: pathogen detection

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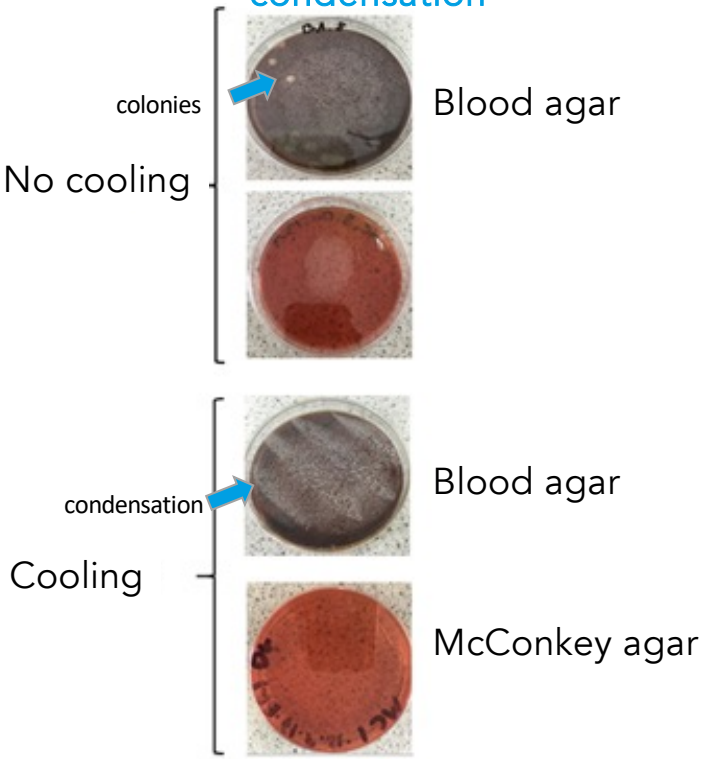
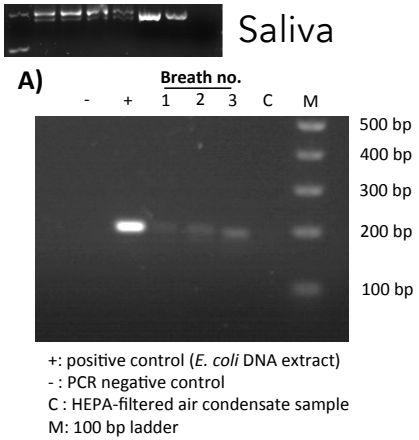
Bacteria & fungi die by dry ice condensation



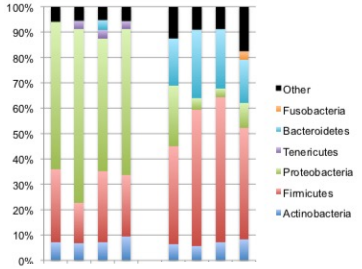
NB: no colonies on plates, white marks are condensation

COVID-19-like test works with 1-3 breaths for bacteria

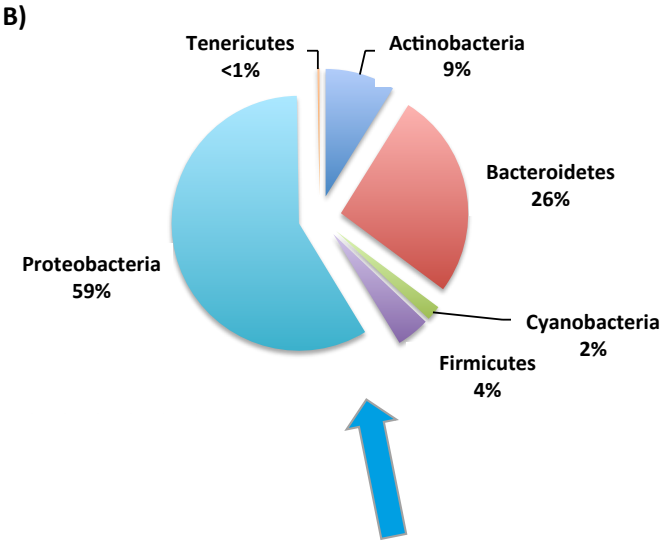


+: positive control (*E. coli* DNA extract)
- : PCR negative control
C : HEPA-filtered air condensate sample
M: 100 bp ladder

EBC is distinct to saliva



breath saliva



Closest sample like this comes from lung surgery samples only (60% proteobacteria)
(Sze MA *et al. Am. J. Resp. Crit. Care Med.* 2012)

Our focus: unmet need in lung disease

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Asthma in <5-year-old children

"25% of all children need this"

"No way to do this right now"

"You could solve our problem"

Dr. Louise Fleming
Senior Clinical Lecturer
National Heart & Lung Institute
Imperial College
Nov 2019

Imperial College London

Lung infections in elderly

"Big drawbacks with other methods: CT scans, nasal swabs only for flu"

"We take educated guesses"

"Expensive treatments with no tests and huge side effects"

"Current format is fit for purpose"

Dr. Wonnie Ryu M.D.
Assistant Professor
Pulmonary Critical Care Clinician
Yale University
Jul 2019

Yale University

Our focus: unmet need in lung disease

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- 0-5 year old wheeze
  - Market size: visits *per annum*
    - UK: 217,000
    - World: 14 million[3]
  - 1/3 of all children <3yo[1]
  - 75% of paediatric appointments[2]
- Healthy ageing (Respiratory infections)
  - 4.1% of all UK hospital admissions [4]
  - 65yo+ market size:
    - 620,000 uses per week
  - 12.4 million (UK) above 65 years[5,6]
    - >120,500 cases *per annum*
    - 5% in sheltered housing
    - 75yo+: No. 1 cause

1. Martinez FD NEJM 1995; 2. Brand PL ERJ 2014; 3. USA, EU, Canada, Japan; China: anticipated higher due to pollution; 4. BLF, 2016; 5. ONS 2019; 6. LGA 2017.

PBM-HALE™: the platform

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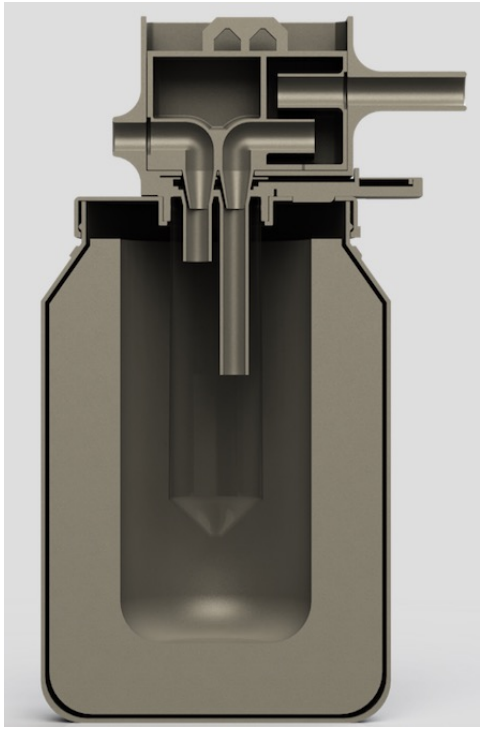

EBC collector:

- Volatiles and
- Proteins.
- DNA.
- RNA.
- Lipids.
- Medications.

Solves key problems:

- Reproducibility.
- Contamination.
- Sample loss.
- Safety.

Cold Chain Dependent:

- Uses dry ice powder (CO_2) to collect sample reliably.
- Dry ice replenished every 1 hr from compressed gas cylinder.
- Sample needs on the spot test or frozen transfer to lab.

WO2017153755A1: exhaled breath collector – granted; WO2019053423A1: cascade impactor array – granted.

PBM-HALE™: the platform

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Path to removing the cold chain:

- Proprietary coating to remove need for dry ice.
- Stabilisation material to remove freezer storage.

Experiments under way

WO2017153755A1: exhaled breath collector – granted; WO2019053423A1: cascade impactor array – granted.

PBM-HALE™: Computational flow modelling

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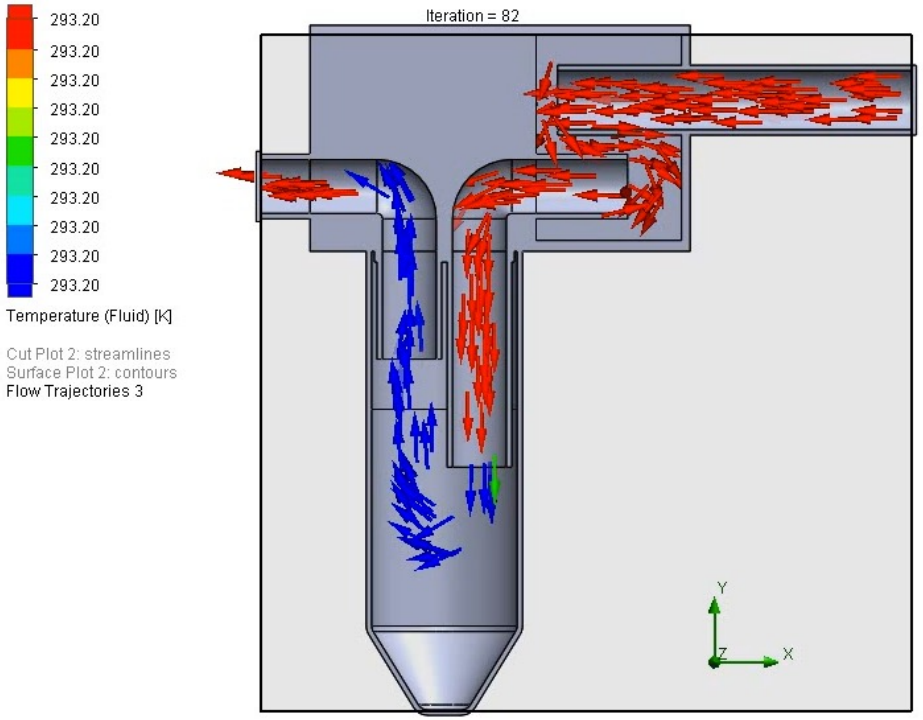

WO2017153755A1: exhaled breath collector – granted; WO2019053423A1: cascade impactor array – granted.

Prototype: Highly consistent sampling

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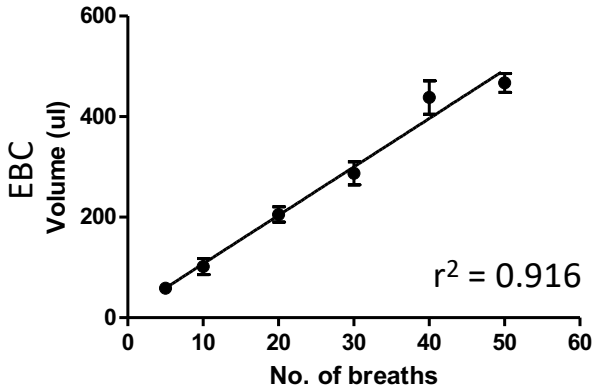

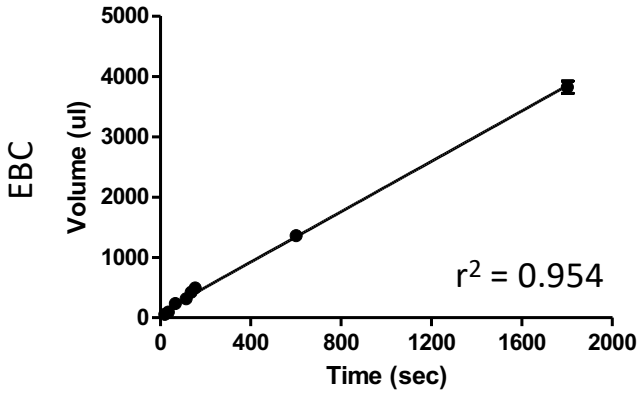

Whether 5 breaths (25 sec, e.g. screening) or half an hour of sampling (e.g. discovery)

R^2 range: 0.88 to 0.95, n = 5.

Data by John Henderson

Prototype: no salivary contamination

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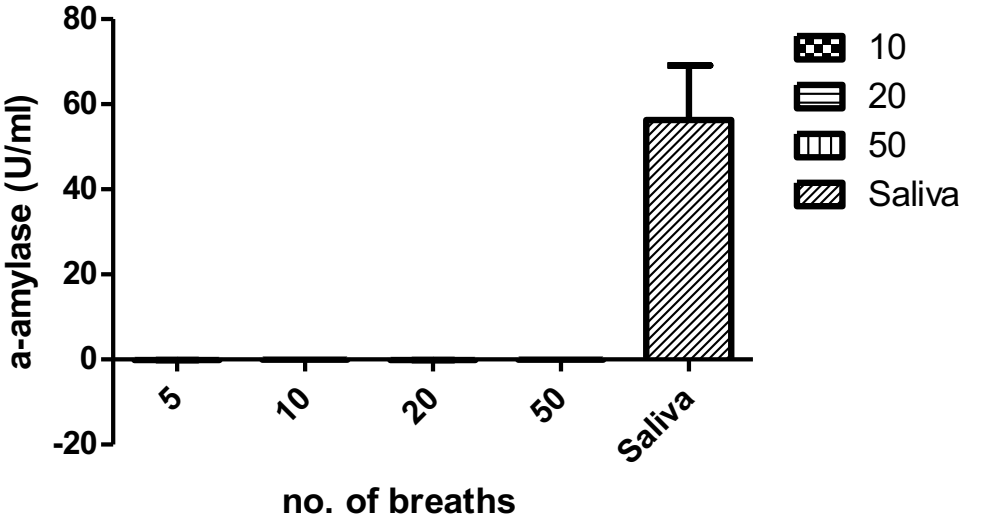

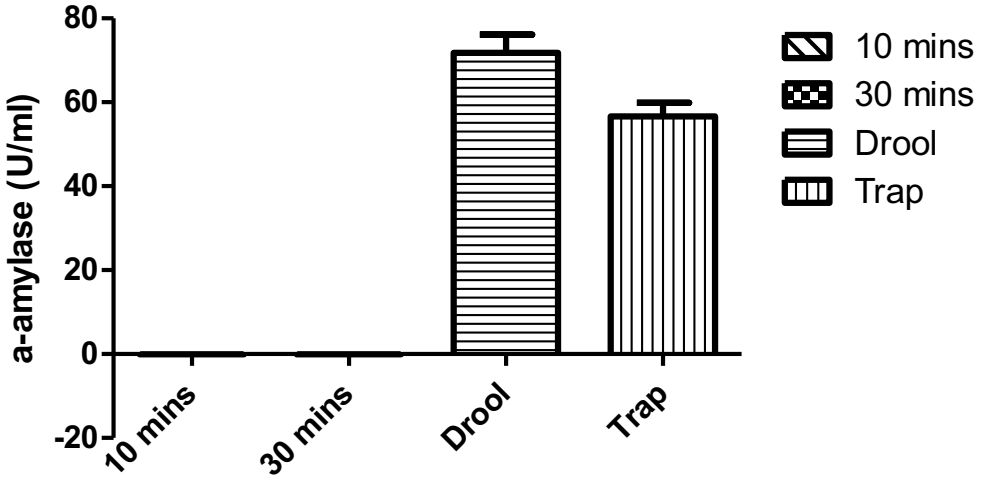

Saliva enzyme levels at least 5000x above assay limit of detection

n = 5.

Data by John Henderson

Prototype: dry ice eliminates viability

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Condensation

-ve control | Dry ice (-78.5°C) | Wet ice (0°C) | Saliva

2 min sampling period

n = 5, blood agar

Data by John Henderson

Prototype: dry ice eliminates viability

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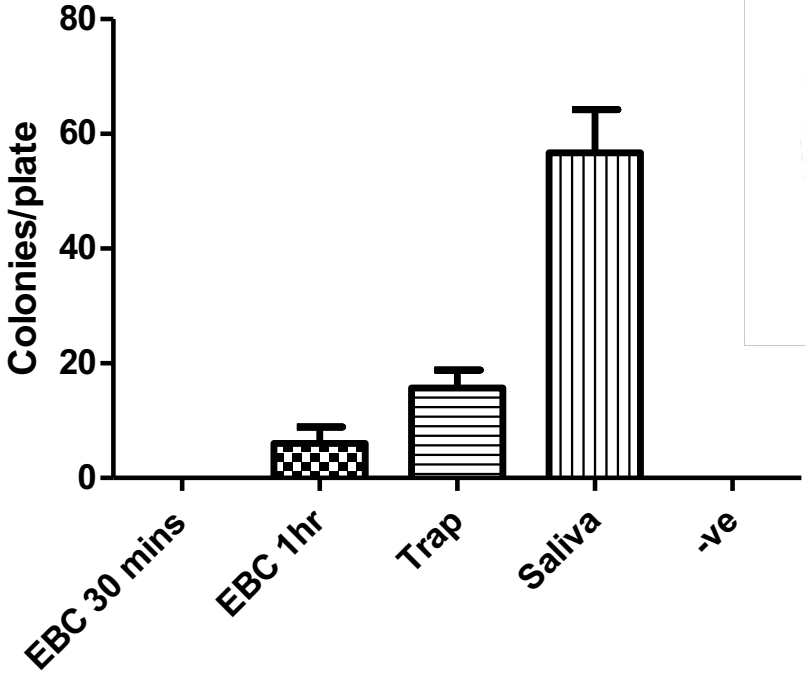

Cooling efficiency lost after ~40 min continuous sampling.
n = 3.

Data by John Henderson

Prototype: consistent [protein] in EBC

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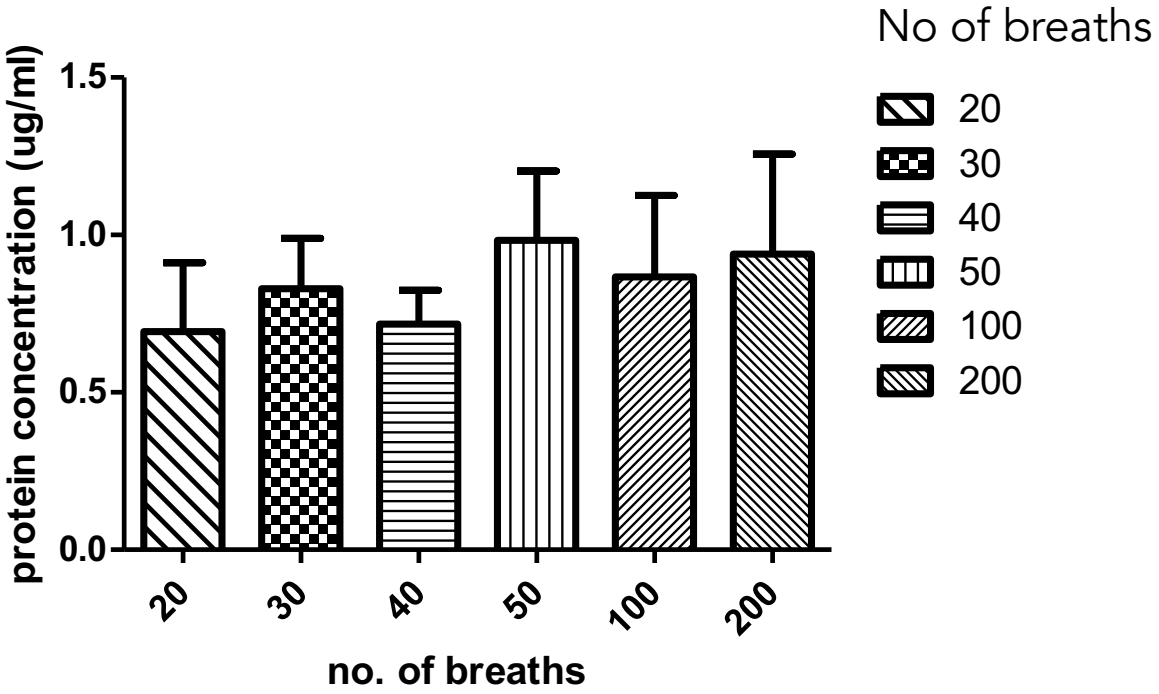

Requires 5x concentration by lyophilization

n=5

Data by John Henderson

Prototype: consistent [RNA] in EBC

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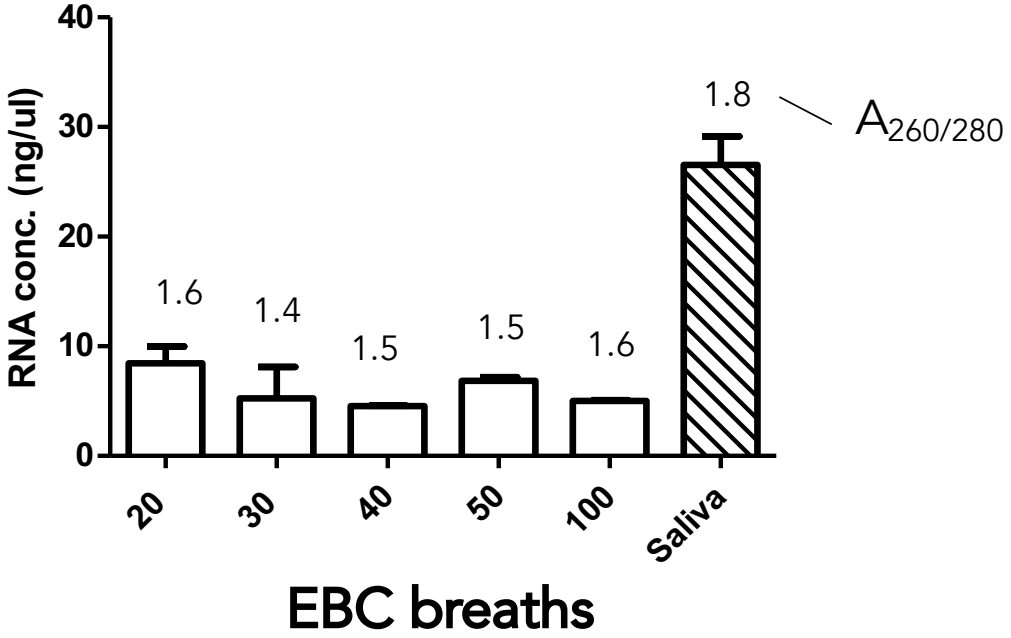

Increased EBC sampling does not increase [RNA].

EBC volume extracted by Trizol matched with volume obtained after 20 breaths.

n=6

Data by John Henderson

Prototype: 18S in EBC RNA

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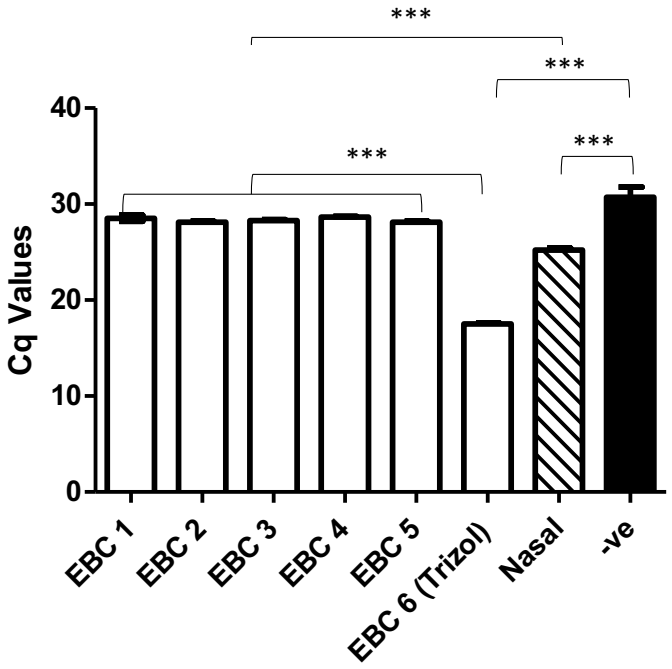
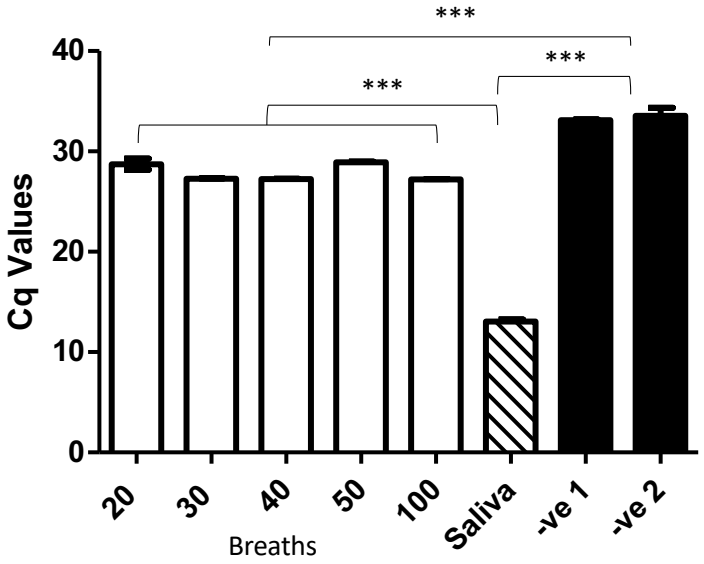


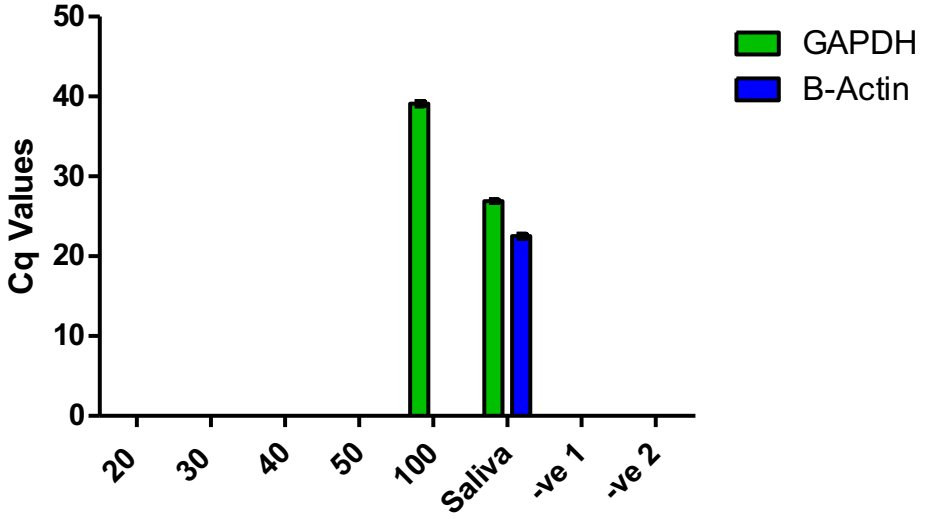
2 step SYBR Gold RT-qPCR (triplicate)
EBC1-5: RNeasy kit 20 breaths
EBC6: Trizol 30 min sample
Nasal = swab.



2 step SYBR Gold RT-qPCR (triplicate)
-ve 1: No RT control
-ve 2: no cDNA
EBC volume normalized to 20 breaths

Data by John Henderson

Prototype: GAPDH & β-actin in EBC RNA



2 step SYBR Gold RT-qPCR (triplicate)

-ve 1: No RT control

-ve 2: no cDNA

EBC volume normalized to 20 breaths

Either low host [RNA] or 18S all fungal.

n=5

Data by John Henderson

Prototype: Metabolomics in EBC

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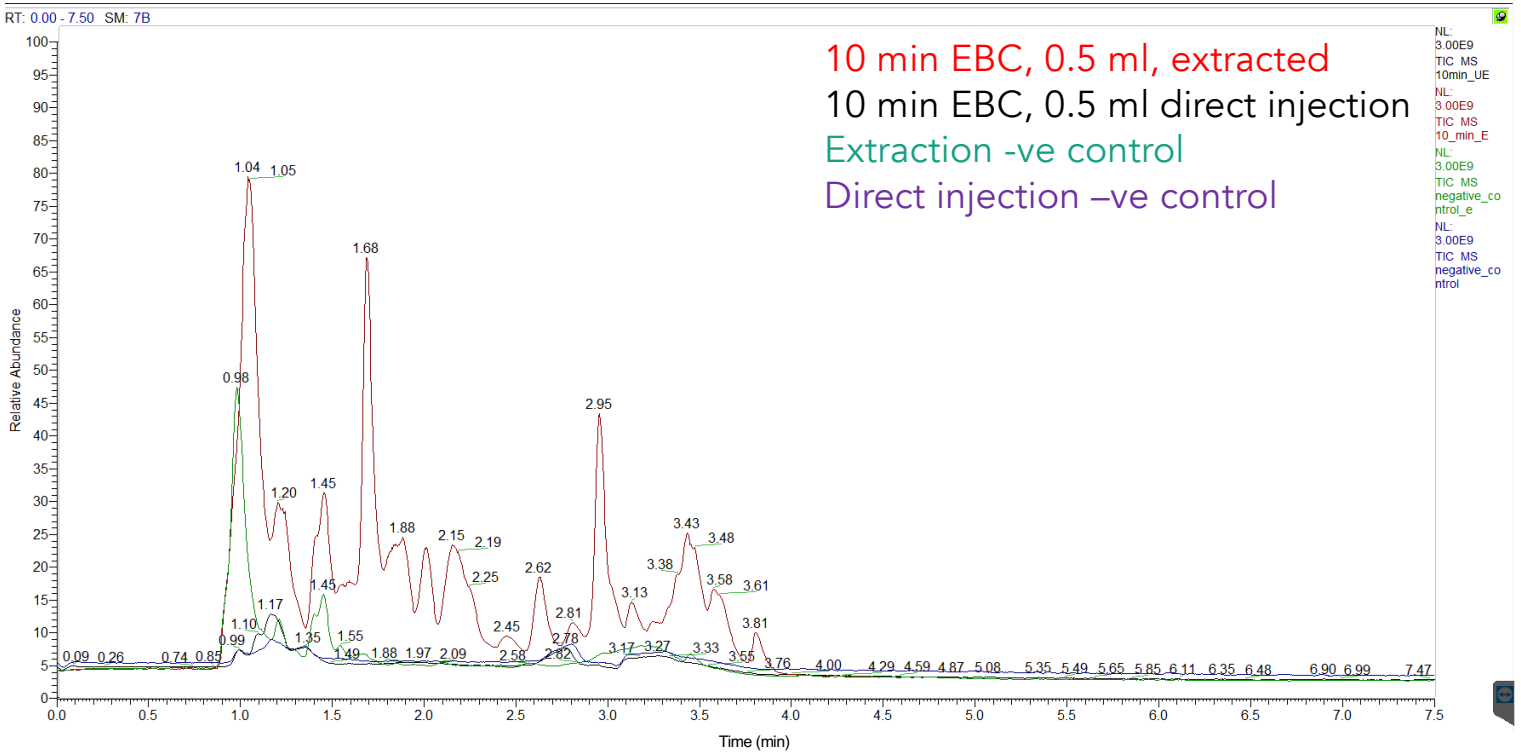

Metabolite profile in EBC after 5x concentration by lyophilization

n=5

Data generated by John Henderson and the Northumbria University Metabolomics Core Service

Prototype: Metabolomics in EBC

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Compound	RMM (g/mol)	RT [min]	Relative ion abundance
1-hexadecyl-glycero-3-phosphate	396.3	1.002	810,094
monoacylglyceride	352.3	1.02	281,866
LysoPA	410.2	1.032	968,316
Palmitoleoylethanolamde	297.3	1.047	187,282
eicosatetraenoate	335.2	1.054	348,544
Linoleamide	279.3	1.061	216,809
Cuscohygrine	224.2	1.067	723,759
N-Decanoylglycine	229.2	1.156	2,612,124
N-Nonanoylglycine	215.2	1.198	1,942,872
cis-3-Hexenyl b-primeveroside	394.2	1.221	160,089
N-Lauroylglycine	257.2	1.923	286,977
N-Undecanoylglycine	243.2	2.072	227,826
phosphatidylethanolamine	837.5	2.388	381,518
Gambogic acid	628.3	2.536	416,778
2-Hexenoylcarnitine	257.2	3.062	994,821
L-argininium	175.1	3.367	502,141
N-Acetylputrescine	130.1	3.519	192,382

Data generated by John Henderson at the Northumbria University Metabolomics Core Service

Compounds detected by MS1:

- C6-C24 fatty acids.
- Phospholipids & precursors.
- Glycans.
- Medications.
- Drugs of abuse.
- Dietary compounds.

Additionally:

- *20 multiple HDBM hits.*
- *104 novel compounds.*

Prototype: 16S microbiomics of EBC

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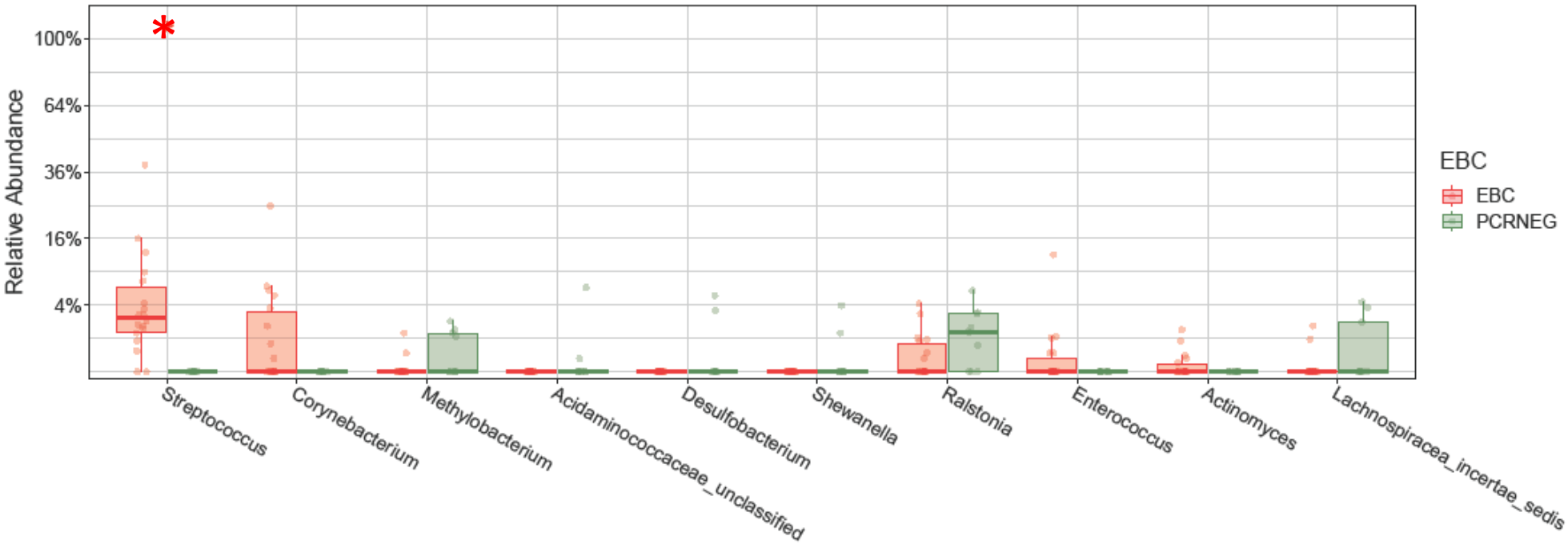

Higher DNA content vs background / kit controls

Detection of *Streptococcus* (FDR q = 0.019) w/out extraction.

Data by John Henderson, Andrew Nelson

Data generated at the Northumbria University NUOmics Core Service

Prototype: detection of virus aerosol

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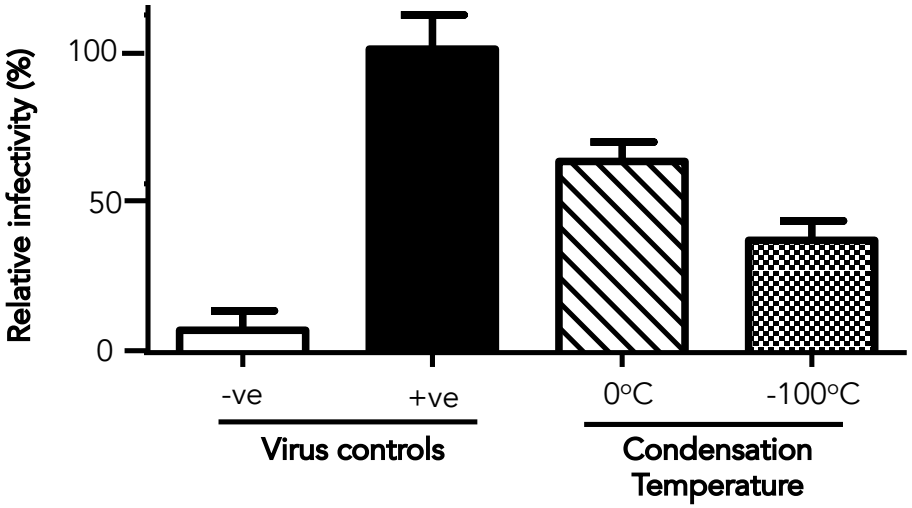
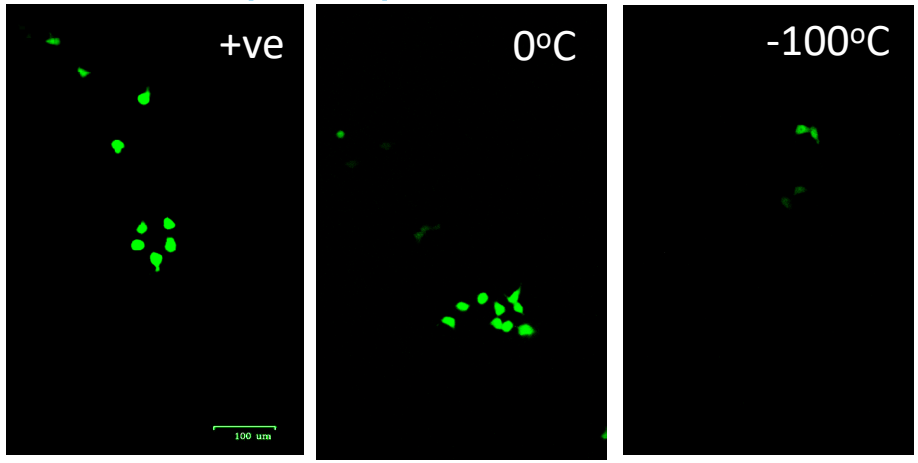
Efficient capture of aerosolized virus; dry ice halves infection risk.
25 infectious virions over 15 min sampling period.





GFP-expressing VSV-pseudotyped lentivirus at MOI 0.01 nebulized using PARI TurboBoy SX and captured using PBM-HALE™. Condensates seeded on 10,000 HEK-293T's and GFP expression measured at 72hrs by FACS, visualized by fluorescent microscopy. Bar = 100 um.

Data by John Henderson

External validation: Efficient Aerosolised SARS-CoV-2 Particle Capture

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PariBoy Classic nebuliser:

- Mean droplet diameter 3.5 µm
- 67% of mass in < 5 µm
- 5 min sampling

Particle types:

- Polysterene beads (118 nm, -71 mV, diH_2O)
- Neutral liposomes (168 nm, -20 mV, PBS)
- Negative liposomes (188 nm, -72 mV, PBS)
- Lentiviral VLP (MLV; 193 nm, -31 mV, PBS)
- SARS-CoV-2 VLPs (155 nm, -17 V, PBS)

Rudiger Gross, Janis Mueller, Jan Munch
U. Ulm

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- SARS-CoV-2 VLPs[1] (155 nm, -17 V, PBS)

Sampling conditions:

- 5 ml sample
- 5 min nebulization
- ~1.5 ml of dry ice condensate captured

Sample analysis:

- Particle size and concentration
- Fluorescent Nanoparticle tracking analysis (Malvern; 488 nm FluoSpheres, TopFluor liposomes, YFP, or scatter)
- Unfrozen input, Frozen input, Condensate.

1. Based on fbioe.2020.00862 protocol

Rudiger Gross, Janis Mueller, Jan Munch
U. Ulm

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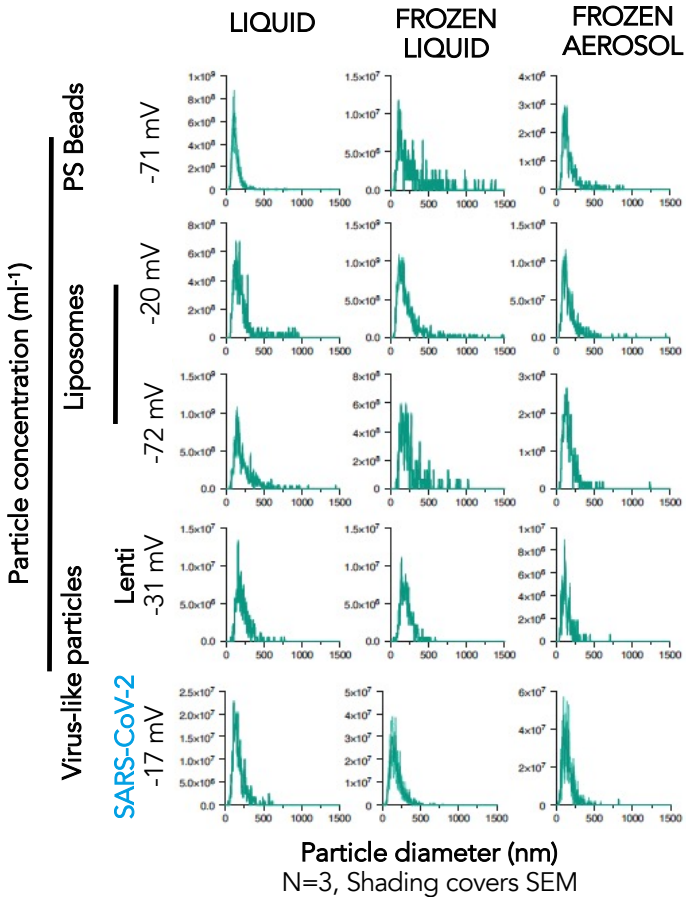

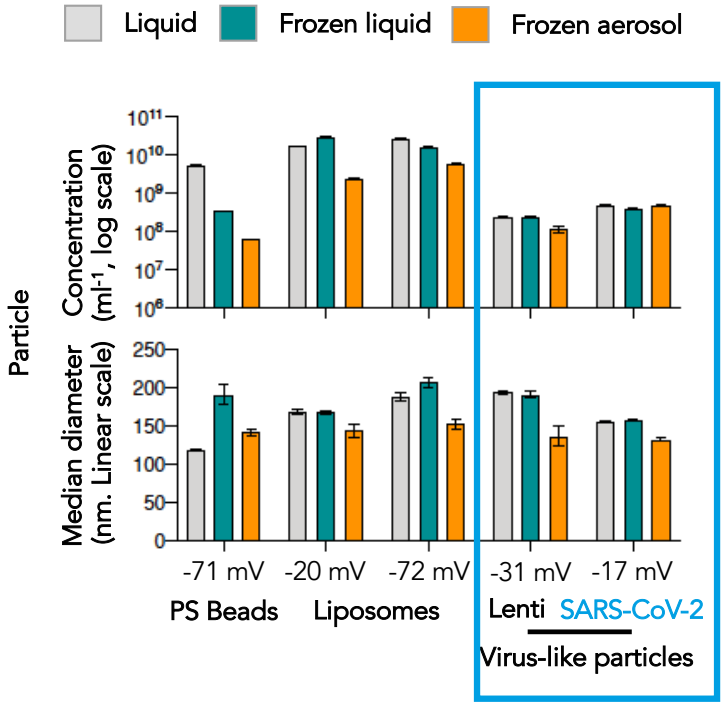

Virtually no loss of VLP size or structure

Aggregation with highly charged particles:

- Drop in concentration
- Rise in particle size
- Beads and liposomes
- Liposome vs VLP stability?

Rudiger Gross, Janis Mueller, Jan Munch
U. Ulm

COVID19 Exploratory Pilot Study

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Inclusion criteria:

- NP swab positive
- Within days 0-5 of symptoms

Study size:

- n=60, 98% power, 10% +ve
- Interim data point: n=30

Samples:

- Tidal breathing
- 30 min
- Aerosol, droplets, NP swab

Analysis:

- Viral load by RT-PCR
- Viral load by LFT
- Infectious virus

COVID19 Exploratory Pilot Study

Northumbria University NEWCASTLE

Establishment	Pipeline	Feedback	Testing	COVID-19 Data: Returned	COVID-19 Data: Verified
AUT 'AHEPA', Thessaloniki, Greece[1]			X		
UoCUH 'PAGNI', Crete, Greece[2]			X		
EKPA 'Evangelismos', Athens, Greece[3]					
Universidade Federal Mina Gerais, BR					
University of Ulm, Germany[4]					

1. Poor protocol compliance; n=2 samples collected in week 3 patients, <5 min sample, sample not quantified.

2. Inadequate local H&S provisions for HCPs; n=3 samples collected, less than 20% of sample analysed.

3. Ongoing, n=30 interim readout. n=21 collected.

4. In vitro *data with nebulised viruses replicate UNN results in full. Local IRB completed. Federal review under way.*

As of 2nd June 2021

Emerging COVID-19 Pilot Clinical data.

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NO FALSE POSITIVES:

- COVID19 ward testing, unventilated
- COVID19 patients (n=12).
  - Nasal swab negative.
  - Week 2-3 of symptoms, known nasal –ve period.
- Blinded analysis.

RECRUITMENT CHALLENGE:

- Clinician compliance
- Ventilation & health & safety
- Fear
- Surges = no testing
- Clinic attendance at week 2+

- PHE: “we have n=3, help us”

3rd party data in the meantime: exhalant is positive for coronaviruses

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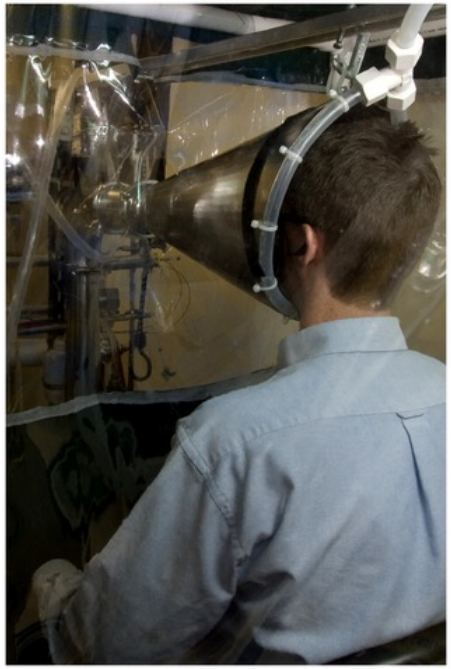
- Leung *et al.* 2020, 30-40% +ve by fraction, n=17.
- Ryan *et al* 2020: 66-93% +ve R-Tube, test dependent, n=16
- Sammadar *et al* 2021: 23-84% masks +ve days 1-4, n=44.
- Zhou *et al.* 2021, Ma *et al.* 2020: 22-27%, n=9, 52, late in clinical stage
- Feng *et al.* 2021, n=21, all -ve, high dilution.



Follow up study: Origin of aerosolized virus

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Inclusion criteria:

- NP swab positive
- Within days 0-5 of symptoms

Study size:

- n=60, 98% power, 10% +ve
- Interim data point: n=30

Samples:

- 6 breath maneuvres (speech, shouting, coughing, etc.)
- Aerosol, droplets, NP swab

Analysis:

- Viral load by RT-PCR
- Viral load by LFT
- Infectious virus

We believe we can detect SARS-CoV-2 in breath.

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- **Fast:** <1 min sample.
- **Simply**: just breathe.
- **>2x safer**: process halves infectivity.
- **More reliably**: Larger sample than nasal swabs.

- **For mass screening**: by mass production of plastic.
- **Where patients are:** using *any* point of need testing system.
- **With current gold standard tests** (Lab RT-PCR, QuRapID®[1], ID NOW®, 5 min LFT/LFD, 4 sec MicroTox BT).

1. Shah K. *et al. Chem. Sci.* 2017

How do I use it?

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1. Device use SOP: https://youtu.be/h6tLt9u-rWU

2. Lay explanation of use: https://youtu.be/TkQEj-KN_os

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