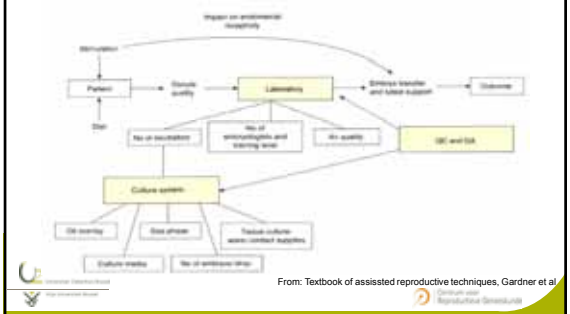
Laboratory setup – important clues

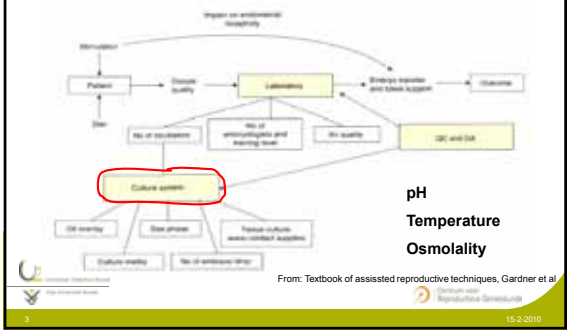
Ronny Janssens

Factors affecting outcome



From: Textbook of assissted reproductive techniques, Gardner et al

Factors affecting outcome – the culture system



pH

Temperature

Osmolality

From: Textbook of assissted reproductive techniques, Gardner et al

1 - pH

pH measurements

- Standard glas pH probes
- ISFET probes
- RI pH meter
- MTG pH meter
- Bloodgas analyser

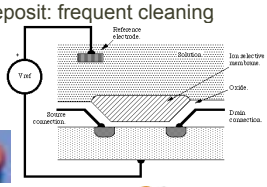
pH measurements – glas electrode

- Calibration temperature = measuring temperature = 37°C
  - → Calibration (buffers ~7 ~9) at 37°C
- - Fragile
- - Needs big volume (min 1 ml) – needs equilibration
- - Not standardised
  - → 2ml in 15 ml tube (R Pool)
  - → 50ml in flask – over night (Don Rieger)
  - → 1 ml in 5 ml tube – over night (UZ Brussel)
- - Measurement under ambient conditions
- - Not suitable for microdrops
- - Does not represent actual culture conditions
- - Leakage of electrolytes over time

- pH HEPES media: measure at 37°C

ISFET (ion sensitive field effect transistor)

- + Measument of small volumes (microdrops) outside incubator
- + Simple - fast
- - Sensor performance drift: frequent calibration
- - Sensitive to protein deposit: frequent cleaning
- - Expensive



RI pH meter

- + Measurement in incubator is possible
- - Slow!
- - Drift over time
- - Difficult/time consuming calibration procedure
- - Does not reflect reality (microdrop culture)

pH Online ™ “fluorescent decay time” pH meter (MTG)

• Continuous pH measurement inside incubator
• Able to monitor pH in up to 10 incubators simultaneously

Disposable Nunc 4-well dish in which one well is fitted with a pH reactive fluorochrome spot

-price (disposable dishes)
-slow (Nunc dish)
+: simple

Bloodgas analyser

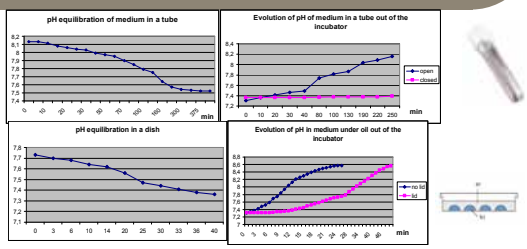
- + Accurate
- - Not suitable for microdrops
- - Sampling/transport errors
- - Not suitable for routine pH controls
- →Method of choice to determine pH of culture media

pH: conclusions

	G-MOPS	HTF-HEPES
Glas electrode	7,31	6,48
Flattrode	7,56	7,64
RI	7,07	7,27
ISFET	-	7,81
Blood gas analyzer	7,22 (7,27 ± 0,05)	7,31 (7,3 – 7,5)

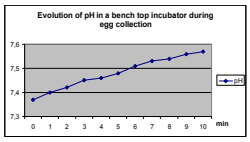
- Difficult (measuring errors - protein deposit on probes, calibration, sampling and equilibration problems)
- Does not detect fast changes in CO_2 concentration
- Not suitable for routine control of incubators
- Usefull to detect manipulation/handling errors (validation of method)
- Do not rely on one method – verify with bloodgas analyzer

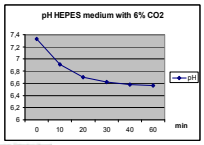
pH: equilibration



Equilibration of culture dishes: 4H
Fast rise of pH in ambient air
Oil = limited protection to pH changes – volume is important

pH: in practice

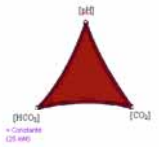




- Bicarbonate buffered medium during oocyte collection
  → Desktopincubator – CO_2
- HEPES buffered medium inside incubator

pH vs CO_2

- Henderson-Hasselbach equation
  pH=pKa + log10 [HCO3-/α pCO2]
- Culture media: 25 mM $NaHCO_3$ + 6% CO_2
- Fast CO_2 changes can not be measured by pH measurement
- pH is not suitable for incubator function control
- Conclusion: measure CO_2



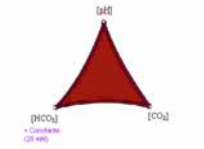
pH vs CO_2 measurements

pH measurement

- Difficult (measuring errors - protein deposit on probes, calibration, sampling and equilibration problems)
- Does not detect fast changes in CO_2 concentration
- Not suitable for routine control

CO_2 measurement

- Reliable, reproducible results (with calibrated devices)
- Fast, easy to repeat
- Does detect fast changes in CO_2 concentration (incubator malfunctioning)
- Method of choice for incubator function control



Fyrite kit

- Accuracy CO_2: 1-2%
- Not suitable for IVF!

CO_2 – function control

- Gas analyser IR
  - Hereaus / Bacharach
  - K-Systems
  - Vaisala
- Calibrate with reference gas
- Measurement of real CO_2 (O_2) Minimum once a week
- Continuous monitoring with independent probes (2006/86/EC (annex I - Equipment and materials C - §2))

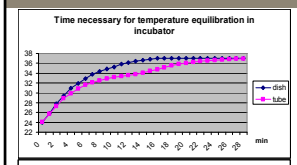
2 - Temperature

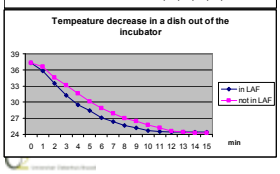
- Culture: 37,0 ± 0,5°C

Temperature

- Digital thermometer
  - → Calibrated thermcouple K
  - → Pt 100

2 - Temperature





Heating: 20 min

Cooling:

- 0.5°C/min

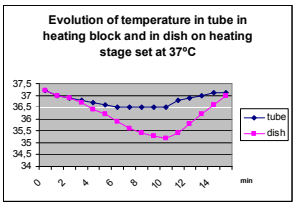
37.0 ± 0.5°C = 1 min!!!

Temperature: heating and cooling

	Optimal T° after (min)	Complete cooling after (min)
3,5 cm culture dishes (3 ml oil)	~ 20	~ 20
Centre Well (500 µl medium + 1 ml oil)	~ 20	~ 15
Centre Well (500 µl medium)	~ 30	~ 15
Nunc (500 µl medium + 400 µl oil)	~ 30	~ 25
Nunc (500 µl medium)	~ 40	~ 20

Temperature: cooling on heated surface

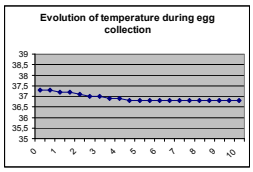
SP = 37.0°C ≠ 37°C in culture



Calibration

Temperature: calibration of equipment

- Calibrated thermometer with thermocouple type K
- Individual fine tuning (equipment – culture dish)
- 37°C in the dish!



Temperature control in the IVF laboratory

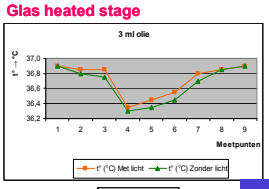
Define setpoints for each dish on heating device

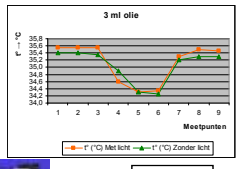
Datum: Toegestane afwijking tov.setpoint: ± 0.5°C

		setpoint	controle	Aktie!
Werkpost 1	Verwarmtafel L mirr (16122)	42.0	T°	
IVF	Tafelincubator OPU (18443)	36.6	T°	
	Verwarmblok OK OPU (14158)	39.0	T°	
Werkpost 2	Verwarmtafel R mirr (17397)	40.1	T°	
IVF	Tafelincubator K-Systems (18445)	36.6	T°	
	Verwarmblok OPU (18840)	39.4	T°	
	Verwarmplaat (30307)	41.6	T°	
Werkpost 3	Tafelincubator K-Systems (14420)	36.9	T°	
evaluatie	Microscoopstafelverwarming (24044)	37.0	T°	
Werkpost 4	Microscoopstafelverwarming linkam(30021)	42.0	T°	
evaluatie	Tafelincubator K-Systems (24421)	37.2	T°	
Werkpost 16	Microscoopstafelverw Hunter (21616)	38.2	T°	
ICSI flow	Tafelincubator K-Systems (24413)	39.5	T°	
Werkpost 17	Microscoopstafelverw Maatuh (30020)	40.1	T°	
ICSI				
Werkpost 8	Tafelincubator K-Systems (24440)	38.0	T°	
Cryo1	Verwarmplaat cryo (12998)	43±1°C	T°	
	Waterbad (08207) eerst aanzetten!	29.9	T°	
Werkpost 9	Waterbad (11762)	42.3	T°	
Cryo2	Tafelincubator K-Systems (24441)	43.1	T°	
Werkpost 10	Verwarmtafel L mirr (17598)	38.9	T°	
ICSI flow	Tafelincubator K-Systems (13417)	36.8	T°	
Werkpost 11	Verwarmtafel R mirr Hunter (21615)	38.2	T°	
ICSI flow	Tafelincubator K-Systems (14436)	36.5	T°	
Werkpost 12	Microscoopstafelverw Tokai (17899)	38.5	T°	
ICSI flow	Warmtafelincubator (24442)	37.6	T°	
Werkpost 13	Microscoopstafelverw aq R (6117)	39.5	T°	
ICSI				
Werkpost 14	Microscoopstafelverw Tokai (21502)	42.6	T°	
ICSI	Tafelincubator K-systems (18439)	37.7	T°	
Werkpost 15	Microscoopstafelverw Rl (24035)	38.4	T°	
ICSI				

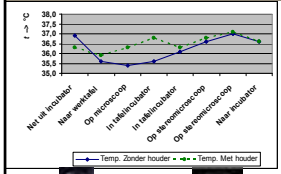
Temperature: ICSI microscope

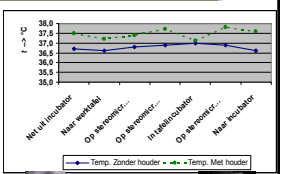
Glas heated stage **Linkam heated stage (metal)**





Temperature outside incubator

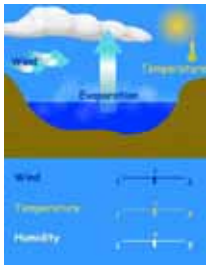




- *fast cooling during transport*
- *Protective potential of metal tray holders??*

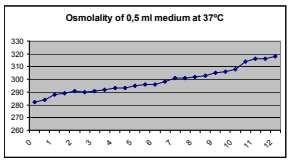
3 - Osmolality

- Culture media: 280-290 mOSM/kg
- Evaporation
- Open culture system vs oil overlay
- Incubator: humidified atmosphere?

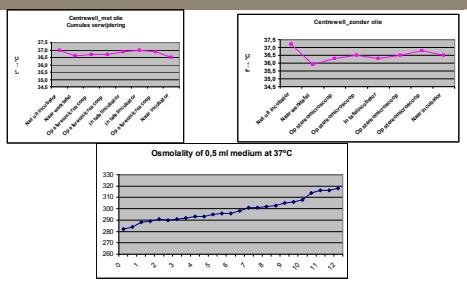


Osmolality

- Volume = 50µl
- Calibration solution 300 mOsm/kg



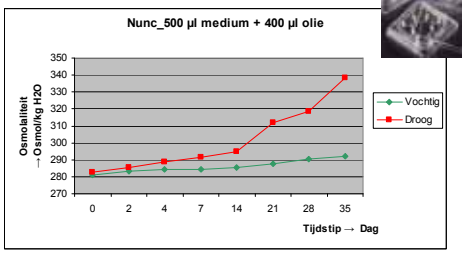
Oil overlay?



YES **1 fast rise in osmolality in open culture systems**

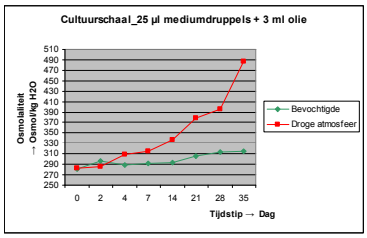
2 protective effect of oil overlay on temperature

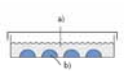
Humidified atmosphere ?



Open culture = YES **Closed culture (oil) = NO (3days)**

Humidified atmosphere?





Open culture = YES **Closed culture (oil) = NO (3days)**

Conclusions

- Oil overlay slows down gas exchange and pH changes – limited protection
- Oil overlay reduces evaporation – osmolality and temperature changes
- pH and temperature maintenance outside incubator is problematic
- Humidification of incubator is not necessary (with oil overlay)

Oxygen – Meintjes et al. Hum Repr vol 24, 2009

- Embryos cultured in a 5% O_2 environment consistently resulted in higher rates of live birth implantation (42.9% versus 30.7%) and live births (57.4% versus 42.6%) when compared with rates among women whose embryos were cultured in an atmospheric O_2 environment.
- Only 7 patients have to be cultured in a reduced O_2 culture environment to result in one additional live birth

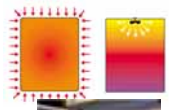
A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program - Meintjes et al. Human Reproduction, Vol.24, No.2 pp. 300–307, 2009

Factors affecting outcome – the incubators

From: Textbook of assissted reproductive techniques, Gardner et al

Standard incubators – requirements?

- Triple gas (6% CO_2 – 5% O_2)
- Large capacity (inertion)
- Heated door
- Stable atmosphere, fast recovery (CO_2 –temp)
  - → Infrared CO_2 sensor
  - → Gas tight split doors
- Air quality: HEPA - VOC filters
- Reliable
  - → Failure Alarm
  - → Possibility to install independent probes
  - → Remote alarm system
  - → UPS
- Easy to clean
- Easy to disinfect

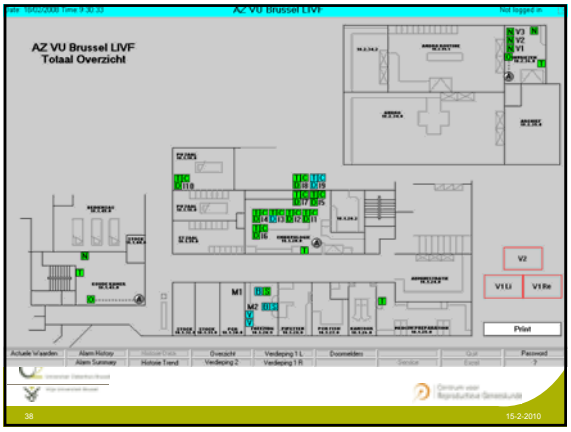


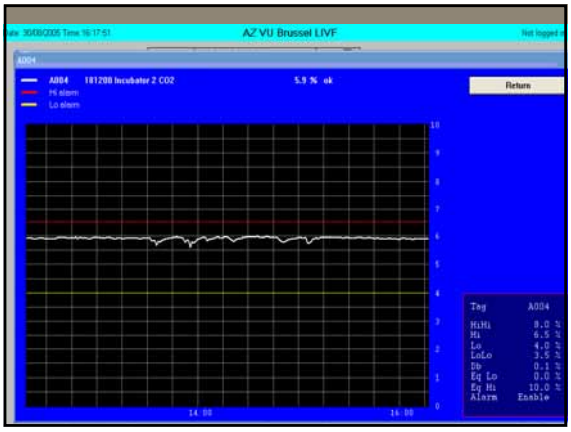
Continuous Monitoring

Requirement of 2006/86/EC (annex I - Equipment and materials C - §2)

- Independent probes
- Autonomous
- Permanent
- Acoustic internal alarm –
- External alarm (SMS)
- Traceability – audit trail

- Incubators (T° – CO_2 – door openings)
- Embryo and sperm bank
- Refrigerators
- Freeze- thawing devices
- Low O_2 alarms



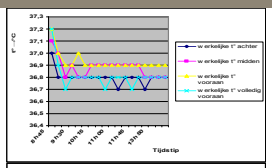


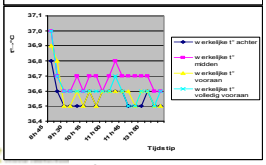
Cleaning

- Frequency: every month
- Procedure
  - → Dismantle
  - → Clean with detergent
  - → Rinse
  - → Decontamination
    - Fertisafe™ (Hum. Rep. vol22 supl1, 2007)
    - Heat sterilization (if available)
  - → Startup
  - → Function control - calibration

Effect of door opening frequency?

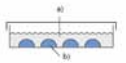
Thermo Forma – “normal” opening frequency





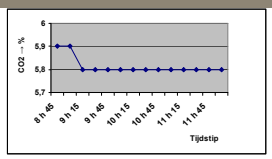
t° TOP

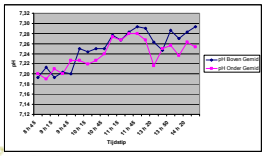
t° BOTTOM

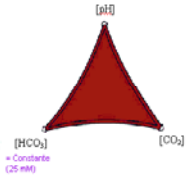


Open.close every 15 minutes for 3h30, then every 30 minutes

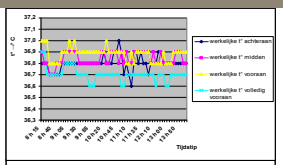
Thermo Forma – “normal” opening frequency



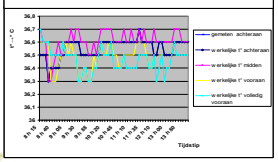




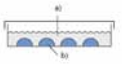
Thermo Forma – high opening frequency



t° TOP



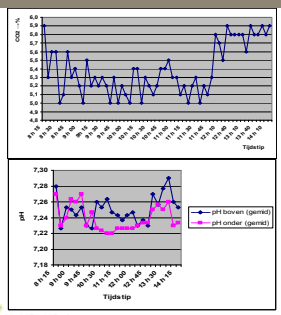
t° BOTTOM

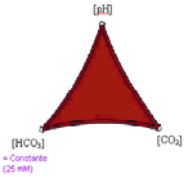


Open.close every 5 minutes for 3h30, then every 10 minutes

46

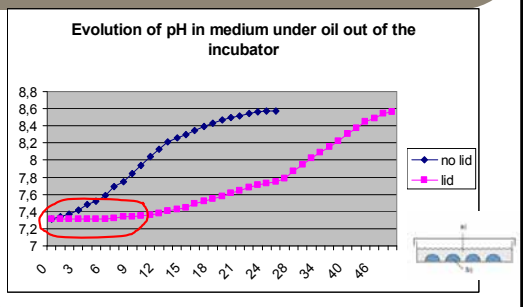
Thermo Forma – high opening frequency





47

pH (25µl culture medium – 3ml oil)

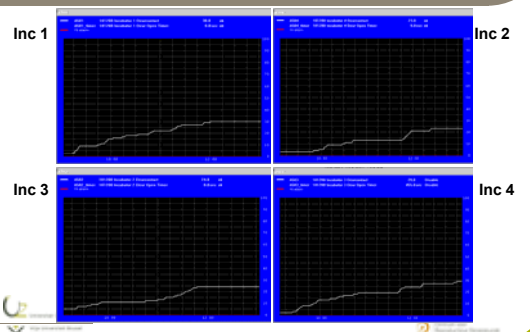


48

Incubators – UZ Brussel

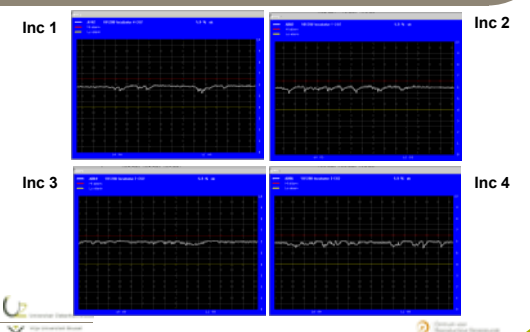
49

Door openings



50

% CO_2



51

Effect of door opening frequency

- Temperature and pH changes do occur but are limited
  - → - 0.3°C
  - → - 0.08 to + 0.05 pH
- Is this relevant?
  - → No one has identified and characterized a precise pH optimum for the culture of human embryos
  - → Temperature optimum???

Recommended pH Ranges for Commercially Available IVF Media (Rakowky, ASRM 2006)

P1	7.1 – 7.3
Quinn's Advantage	7.1 – 7.3
Basal XI	7.2 – 7.35
Global	7.25 – 7.3
G1.3	7.30±0.10
IVF 500	7.35±0.10
HTF	7.40±0.10
Medicult	None specified; "pH tested"

Gardner et al., Textbook of Assisted Reproductive technologies, second edition p20

"The ratio of cases per incubator varies considerably from program to program, and assuredly affects clinical outcome, depending on the number, type, and length of incubator door openings. In principle, the number should be kept to a minimum; we prefer a limit of four cases per incubator."

700 cases – 20 incubators – Old generation Thermoforma

UZ Brussel - 2008

- 4200 retrievals – blastocyst culture
  - → 10 incubators
  - → 8 for culture

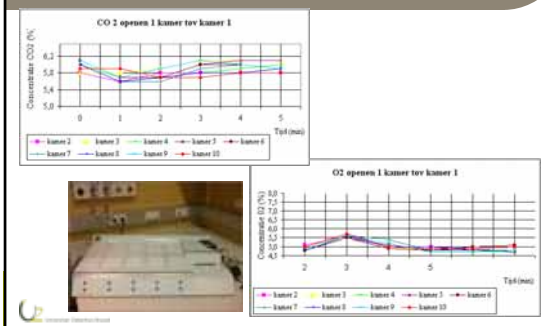
Incubators: conclusions

- Standard incubator
  - → Bigger is better
  - → IR CO_2 sensor
  - → Gas tight split doors are essential
  - → Continuous monitoring is essential
  - → Humidification is not necessary (except open tubes - flasks!)
  - → Effect of high door opening frequency on temperature and pH in a culture dish is limited
- Number of cases per incubator: up to 12
- Mini incubators?
  - → MINC – COOK
  - → G-185 – K-Systems

K-Systems G-185

- Triple gass
- Integrated gas mixing unit
- 10 individual chambers
- Monitoring software
- No humidification!

K-Systems G-185

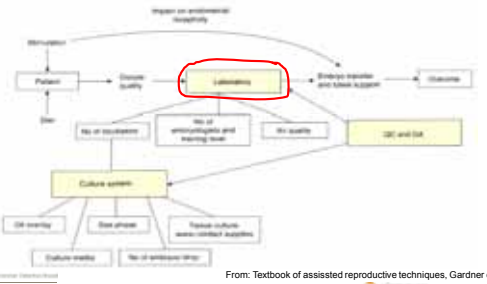


Mini incubator: Conclusions

- Integrated gas mixer
- CO_2 concentration in premixed gas is variable
- More difficult to monitor – safety?

- Allow individual culture
- Less variations in temperature and gas conditions
- Alternative to standard incubators

Factors affecting outcome – the laboratory



From: Textbook of assissted reproductive techniques, Gardner et al

2004/23/EC – 2006/86/EC: GMP Requirements

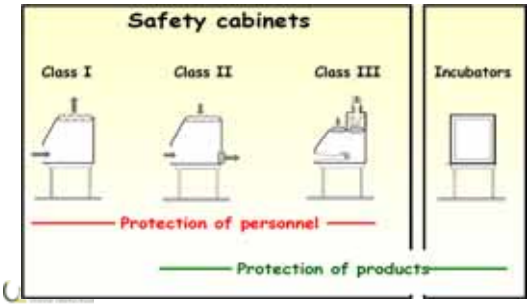
Volume 4 – EU Guidelines for Good Manufacturing Practice Medicinal Products for Human and Veterinary Use – annex 1 (feb. 2008)

- Production in clean areas
- Entry – changing rooms
  - → Personnel
  - → Goods
  - → Designed as airlocks
  - → Flushed with filtered air
  - → Separate for entry and exit desirable
  - → Hand washing facilities
  - → Interlocking system
  - → Visual and/or audible warning system
- Separate areas for operation
  - → Component preparation
  - → Product preparation
  - → Filling etc
- Level of cleanliness
- Filtered air

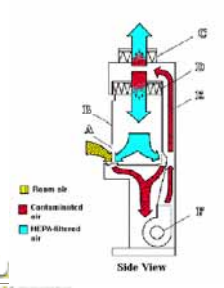
Clean room measurements

- ✓ Pressure differences of the rooms (ISO 14644-1)
- ✓ Air volume & air velocity
- ✓ Recovery time
- ✓ Temperature
- ✓ Humidity
- ✓ Particle counts (ISO 14644)
- ✓ CFU counts (ISO 14698)
  - ✓ Active (air sampler)
  - ✓ Passive (settle plates)
  - ✓ Surfaces (contact plates)
- ✓ *VOC measurement*

Isolation of product from environment: Class II satety cabinets



Class II



Class II biosafety cabinet and IVF: temperature effect

	Period 1 (18/06/2007 – 13/09/2007)		Period 2 (14/09/2007 – 31/10/2007)	
	Standard OPU room	New OPU	Standard OPU room	New OPU
Equipment	Horizontal LAF cabinet	Class II IVF workstation	Horizontal LAF cabinet	Class II IVF workstation
+ hCG/OPU	39.8%	34.6%		

Class II IVF workstation switched off and temperature adjustement!

- Pickering S.J., Braude P.R., Johnson M.H. *et al.* (1990)
- Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril*, **54,** 102-108.

Conclusions

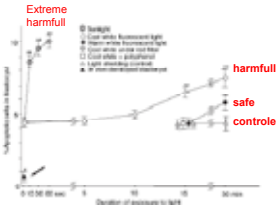
- Clean room technology might be required for new laboratories

- Air quality requirements may compromise IVF pregnancy rates

Light exposure during ART procedures.
Ottosen 2007

- Background intensity: 200-400 lux
- Microscope intensity: 2500-5000 lux
- 95% of total light energy is = microscope halogen lamp
- Harmfull effect: 400-500nm – filter?
  - It has been suggested that the harmful effect of blue light is associated with generation of H2O2 and specific absorption by enzymes applied in the respiratory chain. H2O2 and its metabolites, hydroxyl radicals, are known to cause cellular damage and result in impaired mitochondria function through reaction with amino acids, phospholipids,nucleotides, organic acids.

Effects of light on development of mammalian zygotes. Takenaka 2007



Incidence of apoptotic cells in mouse blastocysts after exposure of zygotes to sunlight (20,000 lx) and cool white and warm white fluorescent light (1,200 lx).

Free radicals

Light: conclusions

- Damage:
  - → Duration
  - → Intensity
  - → Wavelenght (cool > warm)
    - < 300 nm UV absorbed by plastic
    - Near UV 300-400 nm => apoptosis

- **Effects of light on development of mammalian zygotes. Takenaka et al. PNAS, 2007.**
  "development of mouse (and hamster) zygotes to blastocysts is apparently unaffected by exposure to cool white fluorescent light, but they develop to term fetuses less efficiently than those exposed to warm white fluorescent light or those that are not exposed to light at all.
- **Viability of bovine embryos following exposure to the green filtered or wider bandwidth light during in vitro embryo production. Korhonen et al. Hum Rep, Vol.24, 2009.**
  An easy to use and inexpensive green filter seems to reduce the stress caused by light during the IVP procedures without affecting either the accuracy of embryo monitoring or the need to increase the light intensity.

Factors affecting outcome – air quality

From: Textbook of assissted reproductive techniques, Gardner et al

4 - VOC

VOC

- Prevention - Elimination of known/possible sources
  - Alcohol - disinfectants
  - Anesthetic gasses

- Detection
  - **Capturing - Cryo concentration - Gas Chromatography (GC) - Mass Spectroscopy (MS) - Adsorbent tubes (aldehydes) - High performance liquid chromatography (HPLC)**
  - **ACS badge**
  - **Eco sensor**
  - **VOC meters**
- Removal
  - Active charcoal absorption
  - Oxydation (Potassium permanganate)
  - Photo-Catalytic Oxidation

HPLC - Alpha Environmental (www.alphaenvironmental.com)

- Summa canister (6l – 24h sampling) ~ 1800$
- Test for 61 toxic organics
- TO-11 test tube for aldehydes
- Detection limits 1 µg/m^3
- Compare to other laboratories

Advanced Chemical Sensors (www.acsbadges.com)

Cat. No.	
OV-00	Organic Vapor Monitor
OV-005	Acetic Acid
OV-01	Acetone
OV-05	Acetonitrile
OV-055	Acrylamide
OV-06	Acrylonitrile
OV-07	Allyl Alcohol
OV-08	Benzene
OV-09	B-T-E-X
OV-10	Benzyl Chloride
OV-102	Biphenyl
OV-12	1,3 Butadiene
OV-14	Butyl Acetate
OV-15	Butyl Alcohol
OV-157	n-Butylglycidy 1 Ether
OV-16	t-Butylamine
OV-17	Butyl Ether
OV-152	Sec-Butyl Alcohol (2-Butanol)
OV-16	Butyl Cellosolve (2-Butoxyethanol)
OV-20	Carbon Tetrachloride
OV-22	Cellosolve (Ethylene Glycol Monoethyl Ether)
OV-25	Chlorobenzene
OV-30	Chloroform
OV-31	Collodion
OV-311	Cumene
OV-312	m-Cresol
OV-313	Cyclohexane
OV-314	Cyclohexanol

Cat. No.	
OV-315	Cyclohexanone
OV-3150	Cycohexylamine
OV-316	p-Dichlorobenzene
OV-318	1,2-Dichloroethane
OV-319	Dioxane
OV-325	Dimethyl Sulfoxide
OV-326	Dimethyl Formamide
OV-327	Phenylglycidyl Ether
OV-33	Diesel Fuel
OV-333	Dipropylene Glycol Methyl Ether
OV-34	Ethyl Acetate
OV-335	Epichlorohydrin
OV-345	Ethyl Acrylate
OV-35	Ethyl Alcohol
OV-37	Ethyl Benzene
OV-40	Ethyl Ether
OV-41	Ethylene Glycol
OV-415	Formamide
OV-42	Freon 13
OV-45	Gasoline
OV-50	Heptane
OV-55	Hexane
OV-555	Hexylene Glycol
OV-57	Isopropyl Alcohol
OV-575	Isophorone
OV-576	Jet Fuel (JP-8)
OV-577	Limonene
OV-578	Kerosene
OV-577	Limonene
OV-58	2-Methoxyethylacetate
OV-59	Methyl Acrylate
OV-61	Methylamine
OV-62	Methyl t-Butyl Ether
OV-65	Methyl Chloroform (1.1.1-Trichloroethane)

Cat. No.	
OV-70	Methylene Choride
OV-71	Methyl Ethyl Ketone (2-Butanone)
OV-75	Methyl Formate
OV-76	Methyl Isobutyl Ketone
OV-77	Mineral Spirits
OV-772	Morpholine
OV-777	Nitromethane
OV-78	Pentane
OV-79	Pentachlorophenol
OV-80	Perchloroethylene (Tetrachloroethylene)
OV-81	Petroleum Distillates (Naphtha)
OV-86	2-Propanol
OV-88	n-Propyl Bromide
OV-90	Propylene Oxide
OV-91	Pryidine
OV-93	Stoddard Solvent
OV-95	Styrene
OV-96	Tetrahydrofuran
OV-100	Toluene
OV-102	Total Saturated Hydrocarbons
OV-104	1,1,2-Trichloroethane
OV-105	Trichloroethylene
OV-106	Triethylamine
OV-107	1,2,4-Trimethyl Benzene
OV-109	Vinyl Acetate
OV-110	Vinyl Chloride
OV-115	Xylene

MANY OTHERS AVAILABLE

VOC Sensors: Eco sensor C-21

RESPONSE RANGES FOR SOME COMMON VOCs

	First detects	Alarm (First red bar)	TLV*
	ppm	ppm	ppm
Acetone	4-5	20-25	750
Benzene	5-10	25-50	10
Diacetone alcohol	5-10	25-50	50
Formaldehyde	1-5	15-25	0.1
Methylene chloride	8-10	40-50	50
Methyl ethyl ketone	3-5	15-20	200
Perchloroethylene	5	50	50
Toluene	3-5	15-25	50
Trichloroethylene	10-20	50-100	50

*Threshold Limit Value. Average estimate of government industrial hygienists for repeated worker exposure.

Permanent monitoring – 80 dB alarm

4-20mV connection

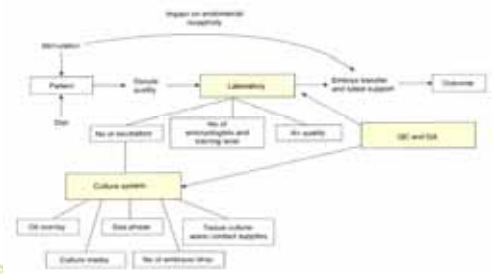
VOC Meter

- High sensitivity: detection limit: 0.1ppm – 0.1 ppb
- Handheld - pin-point the source of VOC
- Screening of equipment and consumables
- Stores data – download to PC

Removal: filtration

- Active carbon - HVAC (expensive)
- CODA filters (active charcoal – permanganate)
  - Laboratory air (effectiveness in clean room?)
  - Gas lines
  - In incubators

Factors affecting outcome – the future?



From: Textbook of assissted reproductive techniques, Gardner et al

The “growing“ Challenge Today

- Increasing regulatory demands - Implementation of new requirements/standards
- Implementation of clean room technology in IVF
- Isolation of product from environment
- Class II LAF is not compatible with accurate temperature controle
- VOC levels
- pH - temperature control outside incubator is suboptimal

The future: - Controlled work environment – isolators?

- Integration of functions: workbench – incubator – microscopes
- 37°C – CO_2 (O_2)regulated – humidification
- Enclosed box = improved environmental control (Temp/pH/pollutants/microbes/particles)
- EU directives compliant
  - → Cellcura
  - → K-Systems
  - → Ruskin Active
  - → Vitrosafe

The team

- Sarah Baes, 2001
  - → Kwaliteitszorg: methode- en toestelvalidatie binnen een IVF laboratorium,
- Annelies De Bisschop, 2007
  - → Controle en optimalisatie van cultuurcondities in IVF,
- Romy Souffreau, 2008
  - → Validatie van de G185 en Biostation CT incubatoren in de reproductieve geneeskunde