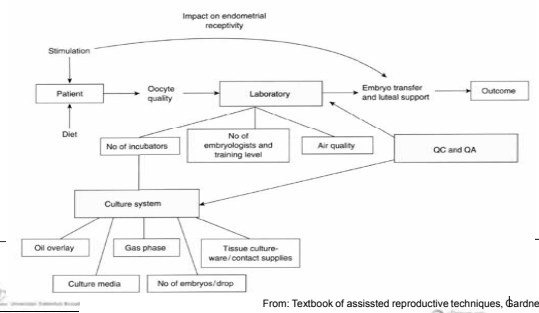
Laboratory setup – important clues

Ronny Janssens

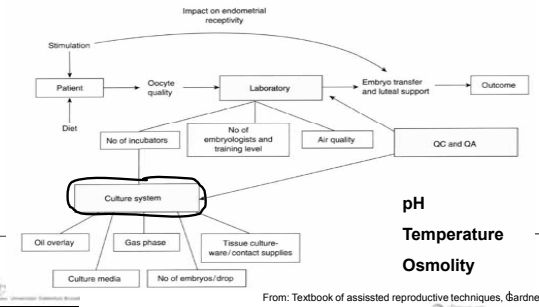
Universitair Ziekenhuis Brussel
Vrije Universiteit Brussel
Centrum voor Reproductieve Geneeskunde

Factors affecting outcome



From: Textbook of assissted reproductive techniques, Gardner et al

Factors affecting outcome – the culture system



pH

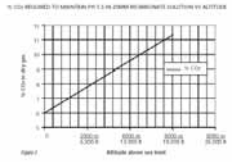
Temperature

Osmolity

From: Textbook of assissted reproductive techniques, Gardner et al

1 - pH

- Henderson-Hasselbach equation
  pH=pKa + log10 [$HCO3^-$/α pCO2]
- Culture media: 25 mM $NaHCO_3$ + 6% CO_2
- HEPES – MOPS buffered media
  → No CO_2– ambient air
  → pH is temperature related (T↑ = pH↓)



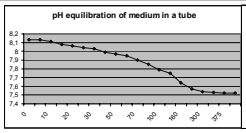
pH measurement

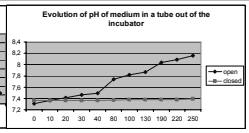
- Standard pH probes - large volumes

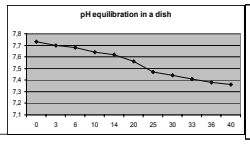

- ISFET probes – suitable for small volumes
- RI pH meter

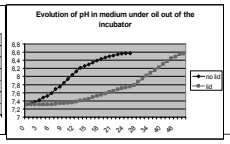
- MTG pH meter
- Bloodgas analyser

pH: equilibration time



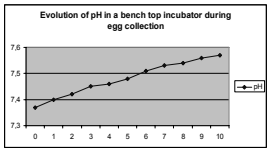


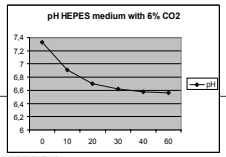




Fast pH rise in ambient air
Oil = limited protection to pH changes – volume is important

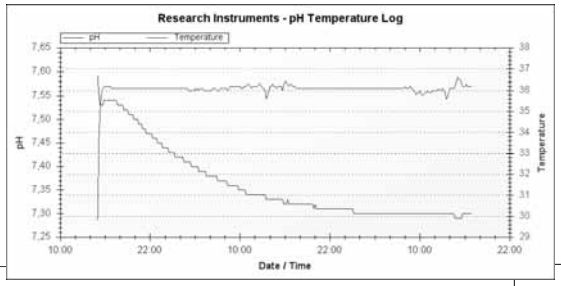
pH: in practice





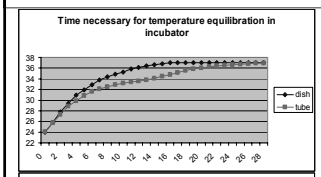
- Bicarbonate buffered medium during oocyte collection
  → Desktopincubator – CO_2
- HEPES buffered medium inside incubator

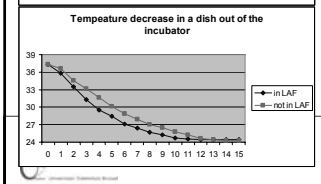
G-Gamete (Vitrolife)



Equilibration time: 30h!

2 - Temperature





Heating: 20 min

Cooling:

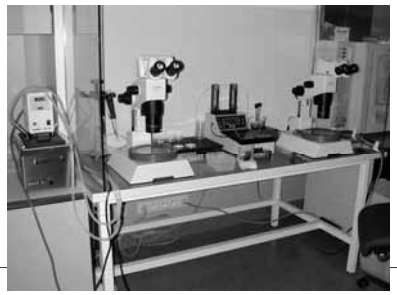
- 0.5°C/min

37.0 ± 0.5°C = 1 min!!!

Temperature: heating and cooling

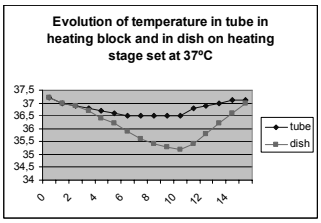
	Optimal T° after (min)	Complete cooling after (min)
3,5 cm culture dishes (3 ml oil) 	~ 20	~ 20
Centre Well (500 µl medium + 1 ml oil) 	~ 20	~ 15
Centre Well (500 µl medium) 	~ 30	~ 15
Nunc (500 µl medium + 400 µl oil) 	~ 30	~ 25
Nunc (500 µl medium) 	~ 40	~ 20

Temperature control



Temperature: cooling on heated surface

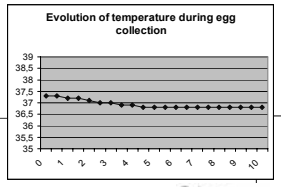
SP = 37.0°C ≠ 37°C in culture



Calibration

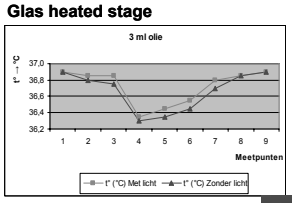
Temperature: calibration of equipment

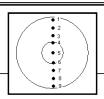
- Calibrated thermometer with thermocouple type K
- Individual fine tuning (equipment – culture dish)
- 37°C in the dish!



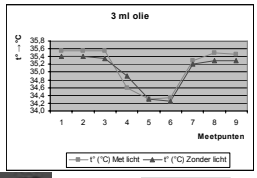
Temperature: ICSI microscope

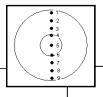
Glas heated stage



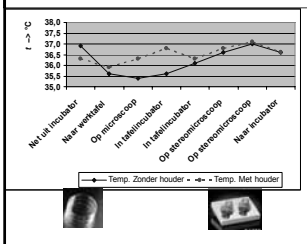


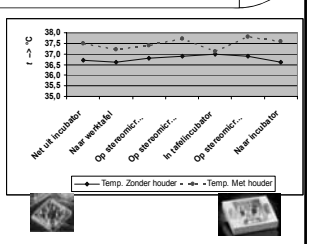
Linkam heated stage (metal)





Temperature outside incubator

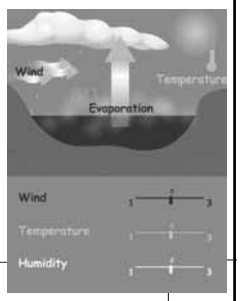




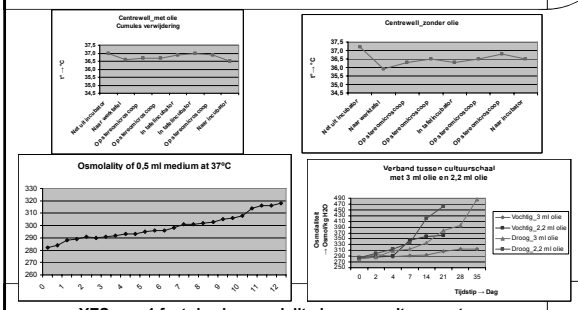
- *fast cooling during transport*
- *Protective potential of metal tray holders??*

3 - Osmolality

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



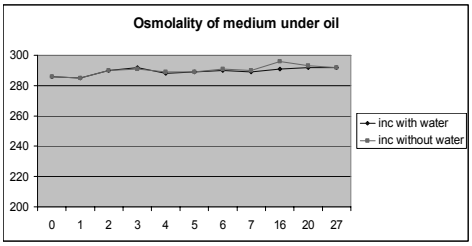
Oil overlay?



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

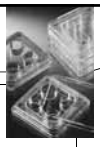
2 protective effect of oil overlay on temperature

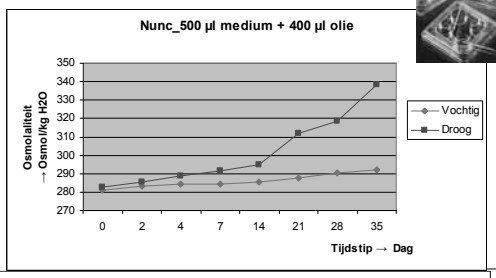
Humidified atmosphere ?



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

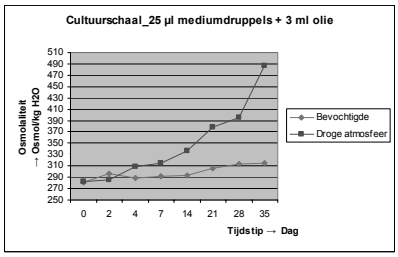
Humidified atmosphere ?

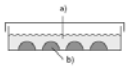




Open culture = YES **Closed culture (oil) = NO (3days)**

Humidified atmosphere?



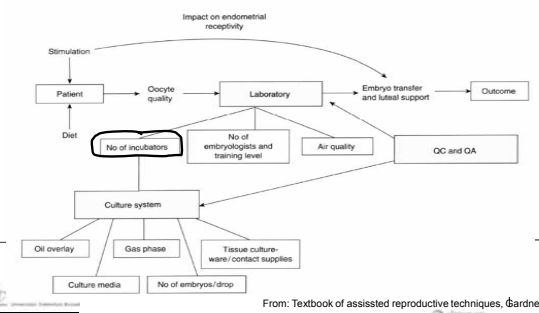


Open culture = YES **Closed culture (oil) = NO (3days)**

Conclusions

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

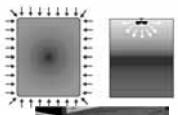
Factors affecting outcome – the incubators



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



"Heracell 240"

	t° (°C)	CO_2 (%)	O_2 (%)	pH
Left	36,9	6,3	5,0	7,28
Right	37,2	6,4	5,1	7,29

	t° (°C)	CO_2 (%)	O_2 (%)	pH
Front	37,0	6,4	5,0	7,29
Back	37,1	6,4	5,1	7,28
Up	37,2	6,4	5,1	7,28
Middle	36,7	6,4	5,0	7,29
Down	37,2	6,3	5,0	7,29

Standard incubators – function control

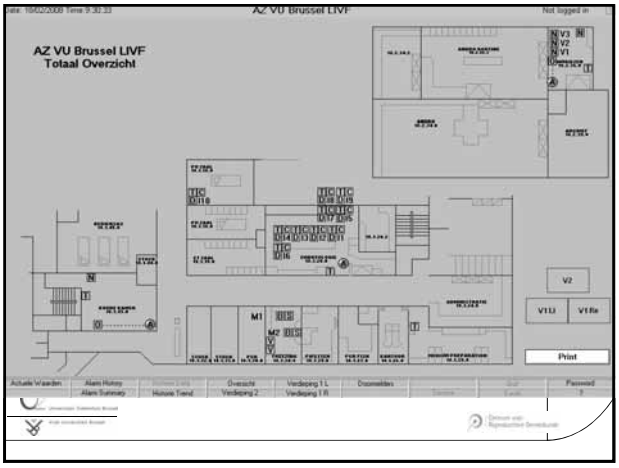
- Calibrated thermometer
  - Thermocouple Type K
- Gas analyser
  - Measurement of real CO_2 (O_2) Minimum once a week
  - Hereaus – K-Systems - Vaisala
- Continuous monitoring

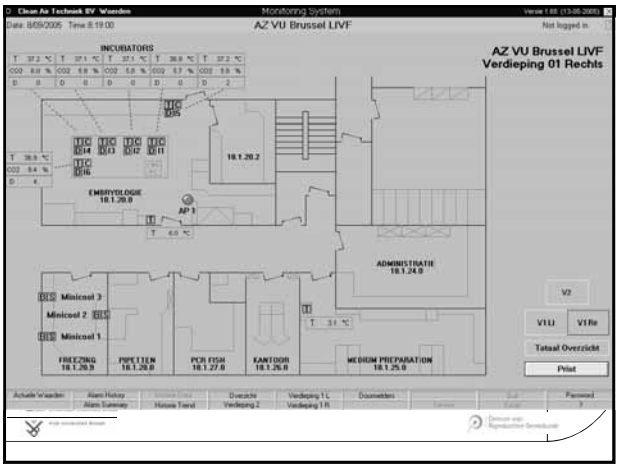
Continuous Monitoring

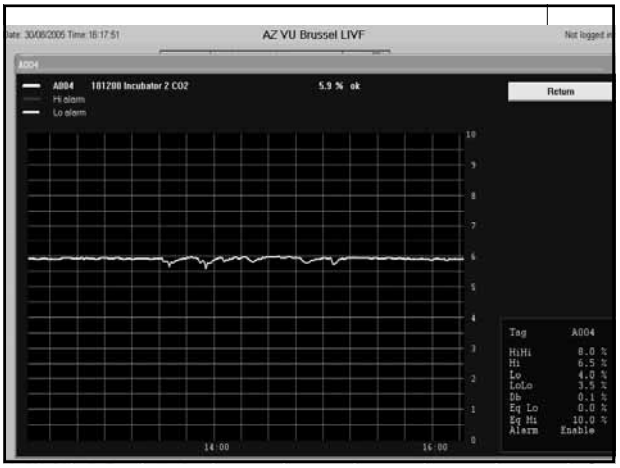
Requirement of 2006/86/EC (annex I - Equipment and materials C - §2)

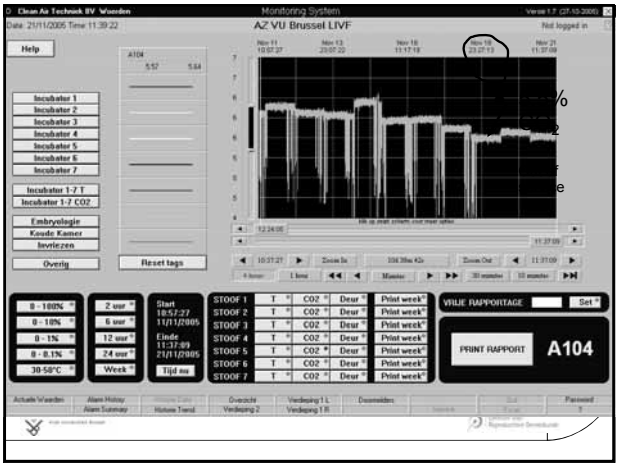
- Independent probes
- Autonomous
- Permanent
- Acoustic internal alarm –
- External alarm (SMS)
- Traceability – audit trail

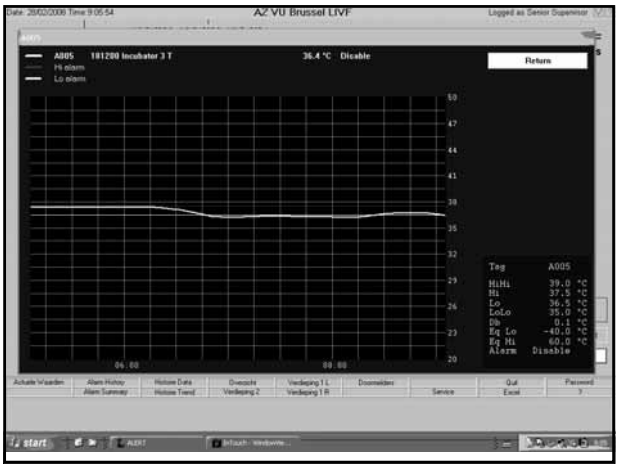
- Incubators (T° – CO_2 – door openings)
- Embryo and sperm bank
- Refrigerators
- Freeze- thawing devices
- Low O_2 alarms




Clean Air Technek BV Woerden
Monitoring System
AZ VU Brussel LIVF
Not logged in
INCUBATORS
AZ VU Brussel LIVF
Verdieping 01 Rechts
EMBRYOLOGIE
18.1.20.0
18.1.20.2
ADMINISTRATIE
18.1.24.0
Minicool 3
Minicool 2
Minicool 1
FREEZING
18.1.26.9
PIPETTEN
18.1.26.0
PCR FISH
18.1.27.0
KANTOOR
18.1.26.0
MEDIUM PREPARATION
18.1.25.0
V2
V1Li
V1Re
Totaal Overzicht
Print


AZ VU Brussel LIVF
A004
A004 181200 Incubator 2 CO2
5.9 % ok
Hi alarm
Lo alarm
Return
14:00
16:00
Tag A004
HiHi 8.0 %
Hi 6.5 %
Lo 4.0 %
LoLo 3.5 %
Db 0.1 %
Eq Lo 0.0 %
Eq Hi 10.0 %
Alarm Enable


Clean Air Technek BV Woerden
Monitoring System
AZ VU Brussel LIVF
Not logged in
Help
Incubator 1
Incubator 2
Incubator 3
Incubator 4
Incubator 5
Incubator 6
Incubator 7
Incubator 1-7 T
Incubator 1-7 CO2
Embryologie
Koude Kamer
Invriezen
Overig
Reset tags
VRIJE RAPPORTAGE
Set
PRINT RAPPORT
A104



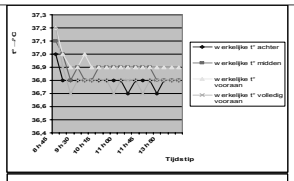
Cleaning

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

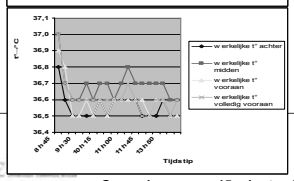
Effect of door opening frequency?

Thermo Forma – “normal” opening frequency

t° TOP

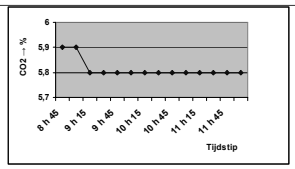


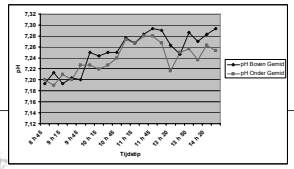
t° BOTTOM

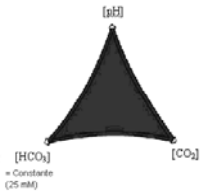


Open.close every 15 minutes for 3h30, then every 30 minutes

Thermo Forma – “normal” opening frequency

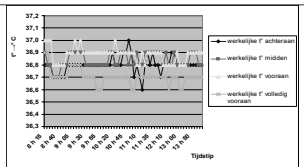




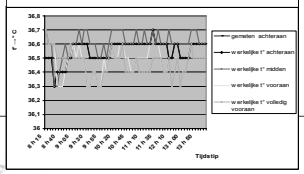


Thermo Forma – high opening frequency

t° TOP

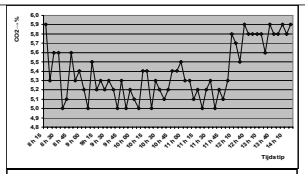


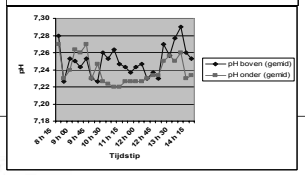
t° BOTTOM

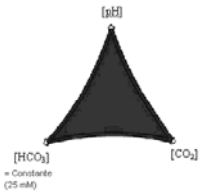


Open.close every 5 minutes for 3h30, then every 10 minutes

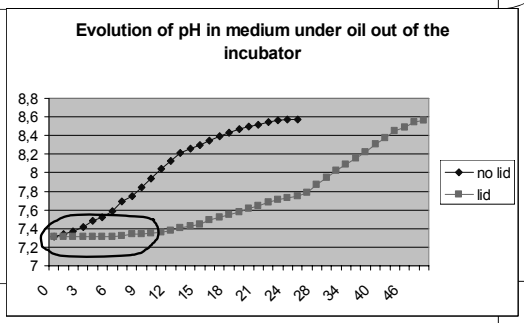
Thermo Forma – high opening frequency







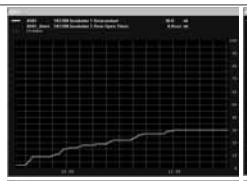
pH (25µl culture medium – 3ml oil)



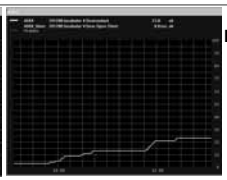
Incubators – UZ Brussel

Door openings

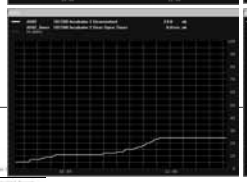
Inc 1



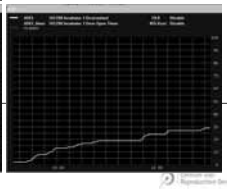
Inc 2



Inc 3



Inc 4



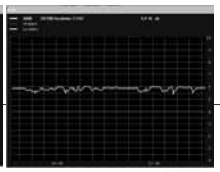
% CO_2

Inc 1

Inc 2

Inc 3

Inc 4



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

UZ Brussel - 2008

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

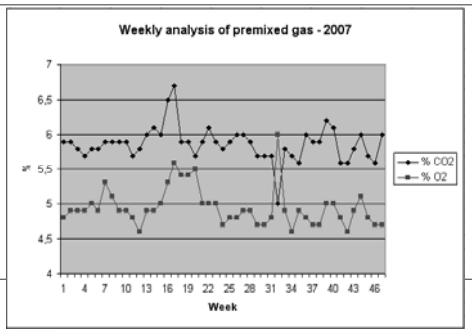
Incubators: conclusions

- 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

MINC (http://www.cookmedical.com)

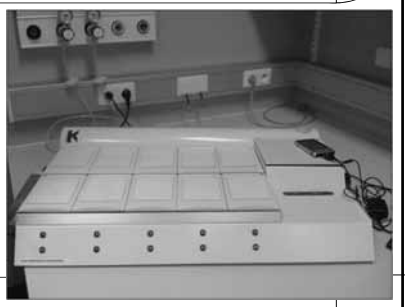
- " it takes only three minutes to reach environmental homeostasis in the MINC versus nearly two hours in other incubators available on the market today".
- (Cooke et al., J Assist Reprod Genet (2002) 19 - Fujiwara et al, J Assist Reprod Genet (2007) 24)
- "the MINC uses minimal amounts of pre-mixed gas to create and maintain a physiological culture environment"
- "the rapid heat transfer provides for a dramatically faster recovery time than other incubators and minimizes embryonic stress"
- " When the lid of the MINC is closed, gas is automatically purged to re-establish the optimal embryonic environment and return the pH level to its normal physiological range. As incubators are opened frequently to monitor for fertilization, a prompt return to normal pH level is vital to the genetic composition of the embryos."

Premixed gas

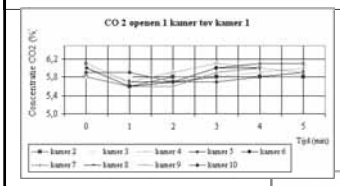


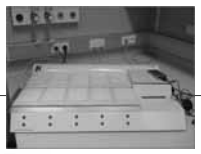
K-Systems G-185

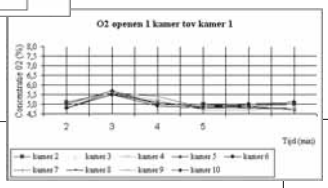
- Triple gass
- Integrated gas mixing unit
- 10 individual chambers
- Monitoring software



K-Systems G-185





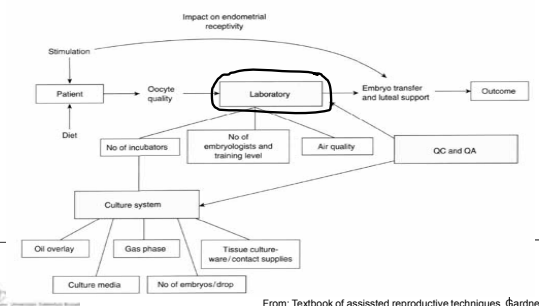


Mini incubator: Conclusions

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

- Allow individual culture
- 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 Classification (GMP)

Maximum permitted number of particles per m^3 equal to or greater than the tabulated size				
	At rest		**In operation**	
Grade	**0.5 µm**	**5.0µm**	**0.5 µm**	**5.0µm**
A	3520	20	3520	20
B	3520	29	352000	2900
C	352000	2900	3520000	29000
D	3520000	29000	Not defined	Not defined

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

Microbial contamination

Recommended limits for microbial contamination

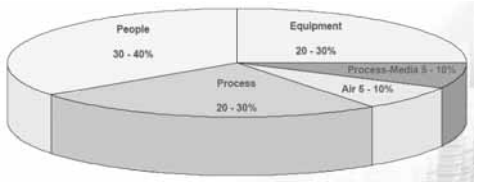
Grade	air sample cfu/m3	settle plates (diameter 90 mm)	contact plates (diameter 55 mm)	glove print 5 fingers
		cfu/4 hours (b)	cfu/plate	cfu/glove
A	<1	<1	<1	<1
B	10	5	5	5
C	100	50	25	-
D	200	100	50	-

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

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*

Cleanroom: sources of contamination



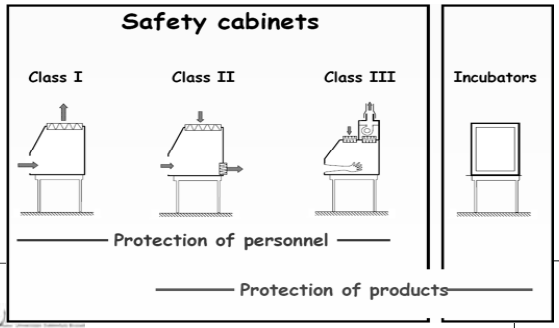
GMP – dress code

- Grade B background
  - High level cleanroom
  - Bunny suits, mask, gloves
  - Two levels of changing rooms, from street to C, from C to B
  - Hermetically sealed pass-troughs for specimens
- Grade C background
  - Trouser suit covering wrists and neck, hair and baird covered
  - Changing room
  - Hermetically sealed pass-troughs for specimens
- Grade D background
  - General protective suit, hair and baird covered

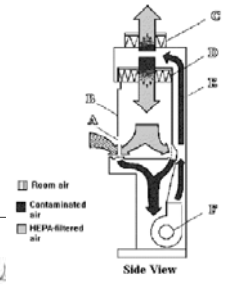
Clean room: Prevention

- Limit
  - → People
  - → Activity
  - → Material
- Cleaning
- Desinfection
  - → Fertisafe™ can be safely used in IVF laboratories, as it was proved to be an effective decontamination solution while being non-toxic for gametes and pre-implantation embryos. Janssens R, ESHRE 2007.

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%	41.1%	47.4%

Class II IVF workstation switched off and temperature adjustement!

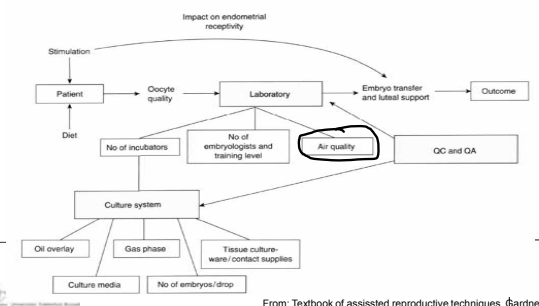
- Pickering S.J., Braude P.R., Johnson M.H. *et al.* (1990)
- Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril*, **54,** 102-108.

Conclusions

- Clean room technology might be required for new laboratories

- Air quality requirements may compromise IVF pregnancy rates

Factors affecting outcome – air quality



From: Textbook of assissted reproductive techniques, Gardner et al

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)

Advanced Chemical Sensors, Inc.
AIHA Accredited Laboratory

Measure workplace exposure or room concentrations of toxic vapors

- Easy to use - Same badge for 15 minute or 8 hour sampling
- All inclusive price for badge, analysis and report
- This is the lowest cost method to measure exposure to toxic vapors
- Meets OSHA and NIOSH accuracy requirements
- Validated Analytical Methods
- AIHA and New York ELAP accredited laboratory
- Fully reviewed QA/QC procedures
- U.S. Patented Badges That Are Analyzed Using OSHA and NIOSH Validated Methods
- Rated Proficient in the NIOSH Proficiency Analysis Testing Program (PAT) for the Analytes Tested

Cat. No.		Cat. No.		Cat. No.	
OV-00	Organic Vapor Monitor	OV-315	Cyclohexanone	OV-70	Methylene Choride
OV-005	Acetic Acid	OV-3150	Cyclohexylamine	OV-71	Methyl Ethyl Ketone (2-Butanone)
OV-01	Acetone	OV-316	p-Dichlorobenzene	OV-75	Methyl Formate
OV-05	Acetonitrile	OV-318	1,2-Dichloroethane	OV-76	Methyl Isobutyl Ketone
OV-055	Acrylamide	OV-319	Dioxane	OV-77	Mineral Spirits
OV-06	Acrylonitrile	OV-325	Dimethyl Sulfoxide	OV-772	Morpholine
OV-07	Allyl Alcohol	OV-326	Dimethyl Formamide	OV-777	Nitromethane
OV-08	Benzene	OV-327	Phenylglycidyl Ether	OV-78	Pentane
OV-09	B-T-E-X	OV-33	Diesel Fuel	OV-79	Pentachlorophenol
OV-10	Benzyl Chloride	OV-333	Dipropylene Glycol Methyl Ether	OV-80	Perchloroethylene (Tetrachloroethylene)
OV-102	Biphenyl	OV-34	Ethyl Acetate	OV-81	Petroleum Distillates (Naphtha)
OV-12	1,3 Butadiene	OV-335	Epichlorohydrin	OV-86	2-Propanol
OV-14	Butyl Acetate	OV-345	Ethyl Acrylate	OV-88	n-Propyl Bromide
OV-15	Butyl Alcohol	OV-35	Ethyl Alcohol	OV-90	Propylene Oxide
OV-157	n-Butylglycidy 1 Ether	OV-37	Ethyl Benzene	OV-91	Prylidine
OV-16	t-Butylamine	OV-40	Ethyl Ether	OV-93	Stoddard Solvent
OV-17	Butyl Ether	OV-41	Ethylene Glycol	OV-95	Styrene
OV-152	Sec-Butyl Alcohol (2-Butanol)	OV-415	Formamide	OV-96	Tetrahydrofuran
OV-16	Butyl Cellosolve (2-Butoxyethanol)	OV-42	Freon 13	OV-100	Toluene
OV-20	Carbon Tetrachloride	OV-45	Gasoline	OV-102	Total Saturated Hydrocarbons
OV-22	Cellosolve (Ethylene Glycol Monoethyl Ether)	OV-50	Heptane	OV-104	1,1,2-Trichloroethane
OV-25	Chlorobenzene	OV-55	Hexane	OV-105	Trichloroethylene
OV-30	Chloroform	OV-555	Hexylene Glycol	OV-106	Triethylamine
OV-31	Collodion	OV-57	Isopropyl Alcohol	OV-107	1,2,4-Trimethyl Benzene
OV-311	Cumene	OV-575	Isophorone	OV-109	Vinyl Acetate
OV-312	m-Cresol	OV-578	Jet Fuel (JP-8)	OV-110	Vinyl Chloride
OV-313	Cyclohexane	OV-577	Limonene	OV-115	Xylene
OV-314	Cyclohexanol	OV-58	2-Methoxyethylacetate		
		OV-59	Methyl Acrylate		***MANY OTHERS AVAILABLE***
		OV-61	Methylamine		
		OV-62	Methyl t-Butyl Ether		
		OV-65	Methyl Chloroform (1,1,1-Tricholorethane)		

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

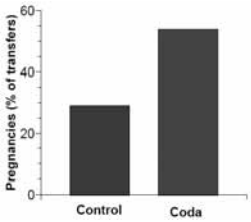
VOC Meter

- 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

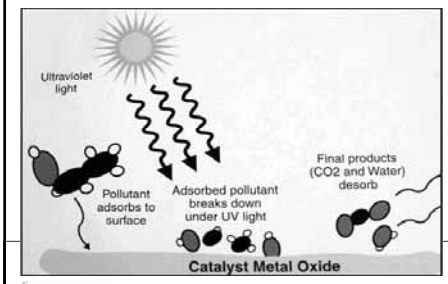
Incubator Filters - Prospectively Randomized Study



There was no observed difference in embryo morphology between the treatment groups.

- Mayer JF, Nehchiri F, Weedon VM, Jones EL, Kalin HL, Oehninger SC, Toner JP, Gibbons WE, Muasher SJ (1999) Prospective randomized crossover analysis of the impact of an IVF incubator air filtration system (Coda genX) on clinical pregnancy rates. *Fertil Steril* **72**, Suppl 1, S42.

Photo-Catalytic Oxidation



CO
N_xO
$CO_2 + H_2O$

Z-IVF AIRe

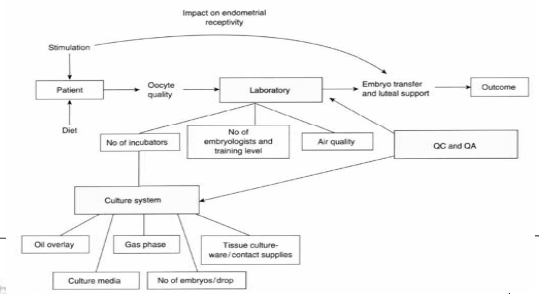
Active Carbon Filter + potassium permanganate

HEPA filtration removes particles >0,3µM

Photo-Catalytic Oxidation: converts toxic compounds into CO2 and water

Ultraviolet light

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

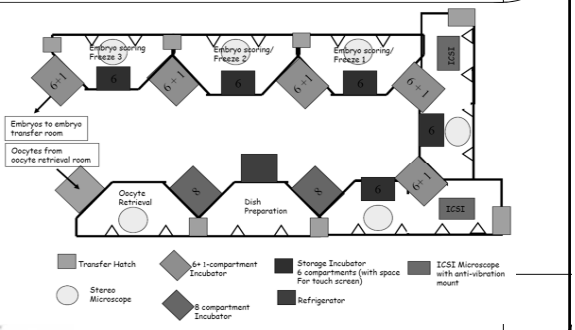
Ac-tive® IVF System: Defined

Assisted Conception Total In Vitro Environment

- Temperature and humidity control
- O_2 and CO_2 control
- HEPA - VOC filtration
- Integrated microscope with Cronus embryo analysis software
- IVF Witness
- Access through especially designed glove ports that do not allow any gas to escape
  - → From oocyte retrieval to embryo transfer in a single workstation
- Modular

Vitrosafe isolators (Newcastle centre of Life)

Vitrosafe UZ Brussel proposal



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