Stroebechmedia
Assisted Reproduction Techniques in Animals

# IVF Manual of Procedures

This manual applies to *in vitro* production of bovine embryos from both
**Ovum Pick Up (OPU) and Slaughterhouse (SH) ovary collection**
The manual also applies to sheep, goat, buffaloes and camelids.
For endangered species, ZOO- and wildlife animals get in touch.
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# IVF Manual of Procedures
# OPU, IVM, IVF and IVC

**Volumes of Media needed**

**The estimated volumes below are calculated by assuming:**

For slaughterhouse an average of 8 COC's (Cumulus Oocyte Complexes) are aspirated per ovary, i.e., a collection of 20 cows, 40 ovaries are equivalent to approximately 320 oocytes
And in average 12 COC's per OPU session (Ovum Pick-up).

| Media | REF# | Slaughterhouse SH | 10 OPU's |
|---|---|---|---|
| OPU Medium<br>For Ovum Pick Up | 2.01.250 | 0 | 5 liters |
| WASH Medium<br>For Oocyte/Embryo Wash | 1.02.050 | 50 ml | 50 ml |
| IVM Medium<br>For in vitro Maturation of Oocytes | 1.03.020 | 20 ml | 20 ml |
| H-IVM Medium<br>For in vitro maturation of oocytes outside the incubator | 1.04.020 | 20 ml | 20 ml |
| SEMEN WASH Medium<br>For Semen Preparation Non-Capacitating | 1.05.050 | 20-30 ml | 8-12 ml/bull |
| IVF Medium<br>For in vitro Fertilization | 1.06.020 | 10-20 ml | 10-20 ml |
| IVC Medium<br>For in vitro Culture of Embryos | 1.07.020 | 10-20 ml | 10-20 ml |
| Stroebech OIL<br>Peroxide tested Pre-washed Oil | 2.08.050 | 10-20 ml | 20-30 ml |
| HOLDING Medium<br>For Transfer, Biopsy and Holding of Embryos | 1.09.020 | 10-20 ml | 10-20 ml |
| Heparin 5000 IU/ml | | 150 µl/50 ml tube | Included in OPU medium |

IMPORTANT

*In vitro* produced embryos are much more fragile and sensitive to pH and temperature fluctuations and not as robust as *in vivo* produced (ET) embryos



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# General Information

## Culture conditions

**Incubator temperature** should be **38.8°C**
All heated stages should be **35°C**, not more!
Equilibrate all media in the incubator to **38.8°C Semen Wash Medium and OPU Medium should only be warmed**

**Gas concentrations**
**The media contain 25-27 mM bicarbonate (HCO3- ) corresponding to a pH 7.3-7.4 see appendix 2**
**Maturation (IVM) 6-6.5% $CO_2$** humidified atmospheric air **(21% $O_2$)**
**Fertilization (IVF) 6-6.5% $CO_2$** humidified atmospheric air **(21% $O_2$)**
**Culture (IVC) 6-6.5% $CO_2$ and 6-9% $O_2$** - Bovine embryos require **low oxygen for IVC**

***If the lab is located above sea level, you will need to adjust the CO2/O2 concentrations accordingly - See appendix 3***

## Media dish preparations

**Maturation:** in 500 µl 4WP **without** oil overlay or in vials 800-1000 µl Note: Many vials are toxic

**Fertilization:** in drops (no less than 100 µl) with oil overlay or in 500 µl 4WP without oil overlay

**Culture:** in drops (no less than 100 µl) with oil overlay or in 500 µl 4WP **with** oil overlay and no change of media

**Rinse dishes:** always rinse oocytes/embryos once in the corresponding final medium in order not to dilute medium that is to be incubated

When **oil overlay** is used in 4WP, rotate the lid. Just one oil drop between the lid and the dish will seal them together and prevent $CO_2$ equilibration of the medium resulting in embryo death. Remember: The quality of oil is key to success!

Do **never aliquot any media** into plastic vials for storage
Remove lid when warming and equilibrating media in glass bottles, except for Semen Wash Medium
Media in 4WP and petri dishes should be prepared minimum 30 minutes prior to use **for $CO_2$/$O_2$ equilibration**

**Micro drop (100 µl) preparation:** first make a 10 µl drop medium, cover with plenty of oil overlay, then inside the drop under oil supplement the remaining medium amount. Drops will now be round and not flat Make sure there is a minimum of 3 mm oil above the drop

## Disposables

Use polystyrene plastics that are embryo-grade quality, cyto- and endotoxin, pyrogen- and RNase free.
Note that cell culture grade **is not** good enough! Use 4-well plates (4WP) with a culture area per well of 1.9 $cm_2$.
Use only filtered pipette tips to avoid risk of contamination of pipettes, which may be a hidden source of continuous infection.


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# Day -1: Maturation IVM

## Oocyte aspiration by OPU or SH Ovary Collection

**Media for equilibration and warming:**
**OPU Medium, WASH Medium, IVM Medium**
Or **H-IVM Medium** in case the COC's are to be matured during transportation.
Do never hold oocytes in the OPU medium longer than necessary and max 30 minutes.
If your IVF laboratory is not within 30 minutes reach, start the maturation in vials at the OPU site.

The H-IVM Medium is HEPES buffered and does not require $CO_2$ equilibration and only needs warming to 38.8°C prior to use.
The H-IVM Medium can also be used in a $CO_2$ gas atmosphere as it contains bicarbonate.

**In the IVF laboratory**

- Warm the WASH Medium
- Prepare and pre-equilibrate IVM Medium in 4WP wells or in maturation transportation vials
- Prepare a 35 mm petri dish (SH) or separate 4WP's (OPU) with IVM Medium for rinsing the COC's prior to transfer to the IVM wells/vials
- Warm bottles of 0.9% saline for washing of the ovaries (31-33°C)
- Add 150 µl of Heparin to 50 ml conical tubes

***If the tubes have a bad smell, take off the lid under a laminar flow hood and let air out 2 hours before adding Heparin***

## Ovum Pick Up (OPU)

Preheat OPU medium to 37°C.

Make sure that the aspiration tube warmer is **not too hot**, it should not be more than 37°C, however, it is often much warmer. Place a thermometer inside a tube with oil in the tube warmer to verify the temperature.

Transport the oocytes to the laboratory preferably after each OPU. Keep the oocytes warm at all times.
Pour OPU content into a filtered dish and wash with OPU Medium until content is clear. Make sure the dish/filter does not dry out at any time. Search for oocytes and wash them through the WASH Medium to get rid of debris.
Rinse once in IVM Medium prior to transferring oocytes into final maturation, as quickly as possible.
No more than 20 oocytes per vial if maturation takes place during transportation. Place vials at 38.8°C in a preheated shipper. Validate the shipper has the correct- and a stable temperature.

***Oocytes should be surrounded by many layers of cumulus cells. If many are naked: adjust vacuum pump pressure and check size of the aspiration tubes. Too narrow tubes will strip the cumulus cells off or, more likely, too wide tubes will also strip the oocytes due to turbulence.***


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# Slaughterhouse (SH)

Preheat water in thermo container to 31-33°C. Collect ovaries in a food grade plastic bag and place in container. Do not let ovaries come into contact with the surrounding water nor PBS/Saline! The follicular fluid in the follicles will be depleted of amino acids and other compounds necessary for the oocytes. The optimal embryo development is obtained when the ovaries are left at 31-33°C for 2-4 hours after collection and prior to aspiration.

Set heating plate to 35°C and connect the aspiration device to the vacuum pump.

***Aspiration by vacuum pump is much better than slicing, as you avoid getting too many oocytes that don't have developmental competence.***

- Record time of arrival and temperature of the water in the ovary thermos container
- Empty water out of container and remove the ovaries from the plastic bag and place in container again
- Wash the ovaries in warm saline and store in clean saline in the thermos container during aspiration
- Measure temperature again – should be 31-33°C
- Orient a sharp 18-gauge needle with the opening facing downwards
- Aspirate follicles ranging from 2-20 mm and no more than 20 -25 ml follicular fluid per tube
- Note the number of ovaries aspirated per tube

When all ovaries have been aspirated prepare 9 cm dishes with squared lines on bottom of dish (gridded) by adding 6-8 ml of WASH medium for oocyte search. Prepare one gridded dish per conical tube.

- Add 3 ml of WASH Medium to each of three 35 mm Petri dish labeled 1, 2, 3
- Remove settled COC's (pellet) from the bottom of the conical tube with a plastic disposable pipette in a volume of ~2 ml and place into the gridded dish for COC collection
- Using a systematic pattern of searching, place all COC's into dish #1 with WASH Medium
- Aspirate the same tube again for a second pellet and repeat above procedure
- Aspirate the remaining tubes as above, collecting all oocytes to dish # 1

***Do NOT waste time discarding potential "poor" oocytes. Give all the benefit of the doubt. You may have evaluated wrong and "poor" oocytes do not have a negative effect on the other oocytes!***

- Wash all COC's through dish marked #2
- Transfer and count the oocytes from dish #2 to dish #3
- For an experiment that requires more groups, randomize the oocytes at this point
- From dish #3 rinse the COC's once in the IVM Medium dish/well prior to transferring to the final maturation dish/well. Incubate no more than 45 COC's per well per 500 µl medium.
- Mature for 21-24 hours in $CO_2$ incubator


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# Day 0: Fertilization IVF

**Media to be equilibrated and warmed**
**Equilibrate the IVF Medium and warm the Semen Wash Medium** in the bottle and with lid on
**(IVC Medium** with **Oil** overlay if overnight equilibration)

- Prepare and pre-equilibrate IVF Medium
- Prepare the number of wells corresponding to wells of matured oocytes or number of bulls to be used
- Prepare one dish per donor with IVF Medium for rinse
- Add 400 µl IVF Medium to all 4-wells of the 4WP with 500 µl being final volume (60 µl volume of oocyte plus ~40 µl volume of washed semen)
- **OR 100 µl micro drops:** Add 10 µl IVF Medium, cover with oil, then add 70 µl IVF Medium (10 µl medium containing oocytes and 10 µl of washed semen will be added to achieve a total volume of **100 µl**)

***Make drops in 4WP's because drops in a petri dish risk floating together and potentially mix donors***

**Preparation of Matured Oocytes**

- After 21-24 hours of IVM, remove matured oocytes from the incubator and evaluate and note cumulus expansion
- Rinse oocytes gently **and only once** with a 60 µl pipette through the equilibrated IVF Medium rinse dish/well
- Do not divide oocytes at this time or disrupt the expanded cumulus cell mass
- Move oocytes into the designated IVF wells/micro-drops and incubate while preparing Semen

**Semen Preparation**

- Prepare a thermos container with 37°C warm water
- Label as many 15 ml centrifuge tubes as semen straws to be thawed
- Add 4 ml Semen Wash Medium per 15 ml centrifuge tube – keep warm
- Verify name of bull and quickly remove desired number of semen straws from $LN_2$ and place into the thermos container

***If a straw need to be cut in half –make sure that the straw is kept under liquid nitrogen at all times and leave the name of the bull on the unused half***

- Dry straw(s) with sterile gauze and place in LAF bench or on a warm surface at 35°C stage
- Cut the end of the straw opposite from the cotton plug and place the straw just below medium surface inside tube.
- Cut the other end of straw off (the cotton plug) and allow semen to flow into the 4 ml Semen Wash Medium tube
- Place a drop of semen from the straw on **a warm** microscope slide, add coverslip and check the motility immediately
- Centrifuge using a fixed bucket and non-refrigerated centrifuge for 5 minutes at 328xg (RCF)

***Do not confuse with RPM, which is rounds per minutes, and depends on length of rotor arm, whereas RCF is the relative centrifugal force (g)***

***Make sure centrifuge tubes are conical and that centrifuge is not with swing arms but fixed buckets to get optimal pellet***

- Remove supernatant with a sterile disposable 5 ml pipette and leave ~400 µl of the suspension and re-suspend pellet.

***Remove supernatant immediately because swim-up occurs as soon as centrifugation stops***

- Add again 2-4 ml pre-warmed Semen Wash Medium to the 400 µl sperm suspension and centrifuge for 5 minutes at 328xg (RCF) to wash semen a second time
- Remove supernatant as described above and leave ~200- 400 µl and re-suspend pellet again to the final sperm suspension
- Calculate the concentration in the sperm suspension in order to calculate the correct volume to use for fertilizing

**See Appendix 1 on how to calculate what volume sperm suspension to add to the IVF well or drop, respectively, to obtain 2 x $10^6$ spermatozoa/ml per well/drop, which is the optimal fertilization concentration. Example illustrated by using the Makler chamber counting device**

**Fertilization**

- Take the IVF plates containing the matured oocytes from the incubator and fertilize by adding the calculated volume of sperm suspension and incubate overnight

***If sperm motility and/or concentrations are low (<50%), double the calculated volume of sperm suspension to fertilize with to compensate. Incubate for 1 hour, and then remove twice the volume you added and replace with freshly equilibrated IVF medium. Incubate again.***
***Be careful not to aspirate any oocytes***

***Short protocol, i.e., fertilization for just some hours does not work as it does in human***

Consider making the IVC plates on fertilization day to equilibrate overnight.


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# Day 1: Culture IVC

**Media to be preheated and equilibrated:**
**WASH Medium, IVC Medium, Stroebech OIL**

- Prepare and pre-equilibrate IVC Medium in the desired number of wells for IVC for final culture
- (no more than 30 inseminated oocytes/500 µl)
- Prepare the same number of wells per 4WP or dishes with IVC Medium for rinse
- Add 500 µl IVC Medium to each well of the 4WP and cover with 400 µl oil
- **FOR 100 µl micro drops:** Add 10 µl IVC Medium, cover with oil, then add 90 µl IVC Medium into the 10 µl drop now covered with oil – this method ensures the drops get round and not flat
- (no more than 10 inseminated oocytes/100 µl)
- Preheat WASH Medium

**Denudation - Removal of cumulus cells from the inseminated oocyte**

**OPU:** Denudate in WASH Medium in drops or in 500 µl wells.
Consider to vortex if you have 15 or more inseminated oocytes. Otherwise denudate with pipette.

- Remove the IVF dishes from the incubator – note that cumulus cells have been disrupted from the spermatozoa activity
- Rinse the inseminated oocytes free of oil through several WASH Medium drops
- Use a denudation pipette 125-145 µm - the preferred method
- Or denudate oocytes vigorously with a 200 µl pipette
- Wash the denudated oocytes through the dishes/wells
- Count the denudated inseminated oocytes and record in the IVC worksheet
- Rinse once in the respective equilibrated IVC medium dish before placement to the 4WP/drop with IVC medium

***Do not use the same wash and/or rinse drops or wells for different donors***

**SH:** Vortex. Use polystyrene plastic for vortexing because it is a harder plastic than the polypropylene centrifuge tubes to ensure the oocytes will be completely denudated.
Be sure that two tubes are hitting hard against each other during vortexing.
For 1-2 min max speed otherwise the cumulus cells remain attached to the zona pellucida.


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**Prepare two sets of 10 ml tubes and dishes for the SH experiment**

If more than two groups are tested prepare wash tubes and dishes corresponding to the number of groups of inseminated oocytes to be denudated.

- Add 1 ml of WASH Medium to two 10 ml vortexing tubes
- Prepare two empty 35 mm Petri dishes both labeled #1
- Add 3 ml WASH Medium to four 35 mm Petri dishes, two labeled #2 and two labeled #3
- Remove the IVF dishes from the incubator – note that cumulus cells have been disrupted from the spermatozoa activity
- Transfer all the inseminated oocytes to the 10 ml tube(s) containing 1 ml WASH Medium
- Vortex for 1-2 min at high speed and pour the content of the 10 ml tube into the empty dish #1
- Add 1-2 ml WASH Medium to the 10 ml tube; pour off again into the same 35 mm dish
- Remove the rest of the medium from the tube with a pipette
- Transfer all the denudated oocytes to dish #2 containing WASH Medium
- Work fast without evaluating individual oocytes
- Wash a second time by transferring the oocytes from dish #2 to dish #3
- For an experiment that requires more groups, randomize the inseminated oocytes at this point
- Count the inseminated oocytes during this last wash step, and finally, rinse once in the equilibrated IVC Medium dish before the final transfer to IVC Medium for culture. Rinse or change pipette tip after each transfer through oil in order not to accumulate too much oil in the IVC Medium rinse dish
- Usually, ~ 30 inseminated oocytes are added to each 500 µl well

***Note that cumulus cells will impair embryo development***
***so only few should remain attached to zona pellucida for culture.***

***Do NOT waste time discarding potential unfertilized oocytes. Give all the benefit of the doubt.***
***You may have evaluated wrong and unfertilized oocytes do not have a negative effect on the other oocytes!***

Write down exactly how many inseminated oocytes that have been placed in each well. Write it on the lid and in the worksheet. Incubate.

***Assess the motility of the sperm in the empty wells/drops – there should still be some activity to detect.***
***If no motile spermatozoa are left, consider sub-optimal conditions in the laboratory***


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# POST IVC

Cleavage can be checked 48 hours after – but **embryos are best left alone** until after morula compaction.

- Transfer at day 7 – but check day 6 where some embryos have already reached the blastocyst stage
- Use HOLDING Medium for transfer immediately
- If transporting the embryos to recipients preferably use vials and load at the farm do not transport embryos in straws

***IVF embryos are NOT in vivo embryos, but more fragile and sensitive to pH and temperature fluctuations***

## We are here to help – get in touch!

## Remember:
## “Luck never comes to the unprepared!” ☺



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# APPENDIX 1.

## Calculation of sperm suspension volume to use for fertilizing in 100µl drops

The dilution is 1:2 as 25 µl of sperm suspension has been mixed with 25 µl of cold water
And the number of sperm counted in 100 squares must be divided by 10 (in Makler chamber), and multiplied by 2, to give the actual sperm concentration x$10^6$/ml

### EXAMPLE

You want a final concentration of 2.0x$10^6$ sperm/ml for fertilization so you calculate:
The IVF well already contains 80 µl of fertilization medium + 10 µl IVF medium when the oocytes were transferred, so, addition of approximately 10 µl of sperm suspension would give a total volume of 100 µl

$$C1xV1=C2xV2 \quad 26 \times 10^6 \times volume_{sperm} = 2.0 \times 10^6 \times 100\mu l$$

$$\frac{C2 \times V2}{C1} = V1 \qquad volume_{sperm} = \frac{2.0 \times 10^6 \times 100\ \mu l}{26 \times 10^6} = 7.7\mu l$$

## Calculation of sperm suspension volume to use for fertilizing in 500 µl wells

The dilution is 1:2 as 25 µl of sperm suspension has been mixed with 25 µl of cold water
The number of sperm counted in 100 squares must be divided by 10 (in Makler chamber), and multiplied by 2, to give the actual sperm concentration x$10^6$/ml

### EXAMPLE

You want a final concentration of 2.0x$10^6$ sperm/ml for fertilization so you calculate:
The IVF well already contains 400 µl of fertilization medium + 60 µl IVF medium when the oocytes were transferred, so addition of approximately 40 µl of sperm suspension would give a total volume of 500 µl

$$C1xV1=C2xV2 \qquad 26 \times 10^6 \times volume_{sperm} = 2.0 \times 10^6 \times 500\ \mu l$$

$$\frac{C2 \times V2}{C1} = V1 \qquad volume_{sperm} = \frac{2.0 \times 10^6 \times 500\ \mu l}{26 \times 10^6} = 38.5\ \mu l$$


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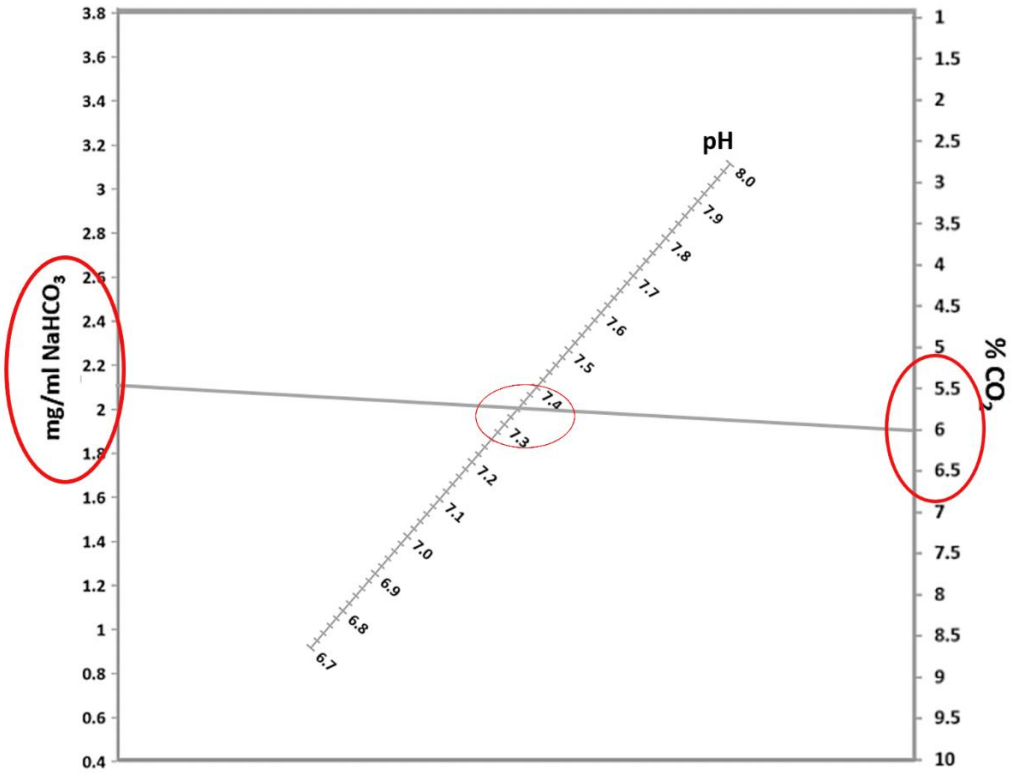
# APPENDIX 2.

## Correlation between Bicarbonate and $CO_2$ Concentrations and the effect on pH




- pH will be 7.35 in a medium with a concentration of 2.1 mg/ml bicarbonate at 6% $CO_2$
- Lower $CO_2$ = increase pH
- Know your incubator gas concentration and your medium bicarbonate concentration rather than 'measuring' pH
- At an Increased altitude, the $O_2$ and $CO_2$ partial pressure is lower
- Lower partial pressure of $CO_2$ means a lower concentration of $CO_2$ inside the incubator
- At 1500m above sea level, an incubator set at 6% $CO_2$will have an actual concentration of only 4.9% $CO_2$

***Stroebech media recommends a $CO_2$ concentration of 6-6.5 %***



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# APPENDIX 3.

Correction table to maintain the same partial pressure in incubator of $CO_2$ and $O_2$ as at sea level

| Altitude above Sea Level (Meter) | Altitude above Sea Level (Atm) at 39 degrees | % $CO_2$ and $O_2$ |
|---|---|---|
| 0 | 1,00 | 6,0 |
| 100 | 0,99 | 6,1 |
| 300 | 0,97 | 6,2 |
| 500 | 0,95 | 6,3 |
| 700 | 0,93 | 6,5 |
| 900 | 0,91 | 6,6 |
| 1100 | 0,89 | 6,7 |
| 1300 | 0,87 | 6,9 |
| 1500 | 0,85 | 7,1 |
| 1700 | 0,83 | 7,2 |
| 1900 | 0,81 | 7,4 |
| 2100 | 0,79 | 7,6 |
| 2300 | 0,77 | 7,8 |
| 2500 | 0,76 | 7,9 |
| 2700 | 0,74 | 8,1 |
| 2900 | 0,72 | 8,3 |
| 3100 | 0,70 | 8,6 |
| 3300 | 0,69 | 8,7 |
| 3500 | 0,67 | 9,0 |
| 3700 | 0,66 | 9,1 |
| 3900 | 0,64 | 9,4 |

## EXAMPLE:

Laboratory is **at 1500 m above** sea level where the partial pressure is 0,85 atm. The desired $CO_2$ concentration is 6% then you calculate 6/0,85 atm = 7,1 and your incubator setting **should be 7.1% $CO_2$** in order to have 6% $CO_2$ inside the incubator



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