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Cryoport SRBT Webinar - Practical pH for the IVF Lab
February 28, 2013, 10:00 am

Question Log

I would like to thankThomas Pool, PhD, HCLD and Michael Reed, PhD, HCLD for their assistance, insight and comments to the answers given below – Jason Swain, PhD, HCLD

Q: Any thoughts on elevation and if a potential equation can be created so every clinic can have the most appropriate levels for their geographic location?
Elevation can impact the levels of CO2 needed in the incubator to achieve a desired pH. The higher the altitude, the higher the CO2 level required. I'm not aware of any equation (modification of Henderson Hasselbach) that addresses this though. You will likely have to simply measure the pH to see what CO2 levels are required.

Q: Is a blood gas analyzer appropriated to measure the pH of our culture media?
Yes. If there is access to a blood gas analyzer, this can be used to measure pH. There are portable systems available. However, it should be pointed out that they are expensive and required a cartridge for each use (which can also be expensive if dealing with several incubators/samples). I would also recommend verifying the reliability/repeatability of the results by measuring pH with a meter if one is available.

Q: Would you please the information about the electrode type?
I use a glass, double junction, KCL filled electrode in my lab. This was recommended by the manufacturer for use with samples with protein content or with biologic buffers present. However, other electrodes can also be used. I would recommend contacting your pH meter or electrode manufacturer to get their recommendations. We also use a semi-micro electrode to be able to measure smaller volumes of media and help reduce the associated waste/cost of media. Other "in incubator" pH measuring devices use other technology, which can work as well.

Q: With oil overly, what is the maximum time we should have the embryos outside the incubator?
As quickly as possible is the rule of thumb. I believe Joe Conaghan has presented some data in abstract form showing that pH rises above ~7.4 after around 5 minutes (Steel & Conaghan, Fert & Stert 2008). One also has to consider that it then takes much longer for the pH to re-equilibrate once put back into the incubator…as this isn't an instantaneous event. Another reference is "Real-Time pH Profiling Of Ivf Culture Medium Using An Incubator Device With Continuous Monitoring" J Clinical Embryology Summer 2008 Vol 11, issue 2

Q: Hi, at our lab we achieved optimal pH at 8% CO2. Does the higher CO2 concentration have an effect on fragmentation?
I can't say for certain. I also can't say for certain that the higher CO2, while maintaining the correct pH,

wouldn't potentially be beneficial either. I'm not aware of any data that demonstrates that a higher CO2 level to achieve an appropriate pHo is detrimental (in fact, some very successful labs are present in areas of high elevation, like Denver, that need to use elevated CO2 levels (~7%)and have excellent embryo development and outcomes. You may want to re-zero your incubator to make sure your CO2 reading is correct. This may help alleviate some of your concerns if it is a bit "off".

Q: Any recommended make for a pH probe?
I have probes from two separate companies in my lab and both work well and give similar readings. So, I don't really have a recommendation for a particular manufacturer. It may be safest to use the probe sold by the maker of your pH meter to ensure compatibility of fittings, etc. However, you should be able to interchange the correct probes as well. I use a glass, double junction, KCl filled, semi micro probe…but other probe types can also be used.

Q: Could you please recommend one good electrode for measuring the pH of culture media?
I don't really have a recommendation for a particular manufacturer. It may be safest to use the probe sold by the maker of your pH meter to ensure compatibility of fittings, etc. However, you should be able to interchange the correct probes as well. I use a glass, double junction, KCl filled, semi micro probe, but other probe types can also be used.

Q: How often do you measure pH? Daily, per lot?
We measure our pH with each new lot of media and also on a weekly basis to verify incubator functioning. However, measuring daily is fine as well if it fits in with your lab workflow and budget. It should be noted that CAP is asking for daily incubator checks, independent of the CO2 readouts. If you supplement CO2 measurement daily with a Fryrite or some other system, pH measurement scheduling can fit into your program however you like. Alternatively, daily pH checks can replace the need for independent daily verification of CO2 levels. pH should likely also be checked with each new of lot pre-mixed gas if this is used. Periodic checks in the incubator on various shelves may also be beneficial.

Q: Is there a real need to adjust CO2 in function of the altitude your lab is situated?
Labs at higher elevation often need to use slightly higher CO2 levels to achieve the desired pH. This may also be due, in part, to the method of CO2 measurement. If using Fyrite, corrections to CO2 readings are needed. The following expert is from a Fyrite manual:

The gas concentration read on the FYRITE is directly dependent upon the mass of air in the sample. The aspirator bulb used in the FYRITE is a constant-volume pump, not a constant-mass pump. Altitude, therefore, affects the FYRITE reading due to the air's density changing with altitude, thus requiring higher CO_2 or O_2 concentrations to reach the same mark on the scale.
Use the following table to find the altitude correction (e.g., add the correction to the reading to get the correct concentration).

ALTITUDE CORRECTION TABLE

	Altitude Correction %		
Ft. (Meters)	*20% CO_2*	*21% O_2*	*7% O_2 or CO_2*
1000 (305)	*0.0*	*0.0*	*0.0*

2000 (610)	*0.0*	*0.0*	*0.0*
3000 (914)	*0.1*	*0.1*	*0.0*
4000 (1575)	*0.1*	*0.2*	*0.1*
5000 (1219)	*0.2*	*0.2*	*0.2*
6000 (1829)	*0.2*	*0.3*	*0.2*
7000 (2134)	*0.3*	*0.3*	*0.3*
8000 (2438)	*0.4*	*0.4*	*0.3*
9000 (2743)	*0.4*	*0.5*	*0.4*
10,000 (3048)	*0.5*	*0.6*	*0.5*
11,000 (3353)	*0.6*	*0.7*	*0.5*
12,000 (3658)	*0.7*	*0.8*	*0.6*
13,000 (3962)	*0.7*	*0.8*	*0.6*
14,000 (4267)	*0.8*	*0.9*	*0.7*

Q: What is the suggested corrective action when your media pH is out of range and you need it within the hour, since it takes hours to equilibrate?

If new lots of media are tested prior to implementation, and incubators have been set accordingly, there shouldn't be many instances where pH falls out of range enough to compromise development. Slight daily variation is expected, which is why an acceptable pH range is set. So, if your pH falls out of range by say 0.01 or 0.02 pH units, this may simply be due to probe/meter variation. You could try recalibrating the machine and retesting the sample and see if it is back "in range". We don't like to constantly adjust CO2 levels on our incubators. Doing this daily is likely an exercise in futility. (if your pH is falling out of range too often, there may be issues with consistency of how it's equilibrated/measured, etc). We pH weekly and have a target range of 7.27-7.32. Our acceptable range is slightly wider of 7.25-7.35. We only adjust the CO2 levels when the pH falls out of our acceptable range 2 weeks in a row or immediately if it falls outside of the target range. If the pH is far outside the range, perhaps use of an extra dish from another incubator , or a tube of media with the correct pH from another incubator would be feasible. Your corrective action could be the same as if an incubator fails…move to a new incubator until corrected. (an alternate method could be to keep pre-equilibrated media/dishes in the refrigerator in sealed bags filled with premised gas to give the correct pH. Because pH is already set in these dishes, only temperature needs to be equilibrated. These dishes could then be removed as needed and placed in the incubator, as temperature will equilibrate to 37°C much faster than if they were ungased—recommended by Bavister & Poole 2005. Storing these at the cooler temp may help prevent degredation of components and allow the dishes to be ready for use for several days, rather than making up extra dishes every day and placing them into the incubators.)

A colleague also recommends use of a control chart for plots of incubator pH (see David and Sharon Mortimer's Quality and Risk Management in the IVF Laboratory book). It is just a plot of pH as a function of time. You can put a line for the pH mean you want, an upper limit to the range, and a lower limit to the range, but you can also put warning limits short of the upper and lower limits. Use these as warning indicators to help know when to make adjustments.

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Q: We use PBS only for egg retrievals. Is that acceptable or should we switch?
Various labs still use PBS for retrievals. Some of these labs have very good success rates. My personal recommendation, based on the literature that shows that elevated phosphate is detrimental to embryo development, is to not use phosphate buffered media in my lab. The elevated phosphate compromises metabolism (via the Crabtree effect). In the original publications by Norman Good detailing development of the Good's buffers, like HEPES and MOPS, it was pointed out these buffers were developed due to the limitations and problems associated with using phosphate as a buffer. Thus, I prefer to use a HEPES or MOPS buffered medium that is supplemented with the same/similar energy substrates and additives as my regular culture media. Any added expense due to a media change is likely worth it if it avoids detriment to the oocyte or resulting embryo (would you put an embryo into PBS for culture? Why would you then put an oocyte into PBS?)

Q: A plate made with CO2 equilibrated oil can be used after only 3-4 hours isn't it?
I would say the only way to know this would be to measure it. The CO2 from the incubator still has to diffuse through the oil and reach equilibrium in the culture medium to set the pH. Simply putting pre-equilibrated oil on top of culture media doesn't necessarily mean the media is going to equilibrate faster. Also, leaving oil in the elevated temperature of the incubator for extended periods to "pre-equilibrate" may not be the best practice. This could lead to peroxidation in the oil that could end up compromising embryo development. Just as it's not a good idea to leave media in the incubator for too long, it's not a good idea to leave oil in the incubator for too long. (oil can also act as a sink for VOCs and could be accumulating these if left in the incubator for extended periods. You wouldn't want to then put this oil on your culture media). This is why many labs are now storing their oil at 4°C, to cut down on peroxidation, etc. This idea of limited pre-equilibration time due to potential degradation of media/oil is also addressed in Bavister & Poole, 2005.

Q: Any optimal volume of medium to oil?
Not really. Most people use just enough to cover their microdrops or wells, without running the risk of spilling when moving the dish. Having enough is crucial to prevent evaporation, prevent pH rise, as well as acting as a heat sink. Of note, too much oil will extend the equilibration process as well. When using 4/5 well dishes, common approaches are 300-500µl of oil over ~500ul of media. Volumes will vary for microdrops, depending on the size of the dish, the volume of the microdrop, etc. There are also "embryo specific" dishes now from various companies. Some of these use microwells. These products usually give recommendations on the volume of oil to use.

Q: What about the ATC probe?
The ATC probe helps compensate for the impact of temperature on measuring pH. These can be purchased from the manufacturer of your pH meter. Some probes have this capability in them. Meters often have another input for a separate ATC probe. Some meters let you program them to the appropriate temperature, so you don't need an external probe. Use of temperature compensation ensures the greatest accuracy, since our media are warmed to 37°C and temperature does have an impact.

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Q: Does altitude of the place affect pH widely?
Generally, the higher the altitude, the higher the CO2 level that will be needed. This can be impacted by the method used to measure CO2 concentration (if using Fyrite…see above). Measuring the pH will help determine what CO2 levels is required.

Q: Would you use a bicarb buffered media for embryo transfers? How fast will the pH shift in a small volume of media during ETs (20ul)? How detrimental is this brief shift in pH?
Bicarb buffered media can be used for transfers if you're confident they proceed quickly and smoothly. However, with no oil overlay, in just a minute or so (likely less) the pH will have risen above 7.4 (probably much quicker with such small volumes of media…but it's hard to measure such small volumes). I prefer to use a HEPES or MOPS buffered medium to ensure pH stability. It's hard to say how detrimental this shift may be. However, it is something that can be easily avoided using buffered media. A colleague commented "Before moving to using HEPES media for all embryo transfers, we had a two minute window to load the catheter and deposit embryos into the uterus. 16 years of data using HEPES as the transfer medium has proven the 'old' system is not required". There are no published data to suggest that HEPES or MOPS buffer is detrimental to the embryo during this brief exposure or to outcomes following transfer.

Q: We use phosphate for our freezing media and it works very well.
Phosphate has been used for cryopreservation in the past and it can work. However, the data is pretty clear as to the detrimental impact of elevated phosphate on embryo development. The short exposure period and low temperatures involved with cryopreservation may help mitigate some of the metabolic impact of the phosphate.

Q: Why does pHo change rapidly after removing from the incubator but take >8 hrs to re-equilibrate?
Great question. Joe Conaghan presented some data on this (Steel & Conaghan Fert & Stert 2008). He showed that pH, even with oil overlay, could climb above pH 7.4 after around 5 minutes. However, it took ~64 to 148minutes to re-equilibrate, depending on media/oil volume. So, it doesn't take >8hrs, as the pH doesn't have as far to shift to re-equilibrate (the 8hrs mentioned in the slides was for initial equilibration out of media out of the bottle…which has farther to go to reach equilibrium). Still, it does take longer. This indicates it takes longer for CO2 gas to diffuse from the incubator atmosphere, though the oil, into the culture medium, and then to reach equilibrium with bicarbonate that it does for the CO2 to escape from the culture medium under the oil….which results in the equilibrium shifts rapidly back toward the bicarbonate side of the equation. This may also be due in part to the humidified air of the incubator that can act a sink and could slow CO2 diffusion compare to room atmosphere.

Q: How do you clean a probe after testing media with protein?
I simply rinse my probe with deionized water and then store in my storage solution. However, if your probe is becoming sluggish due to a clogged junction with protein, the probe manual should detail cleaning instructions. This usually entails soaking for various periods in a strong acid and then a strong

base solutions to clear the junction. You should then replace the internal solution of the electrode. We also routinely replace our probe at set times to ensure optimal functioning and rapid readings.

Q: When measure CO2 level with Fyrite, the CO2 level was 6% when O2 level was set for 5%. However, when O2 level change to 20% and CO2 set was not change, CO2 level increased dramatically to more than 7% with Fyrite. Why?

This could be due to chemical or mechanical interferences with the Fyrite. As recommended by the manufacturer, retesting the new environment 3-4 times may help correct any interferences. Regardless, if your pH is still in range, you should be fine as far as your growth conditions. Fyrite readings can vary quite a bit. Use of an alternate CO2 measuring device, using and IR sensor, could be used if you wanted to explore the phenomenon further. Of note, Fyrite is caustic and many labs no longer want the chemical in the laboratory for fear of possible contamination concerns, etc.

Q: Which electrode was more reliable in your experience?

A glass, double-junction, KCl filled electrode has always worked well for me. This is what many companies recommend for our applications. Howeverother types of probes can work as well. I have a semi micro Orion probe from Thermo , as well as another micro electrode from Denver Instruments. Colleagues use electrodes from Cole Palmer and Oakton with good results. Purchasing the probe that is compatible with your particular pH meter is important (correct fittings, etc).

Q: Does reduced O2 alter pH?

Lowering the O2 level of your incubator should not affect the pH of your media. However, it could impact the CO2 readings of a Fyrite or other device device. This is why measuring pH is recommended. You can then simply adjust your incubator CO2 level until your pH is in your desired range.

Q: Did you know of pH changes during retrieval dealing with potential temperature changes during follicle aspiration?

I do not. But this is a good point. pH can change with temperature. Thus, prior phenomena attributed to temperature changes, could be also due, in part, to pH changes. We place a small amount of HEPES or MOPS buffered media into our oocyte collection tubes prior to retrieval and keep these warmed. Though we work as rapidly as possible, there is likely a brief cooling period as the fluid/oocyte travels through the aspiration tubing prior to entering the warmed media of the collection tube. . This helps us ensure we are maintaining pH to the best of our ability. (Of note, the oocytes are somewhat protected by their surrounding cumulus cells)

Q: I had difficulty with the pH meter when using the ATC, so we now use warmed stds to cal and this is working for us, can you see any potential issues with this procedure?

This is likely fine. The elevated temperature will result in a slightly lower pH. You can see this with your standards. When you pH your 7.0 standard at room temp, it will read 7.0. When pHing at 37°C, it will read around 6.98. The pH difference is relatively minor, especially when one considers probe

variance is around 0.02 pH units. However, the further you move away from pH 7.0, the more impact you will see due to temperature. (When you use your 10.0 standard at room temp, it read around 10.0. However, at 37°C it will read around 9.89 or so depending on the buffer solution). So, using an ATC probe, or setting your pH meter to 37°C is going to give you the best accuracy.

Q: How do you measure the pHo of media overlayed with oil?

You can do this using one of the commercially available devices used to measure pH in small volumes within the incubator. I believe RI Instruments and MTG sell such devices. However, I wouldn't recommend placing a pH probe though a layer of oil to try to measure the pH of the media beneath, as this could damage the pH electrode. We simply aliquot ~2ml of media into a small test tube with no oil overlay and place this in the incubator. We then measure the pH in this tube as described in the slides.

Q: In time lapse equipment we have had problem measuring ph, do you know how to measure ph in them?

This will be difficult with the current designs of some of the real-time systems that are enclosed in their own incubator. If the real-time system had a small port to permit the placing of a small test tube of media, this would be one way you could test the pH. A small tube could be placed here overnight to equilibrate, and then removed to verify the pH with a bench top pH meter. Otherwise, there really is no good way to measure the pH for some of these systems, as the volumes are too small and the system doesn't permit use of large dishes or tubes.

If you could place a 4-well dish or larger petri dish in the incubator unit, there are devices that can measure pH inside the incubator. However, I'm not sure either of the devices would fit inside the real-time unit.

Another approach might be to remove one of the culture slides/dishes quickly and try to aliquot some media and test the pH with a blood gas analyzer. There are handheld blood gas analyzers that can be purchased. They do require a cartridge for each use, so this can get expensive. This may be the best route for the time being.

Of note, the small microwells used in the static time lapse systems bring up the issue of localized pH around the embryo. It would be nice to have a way to measuring localized pH in small volumes around the cell, as has been done using microelectrodes for other media components (calcium, etc).

Q: What combination do you use for the premixed gas?

We don't use premixed gas. However, the mixture should be that which gives you your desired pH range…meaning your gas supplier should adjust the CO2 concentration based on your request. You should test the pH of your particular media/protein with your current gas mixture. If the pH is in range, you're all set. If it's low, ask you supplier to raise the CO2 concentration slightly. Trial and error is needed to determine what mixture is needed for you lab/media/protein. If you're using 5% O2, the balanced Nitrogen concentration will then be adjusted accordingly based on the final concentration of CO2. Of note, once you choose a mixture you cannot change it easily, unless you have several mixes

on hand. You can also talk to the manufacturers and ask for smaller volumes of different mixes to establish your mix requirements. It's also important to determine pH for every cylinder to ensure it is mixed correctly.

Q: What pH meter you use?
I have a couple different pH meters. I have an Orion 3 star model from Thermo and another basic model from ISC Bioexpress. I've also used models from Beckman Coulter in the past. All work very well.

Q: We have a dry incubator at the moment and we face problems measuring the pH. Do you have any suggestion how we can overcome this problem?
There shouldn't really be a problem with measuring the pH of a dry incubator. The humidity isn't going to affect the pH of the culture media. You should still be able to place a tube of media inside and then take it out to measure with a pH meter. (Though the media i could evaporate over time if left in the incubator too long…but you should still be able to measure the pH). Now, if your incubator uses a TC CO2 sensor, this can be impacted by humidity. So, this could possibly impact your CO2 readings. However, you should still be able to adjust your CO2 to give you your desired pH. You could always use a device to measure pH inside the incubator as well. These permit media to be placed in dishes and covered with oil. This would presumably address evaporation concerns if that was your issue. These devices are available from MTG and RI Instruments. The accuracy of any new device should be validated prior to clinical implementation and adjusting of your incubator gas levels.