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Quality Control in labware manufacturing:

Testing methodologies and stringency parameters

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Labware and reproductive cells

During in vitro fertilization (IVF) treatments, specific culture conditions are required for providing a physiological environment to gametes and embryos. On top of typical incubation parameters including temperature, pH, osmolality and oxygen tension, the quality of the labware used during assisted reproduction technology procedures is pivotal for successful treatments. More specifically, device sterility and lack of gamete and embryo toxicity are necessary for minimizing the impact of external factors on gamete viability and embryo development.

Why testing labware?

At first look, the disposable devices used in the IVF laboratory may appear similar. However, aside from variations in shapes and logos, substantial differences in the quality of plastics, manufacturing, packaging, and sterilization methods across various brands and product lines can affect significantly the overall performance of a product. Ensuring that products meet sterility and embryo toxicity standards through rigorous quality checks before market release is of utmost importance. Labware testing provides reassurance that plastics and derivates of sterilization and packaging processes have minimal impact on gametes' physiology and embryo development processes.

Biological sterility

The presence of bacteria, fungi, or viruses in the labware can severely affect gametes' viability and embryos' developmental competence, as well as compromise the incubation system. To radically minimize the risk of infections, medical devices require sterility assurance levels (SAL) of 10-6, which means that the chances of a pathogen (i.e., bacterium or virus) to survive the sterilization process is one in a million. To achieve this level of sterility, after manufacturing and packaging, the device is exposed to gamma radiations. Nonetheless, toxins affecting gametes and embryos' physiology can be released by certain microorganisms following their destruction (i.e., lipopolysaccharides (LPS) from gram negative bacteria). Labware is tested for the presence of these compounds by performing Limulus Amoebocyte Lysate (LAL) test.

Chemical toxicity

Some of the raw materials employed in manufacturing of plastic labware can generate a toxic environment for the biological specimen cultured within and have a direct effect on gamete and embryo developmental processes, reducing their viability or impairing their biological mechanisms. This may translate in suboptimal development or in atresia of the gamete or developing organism, eventually reducing the overall performance of the IVF laboratory as well as cumulative clinical outcomes. Toxic effects derived from the material used for manufacturing are tested by performing Sperm Motility Assay (SMA) test and Mouse Embryo Assay (MEA) test.

Quality control tests

In general, three main tests are used to assess the sterility and toxicity of products: the Sperm Motility Assay (SMA), Limulus Amoebocyte Lysate (LAL) test, and Mouse Embryo Assay (MEA). However, despite falling under the same description, significant variations in the way these tests are conducted and their stringency criteria can be found across different brands and product lines. As these methodologies are not standardized yet, it becomes the responsibility of the manufacturer to disclose the criteria used for each test.

Sperm Motility Assay test - SMA

The SMA test checks whether the exposure to the tested product influences the viability of the biological specimen. Since the first IVF attempts in the late '70s, sperm motility has been regarded as a good indicator for assessing the impact of products' characteristics on the overall physiological state of cells. To assess the toxicity level of the product, the percentage of sperm cells displaying a

forward progression is used as the test's outcome measure. To minimize samples' intrinsic differences and standardize the testing procedure, only fresh sperm samples within the normal range for concentration, progressive motility, and morphology (>15 M/mL, >32%, and >4%, respectively) according to the World Health Organization (WHO, 2010) are enrolled in the test (**Figure 1**). Each sample is processed through gradient separation and subsequently washed in specifically formulated solutions for gamete processing. To ensure that the quality of the sample is maintained throughout the preparation procedure, the processed sample is assessed for concentration and percentage of sperm cells with forward progression (acceptance >90% motility). The sample is then split into two experimental groups: half of the sample is exposed to the tested product (Test group), the other half is maintained in a reference product of similar type (Control group). The samples are exposed to the product (and reference product) for 8 hours at 37˚C. The percentage of sperm cells with forward progression is then assessed in both the Test and Control groups. Sperm motility can be assessed automatically by computerized counters or manually by a certified operator. Technical and biological replicates can be increased to validate test outcome.

The ratio between sperm motility rate from the Test and Control groups determines the Sperm Motility Index (SMI). The larger the difference between the motility rate of the Test and Control groups, the lower the SMI. Motility of the group can be assessed at different time intervals (e.g., 4hrs, 8hrs, and 24hrs), with longer intervals increasing stringency of the test. Generally, the acceptance threshold for a product lot is typically set at SMI >0.75 at 8hrs incubation (**Table 1**).

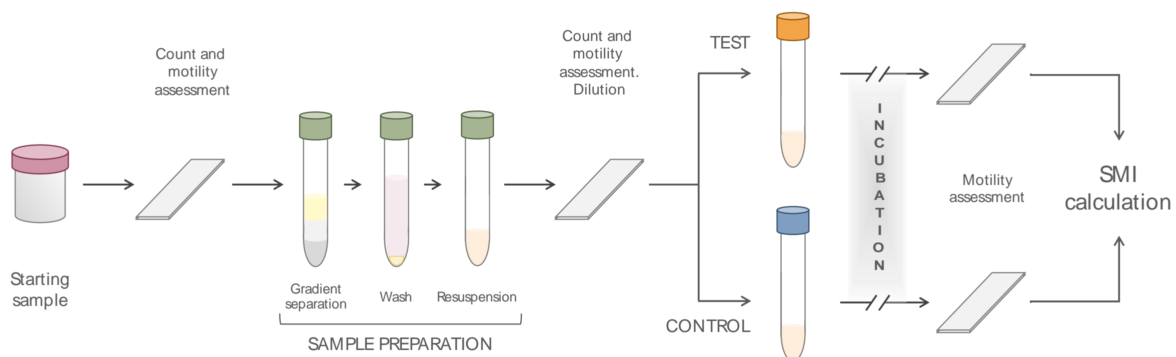

*Figure 1 - **Sample processing for SMA testing** – Only good quality semen samples are enrolled for SMA testing. After conventional gradient separation, samples are reassessed and split into test and control at a standard concentration (e.g., 10M/mL). The sample is cultured in the tested product and, in parallel, in a control product. After 8hrs of incubation at 37°C, sperm motility is assessed in both samples. The ratio between the sperm motility rate of the test and the control provides the Sperm Motility Index (SMI).*

BIRR monitors sperm motility and SMI up to 24hrs incubation intervals with an acceptance threshold of SMI >0.75 at 8hrs. Despite SMI values at 8hrs, if SMI drops below the 0.75 threshold at the 24hrs assessment, then the test is repeated to check for results consistency. Should the outcome at 24hrs remain suboptimal, investigation on the product lot is started.

***Table 1 - Examples of SMA outcomes** – SMI < 0.85 at 8hrs incubation result in product unsuitability for IVF processes.*

	Pre exposure motility	Sample	Post exposure motility	SMI	OUTCOME
Example 1	80%	Test	55%	**0.73** (55 / 75)	REJECTED
		Control	75%		
Example 2	90%	Test	75%	**0.93** (75 / 80)	ACCEPTED
		Control	80%		

Limulus Amoebocyte Lysate test – LAL

Labware is routinely sterilized by gamma irradiation. This process destroys all possible organisms present on the product or within its material. However, inner components (i.e., liposaccharides, LPS) of the extinguished organisms may still be active and interfere with gametes and embryos' viability and developmental competence. Presence of endotoxins is routinely assessed through Limulus Amoebocyte Lysate (LAL) test. LAL test can be performed following three methodologies: the gel-clot technique, where the presence of the endotoxin produces clotting of the lysate; the turbidimetric technique, where the presence of the endotoxin is detected through the appearance of turbidity in the lysate solution; and the chromogenic technique where the interaction between the endotoxin and the lysate causes the appearance of color in the solution. The latter of these techniques is the one conventionally used for testing labware. A pre-warmed solution containing LAL and purified endotoxin-free water is exposed to the product for at least 1hr. This process can be carried out in different ways: 1) the device is filled with the testing solution, or 2) the device is fully submerged in it (**Figure 2**). The first approach requires less rinsing solution however, it tests only the inner surface of the device. The second approach requires more rinsing volume, but it also provides a more comprehensive assessment as it tests the presence of endotoxins on the whole device, including its exterior surfaces. After 1hr exposure to the device (whether through filling or submersion), chromogenic emissions of the testing solution are measured by a spectrophotometer.

In parallel, control solutions at increasing concentrations of endotoxin units (EU) per mL (e.g., 0.05 EU/mL, 0.5 EU/mL, and 5 EU/mL) are also measured to generate standard calibration curves

subsequently employed to determine the concentration of endotoxins in the tested solution. The total number of endotoxin units is calculated by multiplying EU concentration (EU/mL) by the volume of rinsing solution (mL). The number of endotoxin units per device is then extracted by dividing the total number of endotoxins detected by the number of devices exposed to the testing solution. Golden standard analysis has a detection limit of 0.005 EU/mL, therefore minimum detection thresholds per unit depend on the rinsing volume employed for testing. If the submersion method is employed, more rinsing volume of testing solution is required and, maintaining the same detection limit of 0.005 EU/mL, minimum detection threshold is generally higher. For example, if 15mL (i.e., filling method) and 300mL (i.e., submersion method) of testing solution are used to assess the same device in triplicates (45mL and 900mL, respectively), then the minimum detection thresholds would be <0.075 EU/device (45mL x 0.005 EU/mL / 3 devices) and <1.5 EU/device (900mL x 0.005 EU/mL / 3 devices), respectively. Although the devices are tested with the same analytical accuracy, the minimum detection level offered by the submersion approach is higher, despite providing a more comprehensive assessment of endotoxins presence as it tests both inner and outer surfaces of the device. Under current medical devices regulation, up to 20 endotoxins per tested unit are allowed.

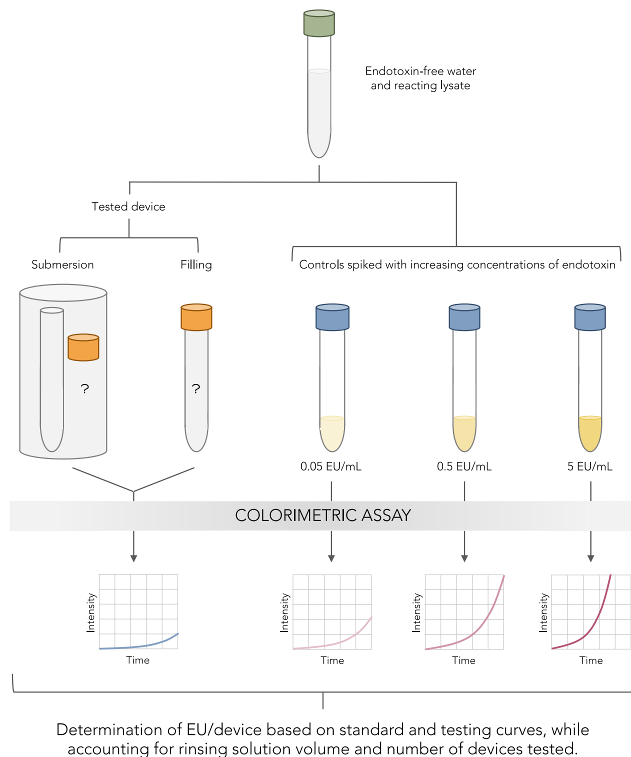

Figure 2 - Sample measurement for LAL testing *– Endotoxin-free water is used to rinse the tested product for at least 1hr. In parallel, controls are spiked with endotoxin at increasing concentration. Comparison of measuring curves obtained through a colorimetric assay allow endotoxin quantification in the tested product.*

Current LAL method employed by BIRR has a detection limit of 0.005 endotoxins per mL tested. Overall minimum detection limits (EU/device) for BIRR products depend on the volume of rinsing solution employed for testing, which is in turn dependent on the size of the device. Being BIRR committed to provide the highest standards of quality assurance, LAL testing protocols are tailored for the product's specific characteristics, to achieve the highest analytical sensitivity while maintaining a comprehensive indication on the device's toxic impact.

Mouse Embryo Assay test - MEA

MEA testing is employed to detect subtle toxicity levels in solutions, oil and disposable devices. This is performed by culturing 1-cell mouse embryos in media that has been exposed to the tested product. Embryo development is monitored until the blastocyst stage (96hrs of culture). Blastocyst formation rate is the main outcome measure of MEA testing. Due to the high sensitivity of embryo developmental processes to exogenous stressors, extended culture in suboptimal conditions (e.g., presence of toxic chemicals) results in reduced embryo viability and developmental rates. Although the concept of MEA is well defined, stringency conditions may vary across different protocols and testing laboratories. Stringency parameters include the time the media is exposed to the tested product (e.g., 2hrs vs. continuous), the strain and number of mouse embryos employed in the test (e.g., sensitive vs. resistant strains), presence/absence of antibiotic or protein supplements in media, and the application of additional qualitative and quantitative outcome measures such as blastocyst quality and number of cells forming the embryo.

Standard MEA

The Standard MEA involves the exposure of culture media to the tested labware for 2 hours (**Figure 3**). This media is then used for incubation of 1-cell mouse embryos until 120hrs of development in a reference dish (Test group). In parallel, 1-cell mouse embryos are cultured in the same type of reference culture dish using media that was not exposed to the testing labware (Control group). For each group, embryo cleavage is assessed after 24hrs and blastocyst formation is assessed after 96hrs of incubation (Day 5 of development).

Extended MEA (MEA+)

The Extended MEA (MEA+) involves continued exposure of 1-cell mouse embryos to the tested labware throughout a 96hrs culture period (Test group) (**Figure 3**). In parallel, 1-cell mouse embryos are cultured in a reference culture dish using media that was not exposed to the testing labware (Control group).

For each group, embryo cleavage is assessed after 24hrs and blastocyst formation is assessed after 96hrs of incubation (Day 5 development). Differently from Standard MEA, Extended MEA involves a longer exposure and direct contact between the embryo and the tested product. This approach increases test stringency, thus increasing the likelihood of detecting any potential labware effects on embryo development. Under current medical devices regulation, a product lot is accepted when MEA testing outcomes (either Standard MEA or Extended MEA) show blastocyst formation rate >80% in the Test group. The presence of blastocyst formation rates <80% in the Control group may

render the test invalid, and a new testing procedure is required. If suboptimal rates are confirmed, the product lot is rejected.

To monitor the effect of the labware to embryo developmental processes, additional measurements can be performed. These include morphological assessment of the blastocysts developed during the test as well as cell counting. Although the measurement and thresholds of these parameters are not set under current medical devices regulations, the application of such additional outcome measures can monitor labware performance with increased accuracy. BIRR employs MEA+, including cell counting and blastocyst morphology evaluation, for all products involving extended specimen exposure (i.e., dishes).

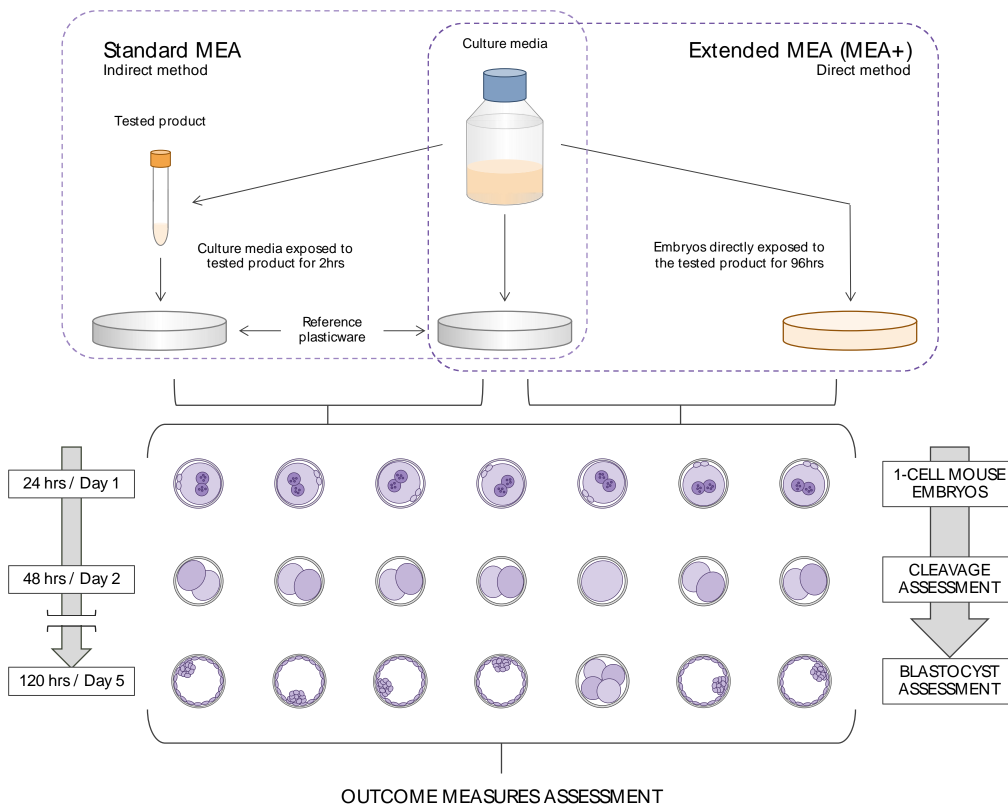

Figure 3 - Sample processing and assessment for Standard and Extended MEA test *– In Standard MEA, 1-cell mouse embryos are cultured for 96hrs in media that was previously exposed to the tested product for a minimum of 2hrs. In Extended MEA (MEA+), 1-cell mouse embryos are cultured for 96hrs in direct exposure to the tested product. Embryological outcomes (cleavage and blastocyst formation rates) are assessed similarly across the two methodologies. Extended MEA can include additional outcome measure such as blastocyst morphology and total cell count. ICM = Inner cell mass; TE = Trophectoderm.*

BIRR approach to Quality Control and Assurance

Being one of the ART disposable device manufacturers to feature a Medical Device Registration for its products (i.e., CE Class IIa sterile Medical Devices in accordance with 93/42 EEC Directive), BIRR pays enormous attention to the quality of its products, both from an engineering and a safety perspective. For its commitment to providing excellent labware for use in IVF laboratories, BIRR employs high stringency parameters during QC validation of its devices. In fact, for SMA testing, BIRR adopts an additional 24hrs threshold, on top of the typically employed at 8hrs. Similarly, for LAL testing, BIRR commits to an acceptance threshold much lower than the 20 EU/device recommended by the regulator. Although this threshold varies according to the size of the product assessed (see LAL section), BIRR's approach offers a sensitive, reliable and comprehensive evaluation of endotoxin levels for each of its products. For MEA testing, BIRR adopts a measured approach, where Standard MEA is performed on the labware that in clinical conditions has a short contact time of <24 hours with gametes or embryos (e.g., tubes, pipettes), while Extended MEA (MEA+) is performed on labware that has a contact time of >24 hours. Although current medical devices regulation only requires Standard MEA acceptance, by conducting MEA+ on all its dishes, BIRR ensures the highest quality of its products, guaranteeing a safe environment for extended embryo culture. Moreover, MEA+ testing also includes the measurement of additional embryological parameters such as blastocyst grade and cell counting.

To facilitate IVF laboratories' QC/QA activities, results of quality tests for each product LOT are available digitally.

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