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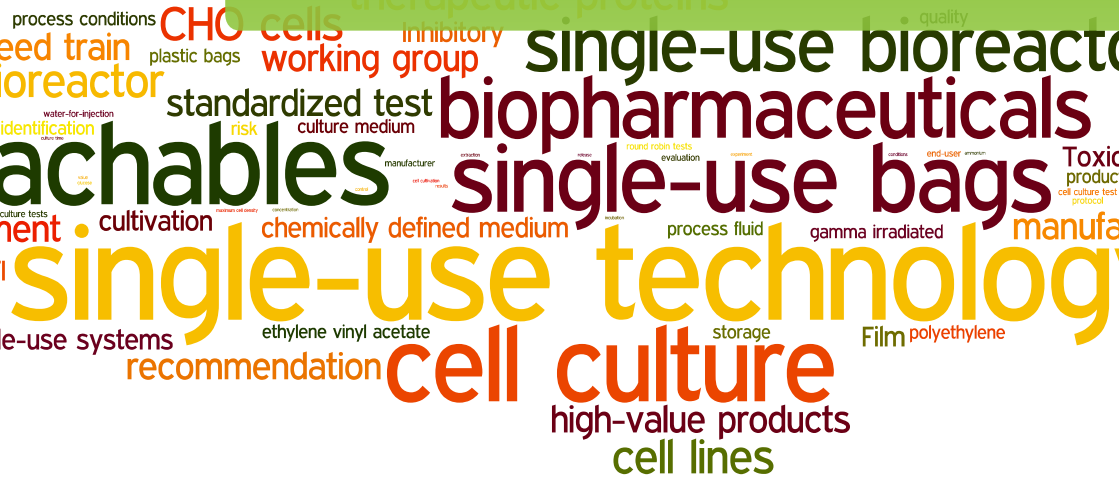
Recommendation for Leachables Studies

Standardized cell culture test for the early identification of critical films



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Recommendation for leachables studies

Standardized cell culture test for the early identification of critical films for CHO cell lines in chemically defined culture media

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Today, single-use technology is used routinely in biopharmaceutical development and manufacturing processes. In addition to single-use filters and storage bags, single-use cell culture bags are well established for use in either wave-mixed or stirred bioreactors in seed trains. Even small and medium volume, high-value, protein-based recombinant products, such as therapeutic proteins and monoclonal antibodies, are now increasingly produced in stirred single-use bioreactors **(1)**. Over the last ten years the greatly increased titer of CHO cell lines, the majority of which are cultivated in chemically defined media **(3-4)**, and the advantages of single-use technology **(2-3)**, have significantly contributed to this trend.

Despite these positive developments, the available single-use systems still have limitations. One of the most cited disadvantages is the risk for release of potentially toxic or inhibitory substances, so called leachables from the plastic bags. The bags are typically made of multi-layer thermoplastic films, incorporating a polyethylene or ethylene vinyl acetate contact layer **(5-6)**, and are normally gamma irradiated. Leachables are chemical compounds that originate from the plastic materials used to fabricate the bags or are generated during gamma irradiation and storage. When in contact with the process fluid (culture medium or culture broth with cells) these compounds might be released from the film. The release of cytotoxic leachables under process conditions is particularly undesirable because these chemicals adversely affect growth and viability of the production cells and, as a consequence, the product titer and potentially even quality **(7)**. As described by Hammond et al. in a paper published in March 2013 **(8)**, the use of a trisarylphosphite processing stabilizer (which could be supplied under the trade name Irgafos 168 as an antioxidant additive) used for production of polyethylene bags, dependent on its concentration, might lead to the formation of bis(2,4-di-tert-butylphenyl)phosphate (bDtBPP). The latter compound reduces the mitochondrial membrane potential of CHO cell lines and inhibits their growth even at low concentrations (0.1 mg L^{-1}). Like Wood et al. **(7)** and Horvath et al. **(9)**, Hammond et al. recommend the use of appropriate screening with mammalian cell cultures, in addition to established leachable studies. Such screening should enable identification of potentially unsatisfactory films early, control and improvement of single-use bags, and facilitate their use.

RECOMMENDATION FOR LEACHABLES STUDIES

Currently the users of single-use cultivation bags implement such cell culture tests themselves using their own cell lines, culture media, and protocols. However, this approach makes comparison of different films difficult and precludes specific recommendations for their use. There is no standardized cell culture test available, ideally based on using one or several of the more sensitive commercial cell lines and chemically defined culture media that satisfies the requirements of the majority of end-users. The availability of such a standardized test would support both bag manufacturers in developing and qualifying new bag films, and end-users in deciding whether to utilize these bags in their processes. To meet this need, a sub-team of the DECHEMA temporary working group "Single-Use Technology in Biopharmaceutical Manufacturing", completed two round robin tests. The objective was to develop a standardized cell culture test that would enable the early identification of non-satisfactory films for cultivation of CHO cell lines in chemically defined culture media.

ROUND ROBIN EXPERIMENT 1

In the first round robin experiment, nine films from seven bag suppliers were evaluated by five users using six recombinant CHO cell lines and six culture media. Each user completed his own cell culture test using shake flasks. The respective culture media were prepared with water-for-injection (WFI) that had been incubated in bags (made of the different film materials) by the Zurich University of Applied Sciences (ZHAW). As is described in detail in (10), WFI incubated in borosilicate bottles was used as control and prepared at the same time as the incubation of the bags took place. The water extraction of the bags was performed for 7 days, at 37°C, static, at 50% fill volume, and protected from light. After extraction, the WFI was transferred to borosilicate bottles.

The final evaluation of the first round robin test was based on a questionnaire completed by each user in which the control data was normalized at 100%. The following variables were evaluated as a function of the culture time: maximum cell density, pH value, cell size, and glucose, glutamine, lactate and ammonium concentrations. All users unanimously reported that seven of the nine evaluated films resulted in data identical to the control for all cell lines and all assessment criteria. However, for two films, a deviation from the control was reported in at least two assessment criteria for one or more cell lines. The release of leachables is believed to be the cause of the deviation from control in both cases.

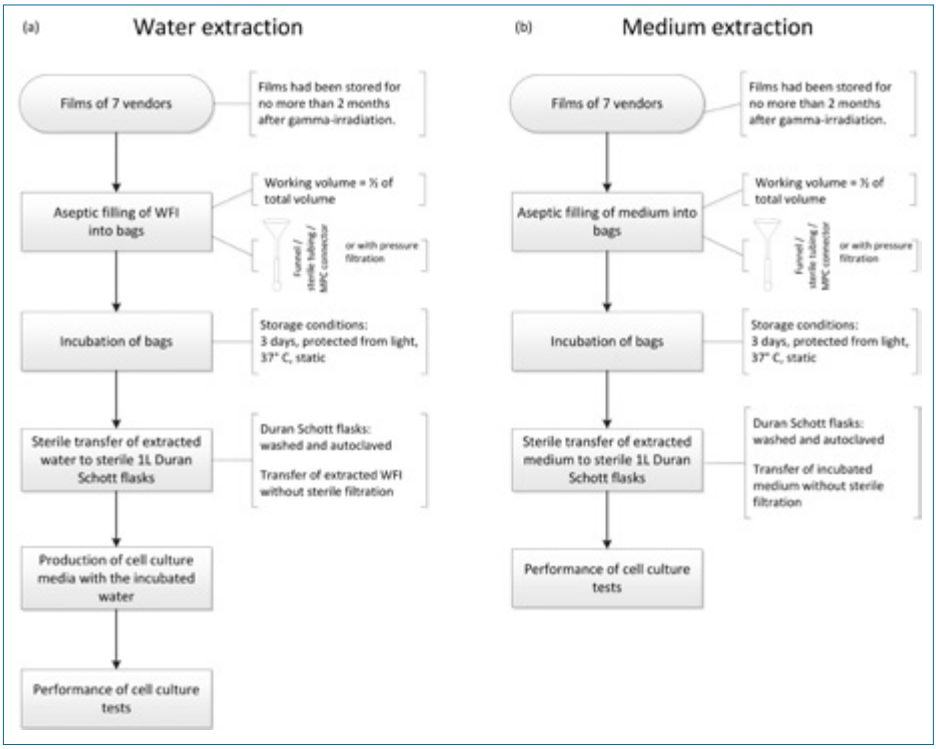
ROUND ROBIN EXPERIMENT 2

The second round robin experiment, which incorporated eleven films obtained from seven bag suppliers (and included some different films to those used in the above experiment), was completed by four users utilizing eight CHO cell lines and seven chemically defined culture media. A negative control was provided for this round robin experiment by one bag supplier for one of the films evaluated. One of the CHO cell lines used was a non-engineered, commercially available cell line (CHO-easyC supplied by Cell Culture Technologies, Switzerland). The other cell lines were genetically modified. The ZHAW used a recombinant model cell line (CHO XM 111-10), which secreted SEAP, the secreted alkaline phosphatase of the placenta.

This cell line was established in the 1990s by the group of Prof. Dr. Martin Fussenegger (ETH Zurich) **(11)** and can be obtained from the Culture Collection, Switzerland (CCOS 837).

In the second round robin experiment, bags made out of the test films and the corresponding evaluation forms were sent directly to the users by the ZHAW, rather than the extracted WFI in glass bottles. The users then completed their cell cultivation experiments, with cell culture medium incubated in the test bags and, in selected cases (three testers with four cell lines) with medium prepared from WFI incubated in the bag. In parallel to the water- and media-extraction in the bags, WFI, and respectively medium, were incubated for three days in borosilicate glass bottles as controls. **Figure 1** provides an overview of the experimental set-up. To further improve the comparability of the results and enhance the value of the collaborative study, additional boundary conditions were specified.

Figure 1. Flow charts outlining round robin experiment 2 for (a) WFI extraction and (b) media extraction



RECOMMENDATION FOR LEACHABLES STUDIES

All bags made of the different films and included in the experiment had been stored for no more than two months after gamma-irradiation, unlike two of the films evaluated in round robin experiment 1. Furthermore, the culture medium was filtered by all users using a 0.22 µm PVDF membrane filter (Gamma Compatible Millipak 20 filter unit 0.22 µm ¼ in. HB / HB w / bell sterile) from Merck Millipore. In addition, the following criteria were applied to analyze the data:

» no deviation or negligible deviation (k), if one of the test cell lines exhibited a negative deviation of ›10% from the control for no more than one of the criteria

Table 1: Results of the cell culture experiments with various cell line and media combinations (a) with extracted medium and (b) with extracted WFI

(a) Extracted medium

Film	Max. cell density									Viabiliby									Cell diameter			
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V1	V2	V3	V4	V5	V6	V7	V8	V9	V1	V2	V3	V4
1	46%	+	20%	16%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	64%	31%	25%	21%	12%	25%	38%	+	61%	+	+	+	+	+	+	+	–	–	+	+	+	+
3	57%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	20%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	52%	21%	+	12%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	39%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	33%	+	25%	17%	+	+	+	+	38%	+	+	+	+	+	+	+	+	–	+	+	+	+
8 (NK)	68%	42%	22%	26%	50%	55%	95%	12%	80%	–	+	+	+	+	+	–	–	–	+	+	+	+
9	20%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	50%	33%	21%	19%	+	+	+	+	57%	+	+	+	+	+	+	+	+	–	+	+	+	+
11	73%	+	23%	17%	13%	+	+	+	47%	–	+	+	+	+	+	+	+	–	+	+	+	+

(b) Extracted WFI

Film	Max. cell density				Viabiliby				Cell diameter				Metabolism				pH				
	V3	V4	V5	V7	V3	V4	V5	V7	V3	V4	V5	V7	V3	V4	V5	V7	V3	V4	V5	V7	
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
2	31%	+	12%	81%	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	–	s
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
4	+	+	12%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
5	21%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
6	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	k
7	+	+	+	43%	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	+	s
8 (NK)	41%	+	55%	89%	+	+	+	–	+	+	+	+	+	+	–	–	+	+	+	–	s
9	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	k
10	33%	+	16%	75%	+	+	+	–	+	+	+	–	+	+	+	–	+	+	+	–	s
11	+	+	12%	31%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	m

» moderate deviation (m), if one of the test cell lines exhibited a negative deviation of >10% from the control for two or three criteria, or several of the test cell lines exhibited a negative deviation of >10% from the control for one, two or three criteria
» strong deviation (s), if one or more of the test cell lines exhibited a deviation of >10% from the control for four or five criteria.

The results of the cell culture tests are summarized in **Table 1a** (extracted medium) and **Table 1b** (extracted WFI). The cell lines used in each set of experiments are summarized in **Table 2**.

Cell diameter					Metabolism									pH									
V5	V6	V7	V8	V9	V1	V2	V3	V4	V5	V6	V7	V8	V9	V1	V2	V3	V4	V5	V6	V7	V8	V9	
+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	m
+	+	+	–	+	–	–	–	–	+	+	–	+	–	+	+	+	+	+	+	–	+	–	s
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	m
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
+	+	+	+	–	+	+	–	–	+	+	+	+	–	+	+	+	+	+	+	–	+	–	s
+	+	–	–	+	–	–	–	–	–	+	–	–	–	+	+	+	+	+	+	–	–	–	s
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	k
+	+	+	+	+	+	–	–	–	+	+	+	+	–	+	+	+	+	+	+	–	+	–	s
+	+	+	+	–	–	+	–	–	+	+	+	+	–	+	+	+	+	+	+	+	+	–	s

Table 2: Overview of the cell types used in each set of experiments

Experiment	V1	V2	V3	V4	V5	V6	V7	V8	V9
Cell type	CHO DG44	CHO DG44	CHO K1	CHO K1	CHO K1	CHO K1	CHO K1	CHO DG44	CHO K1

(+) no deviation, (%) deviation from the control, (-) deviation occurred

(k) no or negligible deviation, (m) moderate deviation, (s) strong deviation

NK negative control

The difference between the results obtained with incubated medium and those obtained with medium produced from incubated WFI was not unexpected. One potential reason for the difference might be that, in addition to the presence of leachables, some medium components such as cholesterol and fatty acids (linoleic acid, oleic acid) interact with the hydrophobic film (the contact layer), resulting in limited cell growth. This phenomenon has been observed by several research groups for serum-free, protein-free and chemically defined culture media, in which serum albumin is not present as a carrier molecule **(12-13)**.

Analysis of the data from the second set of round robin experiments indicated that four of the eleven films showed little or no deviation from the results obtained with the control following medium extraction. Both the presence of leachables and the interaction of medium components with the films can be ruled out for films 3, 4, 6 and 9 for the cell line and medium combinations that were evaluated (assuming that deviations from the maximum viable cell density of ≤ 10 % are rated "+" and that the user in experiment V1 assessed the maximum viable cell densities achieved as acceptable, despite deviations greater than 10%). The results of the experiments based on water extraction indicate that films 1 and 5 exhibited negligible or no contamination with cytotoxic leachables for the tested cell line and medium combinations, as it has to be assumed that WFI has a higher potential to extract cytotoxic substances than medium. In these instances, interactions between the films and media components are more likely to be the root cause of the minor variation when compared to results based on media extraction.

Film 8 was confirmed as a negative control in all cell culture tests using extracted medium. However, when using incubated WFI to prepare medium only three of the cell lines tested showed impaired growth (experiments V3, V5 and V7). The cell line used in experiment V4 did not show any apparent deviations from control when using incubated WFI to prepare cell culture media for any of the tested films. The cell line used in experiment V4 is CHO-easyC, which was therefore deemed to be insufficiently sensitive to pick up any negative effect of a film on cell culture behavior. In contrast, films 2 and 10 exhibited strong deviations for all cell line and media combinations evaluated in experiments V3, V5 and V7 when using incubated WFI to prepare medium. The results of the tested cell line and media combinations in experiment V7 and film 7 are less ambiguous (tests based on media prepared from extracted WFI, **Table 1b**).

CONCLUSIONS

Based on the results of the media and WFI extractions for cell line and media combinations tested in experiments V3, V5 and V7 , three films (2, 8 and 10) were identified, that release substances inhibiting cell growth. Thus, these three test systems consisting of a recombinant CHO cell line and a serum-free medium provide a means of detecting leachables in a cell culture test. Cell line and media combinations used in V5 and V7 are proprietary to the participating user company, whereas experiment V3 is based on the freely available recombinant model cell line CHO XM 111-10.

The culture medium used in experiment V3 comprises approximately 50 components. It is based on the FMX-8 basal medium with certain modifications, the original composition of the FMX-8 medium was published in 1993 **(14-15)**. It contains linoleic acid (12), a potential interaction with film 1 may explain the 20% lower maximum cell count in experiments with extracted medium.

The authors therefore recommend using the CHO XM 111-10 cell line in combination with ChoMaster® HP-1 medium (Cell Culture Technologies, Switzerland) and the procedure outlined in Annex 1 for the early identification of films that are critical for CHO cell culture in serum- and protein-free, or chemically defined culture media.

ACKNOWLEDGEMENTS

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ANNEX 1

Titel	**Operating procedure for a standardized cell culture test to identify critical bag films for use with CHO cell lines**
Purpose	This Standard Operating Procedure describes a cell culture test designed to identify critical films based on using the CHO XM 111-10 cell line and chemically defined minimal medium. This cell culture test should be implemented in addition to appropriate leachable studies.
Scope	Cell culture laboratories
Status	–
Accompanying documents	Instructions for use covering ChoMaster® HP-1 medium und FMX-8 medium produced by the manufacturer of the media, Cell Culture Technologies, Switzerland

CONTENTS

1 DEFINITIONS AND ABBREVIATIONS

Abbreviation	Definition
disp.	disposable
T-75	T-Flask 75 cm^2
WFI	Water for Injection

2 MATERIALS

Table 1: Materials

Name	Manufacturer
Bioprofile 100 plus Analyzer	Nova BioMedical, USA
Cedex HiRes	Roche Diagnostics AG, Switzerland
CHO XM 111-10 (CCOS 837)	Prof. Dr. Martin Fussenegger ETHZ, Switzerland (provided by the Culture Collection of Switzerland)
Duran Schott flask	Duran Group GmbH, Germany
Duran glass funnel	Duran Group GmbH, Germany
Incubator	Brouwer AG, Switzerland
Culture medium ChoMaster® HP-1, 2 L – 20 L kit	Cell Culture Technologies GmbH, Switzerland
Culture medium FMX-8	Cell Culture Technologies GmbH, Switzerland
Membrane filter Gamma Compatible Millipak-20 Filter Unit 0.22 µm ¼ in. HB/HB w/bell sterile	Merck Millipore, USA
Omnifix disposable syringe Luerlock 10 ml	B. Braun Melsungen AG, Germany
Pluronic F68	Sigma Aldrich, Switzerland
pH Meter, 691	Metrohm AG, Switzerland
Syringe filter Millex-GP, 0.22 µm, Polyethersulfone, 33 mm	Merck Millipore, USA
Incubator shakers, Multitron 25 mm Displacement	Infors AG, Switzerland
Shake flasks Polycarbonate 500 mL	Corning, USA
Tetracycline	Sigma Aldrich, Switzerland
Water for Cell Culture Application WFI, 1 L	Lonza, Switzerland
Centrifuge Eppendorf, 5417C	Vaudaux-Eppendorf AG, Switzerland

3 IMPLEMENTATION

Two procedures are described. The procedure for completing extraction studies for bag films using cell culture medium is described in section 3.1 (see also **Figure 1**). The procedure using WFI is described in Section 3.2. Both extractions are recommended to ensure a proper evaluation. The actual cell culture test, which is the same irrespective of which extracting medium is used, is described in Section 3.3.

3.1 Extraction with cell culture medium

3.1.1 Properties of the bag films to be tested

The bag film should be tested no more than 2 months after it has been gamma irradiated. Incorporation of a MPC coupling in the bag is advantageous

3.1.2 Filling of the bags

The bags should be filled in a laminar flow bench.

1. The bag is removed from the packaging under sterile conditions and the connections and terminals are immediately closed. If necessary, any open MPC couplings are closed with sterile lids.

2. The medium, ChoMaster® HP-1, is prepared beforehand according to the manufacturer's instructions (Cell Culture Technologies, Switzerland). Thermolabile substances (for example, glutamine) may be added to the medium as required after extraction.

3. The freshly prepared medium (no older than 3 months) is transferred into the bag using either a 0.22 µm filter or a glass funnel, which is connected to the bag via a hose and a MPC coupling. It is recommended that the surface-area-to-volume ratio (S/V) is between 0.5 and 2 $cm^2\ mL^{-1}$.

4. In addition, a sterile Schott Duran bottle is filled with medium and is used as a control.

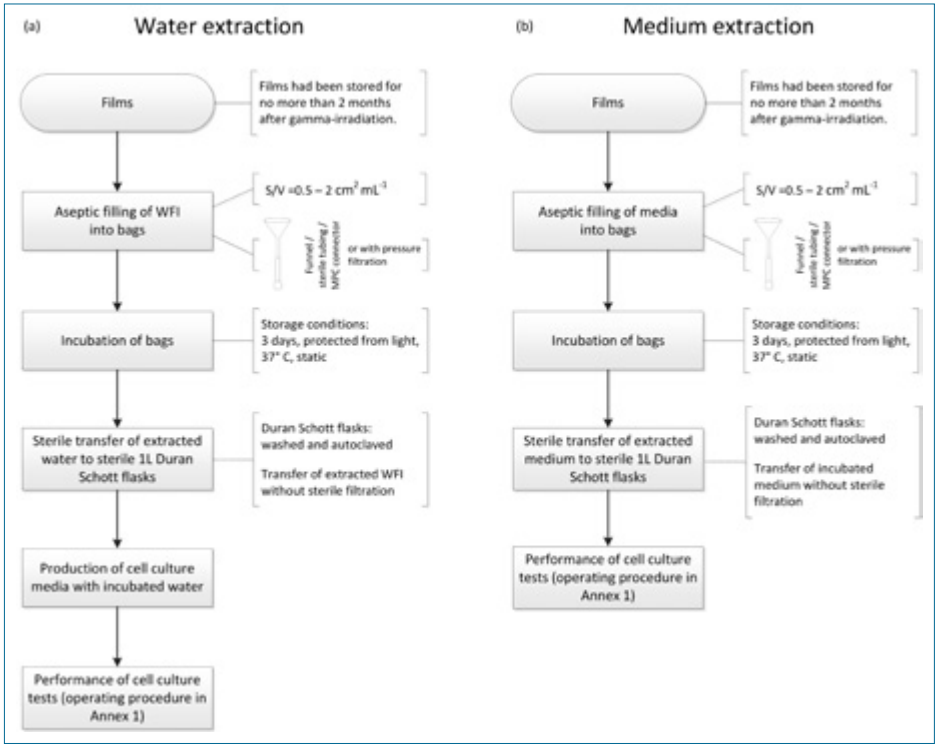
3.1.3 Extraction

1. The bag and the corresponding control are transferred to an incubator and are maintained in a static position in a dark, CO_2 free environment at a temperature of 37°C for three days.

2. After the extraction, the medium is transferred into sterile Duran Schott bottles. The Schott bottles are washed before sterilization to remove any soap residue. The time between the transfer of the extracted medium to the Duran Schott bottles and the actual cell culture tests should not exceed three days. It is important that the bottles are stored in a cool, dark location.

3. Before the cultivation, the medium, Pluronic F68 (0.2 %), and tetracycline (2.5 mg L^{-1}) are added. The tetracycline is not used to suppress contamination, but rather to support the growth of the cell line CHO XM 111-10 (tet-off), and to suppress the expression of the model protein, the secreted alkaline phosphatase.

Figure 1: Flow charts outlining the tests (a) WFI extraction and (b) medium extraction.
S/V surface-area-to volume ratio having taken into account.



3.2 Extraction with WFI

3.2.1 Properties of the cultivation bags to be tested

The bag film should be tested no more than two months after it has been gamma irradiated. Incorporation of a MPC coupling in the bag is advantageous.

3.2.2 Filling of the bags

The bag should be filled in a laminar flow bench.

1. The bag is removed from the packaging under sterile conditions and the connections and terminals are immediately closed. When necessary, all open MPC couplings are closed with sterile lids.

2. The WFI is transferred to the bag through a 0.22 µm filter or through a glass funnel which is connected to the bag via a piece of tubing and a MPC coupling. It is recommended that the surface-area-to-volume ratio is between 0.5 and 2 $cm^2\ mL^{-1}$.

3. In addition, a sterile Schott Duran bottle is filled with WFI to be used as a control.

3.2.3 Extraction

1. The bag and the corresponding control are transferred to an incubator and are maintained in a static position in a dark environment at a temperature of 37 °C for three days.

2. After extraction, the WFI is transferred into Duran Schott flasks, and stored at 4-8 °C until production of the cell culture medium. The Schott flasks are previously washed to remove any soap residue. The time between the filling of the extracted WFI in Duran Schott flasks and the production of the culture medium must not exceed three days.

3. The WFI is now used to make the ChoMaster® HP-1 medium, which is prepared according to the manufacturer's instructions (Cell Culture Technologies, Switzerland).

4. The medium is sterilized by filtration through Millipore Millipak-20 filters into sterile Schott flasks and must be used immediately thereafter for the cell culture tests.

5. Prior to the cultivation, the medium, Pluronic F68 (0.2 %), and tetracycline (2.5 $mg\ L^{-1}$) are added. The tetracycline is not used to suppress contamination, but rather to support the growth of the cell line CHO XM 111-10 (tet-off), and to suppress the expression of the model protein, the secreted alkaline phosphatase.

3.3 Cell culture test

3.3.1 Production of inoculum

CHO XM 111-10 cells (CCOS 837) are used for the cell culture test. After being thawed the cells are transferred to a 75 cm^2 T-flask (T-75) and 10 mL of FMX-8 medium are added. The maintenance culture is kept in a T-75 flask using FMX-8 medium at 37 ° C and 7.5 % CO_2. Passaging is completed every Monday and Friday, during which 20 mL of working volume are inoculated to result in a cell density of 0.2 x 10^6 mL^{-1}. On Wednesdays, 20 mL of medium is added to the working volume of each flask.

The cells of the maintenance culture are propagated (3 passages) prior to the cell culture test (per tested film/reference three to five T-75 flasks). At the time of the next passage, the expanded culture is transferred to a sterile beaker and is supplemented with an equal volume of fresh ChoMaster® HP-1 medium. The cells are held at 37 °C and 7.5 % CO_2 for two to three hours. After settling, the supernatant medium is removed and 50 mL of fresh ChoMaster® HP-1 medium is added. The suspension is then well mixed before a 1 mL sample is withdrawn, which is analyzed by means of a Cedex HiRes or another automated cell counting device. Two to three disposable shake flasks (1 L in size each containing 100 mL of working volume) are inoculated with cells (0.3-0.5 x 10^6 mL^{-1}). The cultivation parameters are listed in **Table 2**. Prior to the start of the cell culture test the contents of the shake flasks are either split up or fed as required (addition of ChoMaster® HP-1 medium until a maximum working volume of 350 mL has been reached). The subsequent cell count should not exceed 2.5 x 10^6 cells mL^{-1}, and the viability of the cells should be › 95%.

On the day of the cell culture test, quantities of fresh medium equivalent to those already contained in the shake flasks are added to each flask respectively. The cells are held for two to three hours at 37 °C and 7.5 % CO_2. After settling, the supernatant medium is removed and 50 mL of fresh ChoMaster® HP-1 medium is added. The suspension is well mixed and a 2 mL sample is withdrawn. The sample is analyzed by means of a Cedex HiRes and a BioProfile 100 plus (or comparable devices).

3.3.2 Performing the cell culture tests

The cell culture test is carried out with CHO XM 111-10 (CCOS 837) cells in disposable 500 mL shake flasks and is run as a batch process with a five day duration. Each cell culture test is carried out in triplicate for each film/reference. The shake flasks are inoculated with 0.2-0.3 x 10^6 cells mL^{-1} with the working volume amounting to 100 mL. The cultivation parameters are listed in **Table 2**.

Samples (each 2 mL) are collected under sterile conditions every 24 hours. The process is monitored by analyzing each sample by means of automated devices such as a Cedex HiRes and a BioProfile 100 plus. The Cedex HiRes allows monitoring of cell density and cell viability (obligatory criteria). Cell diameter can also be measured (optional criterion). Glucose, lactate, glutamine and ammonium concentrations represent obligatory criteria and can be determined with the BioProfile 100 plus. In addition, the pH can be examined.

Table 2: Cultivation parameters for the cell culture test

Parameter	Value
Temperature	37 °C
CO_2	7.5 %
Shaking frequency	120 rpm
Displacement	25 mm
Relative humidity	70 %

When evaluating the results of the experiments, it is recommended that the data should first be normalized with the reference at 100 %. The cell density, viability, and the substrate and metabolite concentrations are all shown as a function of the cell culture time for each evaluated film, the individual data are shown as percentage of the reference data. This should be done with the extracted medium and WFI.

If there is no more than one deviation from the control for the water and extraction media tests for a particular film (taking the results of both extraction media together), the cytotoxicity of the leachables from that film are considered to be negligible. If there is a deviation of ≥10 % in the cell density only for the media extraction, and not for the water extraction, potential interaction between the components in the medium and the bag material should be investigated. But if deviations for more than two criteria are observed, with either medium or WFI extracted, the bag film can be considered as "critical" with regard to cytotoxic leachables. An example of an evaluation matrix is provided in **Table 3**.

Table 3: Evaluation matrix
Evaluation matrix using the example of the experiments with CHO XM 111-10 cells in ChoMaster® HP-1 medium

Film	Max. cell density		Viability		Metabolism		Criterion		
	Medium-extraction	Water-extraction	Medium-extraction	Water-extraction	Medium-extraction	Water-extraction	1	2	3
1	20 %	+	+	+	–	+			
2	25 %	31 %	+	+	–	+			
3	+	+	+	+	+	+			
4	+	+	+	+	+	+			
5	+	21 %	+	+	+	+			
6	+	+	+	+	+	+			
7	25 %	+	+	+	–	+			
8	22 %	41 %	+	+	–	+			
9	+	+	+	+	+	+			
10	21 %	33 %	+	+	–	+			
11	23 %	+	+	+	–	+			

Evaluation criteria:

1 If there is a deviation of ≥ 10 % from the reference in no more than one criterion for all results from the medium and water extraction studies.

2 If there is a deviation of ≥ 10 % in the cell density only for the media extraction, and not for the water extraction, potential interaction between the components in the medium and the film material should be investigated.

3 If there is a deviation of ≥ 10 % from the reference in more than two criteria for all results from the medium and water extraction studies.

Key:

% percent compared to reference

\+ deviation from the reference < 10 %

– deviation from the reference ≥ 10 %

criterion is not met

criterion is met

A bag film can be considered as „non critical“ with regard to leachables if at least two of the three criteria can be designated as “green”.

4 ACCOMPANYING DOCUMENTS

Instructions for use provided by Cell Culture Technologies, Switzerland, the media manufacturer, for the media ChoMaster® HP-1 and FMX-8.

5 INDICATION OF CHANGES

The operating procedure has been newly created.

6 ATTACHMENTS

There are no additional attachments.

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