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Laboratory-scale emulsification process of a recombinant adenovirus vaccine with a water-in-oil-in-water adjuvant

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1 **TITLE:**
2 Laboratory-Scale Emulsification Process of a Recombinant Adenovirus Vaccine with a Water-In-
3 Oil-In-Water Adjuvant
4
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26 **SUMMARY:**
27 This manuscript describes a simple method for the formulation and control of vaccines with a
28 water-in-oil-in-water adjuvant at the laboratory scale, compatible with the safety requirements
29 of live recombinant vaccines.
30
31 **ABSTRACT:**
32 Adjuvants play an important role to enhance the efficacy of vaccines and are often required to
33 direct immune responses toward specific long-term protection. Several vaccination trials have
34 described promising results with the combination of recombinant adenoviruses and water-in-oil-
35 in-water (W/O/W) adjuvants. Specifically, the antibody response elicited by vaccines based on
36 canine adenovirus type 2 (CAV2) vectors steadily increases after being formulated in a W/O/W
37 emulsion. Thus, the production process directly impacts its physical properties, which are crucial
38 to obtain stable, safe, and efficient vaccine emulsions. This article describes a lab-scale process
39 for the formulation of O-206, a W/O/W adjuvant, in a total volume of 1 mL and 10 mL that is
40 compatible with safety requirements of live vaccines based on recombinant adenovirus.
41 Moreover, this article provides reliable and simple quality control analyses of the W/O/W vaccine
42 emulsion formulated with recombinant adenoviruses.
43
44 **INTRODUCTION:**

45 In the context of growing populations, secure access to uncontaminated food while improving
46 animal and human health will become increasingly important. The expression “One World, One
47 Health” describes a multidisciplinary international cooperation associating animal and human
48 health to notably better prevent and control zoonotic agents[1]. Indeed, 60% of emerging
49 infectious diseases in humans are transmitted by animals[2].
50
51 Nowadays, vaccination is still the most effective way to prevent and control infectious diseases
52 in humans and animals. In comparison, only drinking water allows such a reduction in mortality[3].
53 Today, the global effort to contain the COVID-19 pandemic underlies the overriding need for a
54 vaccine. The expected benefits of a vaccine on our public health and society stimulate the
55 development of new vaccines at unprecedented breadth and speed. Among the numerous
56 vaccines in development, traditional approaches (inactivated or live-attenuated virus vaccines)
57 have to cohabit with new vaccine technologies (recombinant protein, DNA or RNA fragment, viral
58 vector, etc.), which are widely used and show promising results[4]. Thus, vaccines carrying the
59 genetic information encoding a foreign antigen, including viral vectors as well as nucleic acids
60 (DNA plasmid or mRNA), are strategies that are increasingly being developed.
61
62 Adjuvants are also expected to play an important role in the efficacy of advanced vaccines by
63 triggering stronger immune responses. The panel of available adjuvants constitutes a wide range
64 of precious tools to enhance and/or shape immune responses toward specific long-term
65 protection. However, there is no universal adjuvant, and their mode of action is still partially
66 understood, as it often relies on several mechanisms. The selection and formulation of adjuvants
67 must consider a wide range of criteria such as the target population (species, age, etc.), the type
68 of antigen, the route of inoculation, and the expected immune mediators of protection. Expected
69 benefits of an adjuvantation include vaccine dose sparing, faster immune response, broadening
70 of immune response profiles, greater magnitude and functionality of antibody responses, or
71 specific targeting of effective T cell responses[5]. Thus, tomorrow’s vaccines are likely to be more
72 sophisticated, combining new vaccine and adjuvant technologies to achieve the best balance
73 between efficacy and safety. The development of new technologies will improve both human
74 and animal health.
75
76 In this article, a protocol to prepare a vaccine formulation containing an adenovirus-vectored
77 vaccine with the oily O-206 adjuvant is proposed. The resulting W/O/W emulsion consists of a
78 continuous aqueous phase within which oil droplets contain a secondary aqueous phase. Stable,
79 fluid, and safe, W/O/W emulsion showed promising results in several vaccination trials,
80 associating human adenovirus type 5 (Ad5) and O-206. Different oily adjuvants and formulations
81 (water-in-oil; oil-in-water; water-in-oil-in-water) were evaluated in mice with a non-replicative
82 recombinant adenovirus vaccine expressing pseudorabies virus gp50 (Ad5-pg50). O-206 based
83 formulation induced higher IgG titers (IgG2a) and stimulated IL6 production, even at low viral
84 vector concentrations[6]. Formulation of O-206 with an Ad5-expressing foot-and-mouth disease
85 virus antigens (Ad5-FMDV) enhanced the antibody response in sheep to a protective level[7].
86 Formulation of O-206 with an Ad5 vector encoding the green fluorescent protein improved GFP
87 expression during the first hours after transduction in bovine migrating DCs. Thus, the adjuvant
88 potentially stimulated the recruitment of DCs at the site of injection, reinforcing antigen uptake

and migration to draining lymph nodes. Beyond that, the frequency of CD4+ T cells increased following calves immunization with Ad5-FMDV and O-206[8].

Initially, W/O/W adjuvant was developed for cattle, swine, and small ruminants in association with non-immunoreactive antigens such as inactivated vaccines, purified proteins, or synthetic peptides. Fluid and easy to use, W/O/W emulsions enhance short- and long-term immune responses against various antigens. Because of the double emulsion structure, the antigens in the outer aqueous phase are immediately available to the immune system, while the antigens in the inner aqueous phase are protected against enzymatic degradation and have a sustained release. Multiphasic emulsions are also known to act through a variety of mechanisms, including a depot effect at the injection site, local inflammation stimulating the recruitment of antigen-presenting cells, and a contribution to the transport of antigens throughout the lymphatic system accompanied by an accumulation of lymphocytes in the draining lymph nodes[9].

The vaccine formulation process with W/O/W adjuvant directly impacts on its physical properties, which are crucial to obtain a stable, safe, and efficient vaccine emulsion. Emulsion stability is highly sensitive to changes and the formulation protocol needs to be optimized for any proposed modification in the production scale. The protocol here details two laboratory processes for the O-206 adjuvant that are suitable for testing small-scale immunization and compatible with the safety requirements of live vaccines based on recombinant adenoviruses. These optimized protocols allow a robust and reproducible formulation. They can be used for other compatible antigens, not interacting with O-206 surfactant. Moreover, they are adapted to the requirements of research laboratories: the first is particularly suitable for the vaccination of rodents, with a formulation volume of 1 mL; the second is well-adapted to the vaccination of large animals such as swine, small ruminants, and cattle, with a formulation volume of 10 mL.

A non-replicative canine adenovirus vector expressing the green fluorescent protein (GFP) is used throughout this study. It is not properly designated as a vaccine vector, but the expression of the reporter gene provides a useful approach for assessing the biological activity of a CAV-derived gene transfer vector.

A reliable and simple quality control analyses of the W/O/W vaccine emulsion formulated with recombinant adenoviruses is also provided. These quality control tests should be considered an integral part of the process.

PROTOCOL:

1. Emulsification process of 1 mL formulation for rodents

1.1. Gently shake the vial of O-206 before opening and transfer 560 µL of O-206 into a 2 mL microtube using a positive displacement pipette.

1.2. Add 440 µL of aqueous phase containing purified canine recombinant adenovirus (CAV2) at the desired concentration in a 2 mL microtube.

133
134 CAUTION: CAV2 recombinant vectors are genetically modified organisms (GMOs) derived from
135 canine adenovirus type 2, classified as a Risk Group II pathogenic microorganism. The handling
136 of CAV2 vectors and the treatment of its waste must meet local biohazard management
137 requirements.
138
139 NOTE: Non replicative canine adenovirus type 2 derived vectors were produced and purified as
140 previously described by Szelechowski, M. et al.[10].
141
142 1.3. Warm both the microtubes containing adjuvant and aqueous phase in a water bath or
143 incubator at 37 °C for at least 20 min.
144
145 1.4. Transfer 440 µL of the pre-warmed aqueous phase in the tube containing 560 µL of O-
146 206. Immediately mix by vortexing at 2,500 rpm ± 50 rpm for 1.5 min at room temperature (18–
147 25 °C).
148
149 NOTE: It is crucial to get a final ratio adjuvant/aqueous phase of 50/50 weight/weight. Because
150 the temperature of the oil and aqueous phases during mixing (32 °C ± 1°C) is a critical step of the
151 process, perform the assembly as fast as possible. Optimal and accurate agitation speed and
152 mixing time are also critical parameters. Use the recommended containers and the process
153 exactly as described here.
154
155 1.5. Cool the emulsion for at least 1 h at 20 °C (or 4 °C if 20 °C storage is not available) with
156 minimal turbulence.
157
158 NOTE: This step is an integral and critical part of the process. Insufficient cooling will affect
159 vaccine stability. The same protocol is suitable for larger formulations of 5 mL, using 15 mL conical
160 tubes.
161
162 **2. Emulsification process of 10 mL formulation for large animals**
163
164 2.1. Transfer 4.6 g of O-206 into a 20 mL gamma sterilized dispersing tube (see **Table of**
165 **Materials**).
166
167 2.2. Prepare 4.6 mL of aqueous phase containing purified canine recombinant adenovirus at
168 the desired concentration in a 15 mL tube.
169
170 CAUTION: CAV2 recombinant vectors are genetically modified organisms (GMOs) derived from
171 canine adenovirus type 2, classified as a Risk Group II pathogenic microorganism. The handling
172 of CAV2 vectors and the treatment of its waste must meet local biohazard management
173 requirements.
174
175 NOTE: Non-replicative canine adenovirus type 2 derived vectors were produced and purified as
176 previously described by Szelechowski, M. et al.[10].

177
178 2.3. Warm both tubes containing O-206 and aqueous phase in an incubator (or a water bath)
179 at 37 °C for at least 20 min.
180
181 2.4. Transfer 4.6 mL of the pre-warmed aqueous phase in the 20 mL dispersing tube containing
182 4.6 g of O-206. Immediately place the 20 mL dispersing tube on the homogenizer and mix at 1,100
183 rpm (speed 3) for 3 min at room temperature (18–25 °C).
184
185 NOTE: It is crucial to get a final ratio adjuvant/aqueous phase of 50/50 weight/weight. Because
186 the temperature of the oil and aqueous phases during mixing (32 °C ± 1°C) is a critical step of the
187 process, perform the assembly as fast as possible. Optimal and accurate agitation speed and
188 mixing time are also critical parameters. Use the recommended containers and the process
189 exactly as described here.
190
191 2.5. Cool the 20 mL dispersing tube containing the emulsion for at least 1 h at 20 °C (or 4 °C if
192 20 °C storage is not available) with minimal turbulence.
193
194 NOTE: This step is an integral and critical part of the process. Insufficient cooling will affect the
195 stability of the vaccine. The same protocol is suitable for larger volumes. For 10– 15 mL, use a 20
196 mL dispersing tube; for 20– 40 mL, use a 50 mL dispersing tube.
197
198 **3. Storage (optional)**
199
200 3.1. If storage is required, transfer the emulsion into a sterile glass vial after the cooling step.
201 Use a pierceable sterile cap to seal it.
202
203 NOTE: The emulsion can be stored for up to 1 week at 4 °C without significant loss of the CAV2
204 vector-mediated gene delivery. To preserve the vaccine, ensure that the packaging materials do
205 not interact physically or chemically with the finished product (e.g., do not use rubber). Elevated
206 temperatures may alter the partition characteristics of the emulsifiers and result in instability.
207
208 **4. Quality control tests**
209
210 NOTE: Perform the quality control tests after one-night storage at 4 °C.
211
212 4.1. Appearance
213
214 4.1.1 On the day of the formulation, keep the emulsion in a transparent tube at 4 °C. Handle
215 the tube with minimal turbulence. Do not shake the emulsion container for this test.
216
217 4.1.2 The next day, arrange a light directed throughout the emulsion in a transparent tube.
218
219 4.1.3 Check for the absence of critical defaults in the appearance of the resulting emulsion
220 (**Figure 1**).

221
222 4.2. Drop test
223
224 4.2.1. Gently shake the vaccine container with the emulsion to ensure that it is well mixed.
225
226 4.2.2. Add a drop of the emulsion in a 50 mL screw cap bottle containing 30–40 mL of water.
227
228 4.2.3. Gently shake and immediately observe the repartition of the droplet in water (**Figure 2**).
229
230 4.3. Microscopic observation
231
232 4.3.1. Gently shake the tube containing the vaccine to ensure it is well mixed.
233
234 4.3.2. Place a small drop of the vaccine in the middle of a Petri dish.
235
236 4.3.3. Carefully place a coverslip on the drop, without introducing air bubbles or crushing it.
237
238 4.3.4. Close the Petri dish and immediately observe the vaccine drop under a microscope using
239 brightfield (**Figure 3**).
240
241 4.4. Biological activity
242
243 4.4.1. A day before inoculation, seed MDCK cells on a 6-well plate. Grow cells at 37 °C in 5% CO_2,
244 in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, supplemented with 7% heat-
245 inactivated fetal calf serum, 1 mM of sodium pyruvate and 100 U/mL of penicillin/100 µg/mL of
246 streptomycin. Allow the cells to become 70%–80% confluent the following day for inoculation.
247
248 4.4.2. On the day of the biological activity assay, gently shake the tube containing the
249 formulated vaccine. Then, add up to 10 µL of the W/O/W emulsion per well in a 6-well plate.
250
251 NOTE: This test is scalable in 24-well plates by adding up to 2 µL of vaccine formulation per well.
252
253 4.4.3. Gently shake the plates and incubate at 37 °C in 5% CO_2 for 24 h.
254
255 4.4.4. The expression of the adenovirus-encoded antigen is sought in transduced cells by
256 immunocytochemistry or other appropriate methods.
257
258 **5. Vaccination**
259
260 NOTE: The vaccine can be administered if the formulation passes at least the appearance,
261 dilution, and microscopic observation tests. Evaluation of biological activity is not mandatory for
262 every formulation. However, it is strongly recommended to ensure, at least once, the biological
263 activity of the formulated viral-vectored vaccine.

5.1. Gently shake the vaccine container with the emulsion.

5.2. If the vaccine is packaged in a container with a pierceable cap, install a vial adapter and mount a syringe with a rubber-free piston. Pull-up the required volume of vaccine and then place the needle.

5.3. Otherwise, place a needle on a syringe with a rubber-free piston, pierce the vial or the cap of the 20 mL dispersing tube and then pull-up the required volume of the vaccine.

5.4. Administer the vaccine formulation *in vivo* directly by the intramuscular or subcutaneous route.

REPRESENTATIVE RESULTS:

Typical results obtained from the appearance test are shown in **Figure 1**. An emulsion is stable if there is no default or non-critical defaults. A default is considered non-critical when the difference of both color and phase is low. Non-critical defaults are hand reversible because the properties of the emulsion are conserved (e.g., whitish phase at the surface). A default is considered critical when the physical properties of the emulsion are permanently altered: when there is a change in droplet size; or when there is a fusion of dispersed droplets (e.g., a layer of oil at the surface or layer of water at the bottom).

The drop test is primordial to detect any change in the type of emulsion from O/W to W/O or vice versa, also known as phase inversion. A drop diffusing immediately into the water reveals an oil-in-water emulsion. On the contrary, the drop remains on the surface as a white ring in oil-in-water emulsion. The drop of the water-in-oil-in-water emulsion floats on the surface while diffusing into the water (**Figure 2**). The conductivity also allows the identification of the type of emulsion. The expected result for the conductivity of O-206 formulated with a saline solution (0.9% NaCl) is 5 $mS \cdot cm^{-1}$.

The microscopic aspect of the emulsion will provide information on its physical properties. An optimized formulation and a good process will lead to the formation of thin and homogeneous droplets, with a median droplet size of about 300 nm. On the contrary, a non-optimized formulation or an incorrect process will result in heterogeneous droplets with large drops. This critical default might cause the breakage of the emulsion (**Figure 3**). In most cases, emulsions with a small droplet size and homogeneous distribution are more stable.

Droplet size in an emulsion of O-206 formulated with a saline solution (0.9% NaCl) follows a normal distribution: 50% of the volume of droplets are smaller than 0.12 µm and 90% are smaller than 0.39 µm (D (v; 0.5) = 0.12 µm and D (v; 0.9) = 0.39 µm) (**Figure 4**).

The viscosity of the emulsion is closely related to the surfactant and its hydrophilic-lipophilic balance, and can be affected by the adjuvant/aqueous phase ratio. Usually, the greater the proportion of the continuous phase compared to the dispersed phase, the lower the viscosity of

the resulting emulsion. Then, the expected viscosity of O-206 formulated with a saline solution (0.9% NaCl) is 30 mPa·s at 20 °C.

The physical stability of W/O/W vaccines is critical to assess the homogeneity and reproducibility of the formulation. The formulation of an emulsion with O-206 is expected to be stable for more than 2 years at 4 °C and more than 6 months at 20 °C.

Formulation with W/O/W adjuvant does not affect the biological properties of CAV2-based vaccines. As an example, 1 mL of W/O/W emulsion is prepared with 10^8 $TCID_{50}$ of a non-replicative canine adenovirus type 2 vector encoding the green fluorescent protein. The non-replicative CAV-GFP vector is a model of such vaccines, and is appropriate for assessing its gene transfer activity. Indeed, expression of the GFP can be easily monitored in MDCK cells transduced with emulsion-formulated CAV-GFP, at a multiplicity of infection of about 1 $TCID_{50}$ per cell (**Figure 5**). CAV2 vectors are stable for years when stored in 10% glycerol (V/V) at -80 °C, but their stability at 4 °C is more limited. Thus, CAV2-based emulsions with O-206 can be stored for up to 1 week at 4 °C without significant loss of biological activity and physical stability.

FIGURE AND TABLE LEGENDS:

Figure 1: Appearance of the emulsion. (**A**) Stable O-206-based emulsion, without any gradient of color or phase separation. (**B**) Unstable O-206 based emulsion, with a sedimentation effect visible through a gradient of color: smaller drops constitute a white layer at the bottom. (**C,D**) Two examples of emulsion breakage, with a clear separation of water and oil phases. These defaults are critical.

Figure 2: Drop test to assess the type of emulsion. (**A**) The drop oil-in-water emulsion immediately diffuses into the water. (**B**) The drop of water-in-oil emulsion stays on the surface. (**C**) The drop of water-in-oil-in-water emulsion both stays on the surface and diffuses into the water.

Figure 3: Microscopic observation of the vaccine formulation. Observation of samples of O-206 water-in-oil-in-water emulsions using bright-field microscopy (200x). (**A**) This stable emulsion was well formulated, and presents homogeneous and thin droplets, with a median size around 300 nm. (**B–D**) These examples of unstable emulsions present heterogeneous and large droplets of oil.

Figure 4: Granulometric repartition in volume of O-206 formulated with a saline solution. (**A**) Representative results of a well-formulated emulsion of O-206 with a saline solution (0.9% NaCl). (**B**) Example of an unstable emulsion of O-206, formulated with a saline solution (0.9% NaCl) at room temperature instead of 32 °C ± 1 °C. Data analyzed with a laser diffraction particle size analyzer.

Figure 5: Biological activity of canine adenovirus vectors. MDCK cells were observed 24 h post inoculation of a non-replicative canine adenovirus type 2 vector encoding the green fluorescent protein freshly formulated in a W/O/W emulsion, using fluorescence microscopy (FITC, 100x). Up

352 to 1 week after formulation, the vector is still able to effectively transduce MDCK cells without
353 significant loss of its biological activity.
354
355

356 **DISCUSSION:**

357 The protocol in this study details two lab-scale processes adapted to the safety requirements of
358 live vaccines based on recombinant adenoviruses formulated with W/O/W adjuvant.
359

360 These optimized protocols allow a robust and reproducible formulation. However, it is crucial to
361 scrupulously respect certain critical steps. Firstly, it is important to obtain a final
362 adjuvant/aqueous phase ratio of 50/50 weight/weight. Secondly, warming of the oil and aqueous
363 phases before their assembly must ensure a homogeneous temperature of 32 °C ± 1 °C during
364 mixing. The lower the final volume of the emulsion, the faster is the cooling of both phases during
365 pipetting and mixing. Therefore, it is advised to preheat both phases and emulsification container
366 to 37 °C. Thirdly, an optimal and accurate stirring speed and mixing time are also critical
367 parameters. The last critical step is the cooling of the emulsion at 20 °C (or 4 °C) for at least 1 h,
368 with minimal turbulence. It is important to wait until after cooling to transfer the emulsion to
369 another container. Any deviation from the above steps could permanently affect the quality of
370 the resulting vaccine emulsion. If storage is required, the vaccine formulation can be stored for
371 up to 1 week at 4 °C, without significant reduction in the biological activity of the CAV2 vector.
372 To preserve the vaccine, the packaging materials should not interact physically or chemically with
373 the finished product (e.g., do not use rubber). High temperatures may alter the partition
374 characteristics of the emulsifiers and result in instability.
375

376 The protocols described herein are also suitable for larger formulation volumes. The 1 mL process
377 is also adapted to 5 mL formulations, using 15 mL conical tubes instead of 2 mL microtubes. The
378 10 mL process is suitable for a wider range of formulation volumes. For 10–15 mL, use the 20 mL
379 dispersing tubes; for 20–40 mL, use the 50 mL dispersing tubes.
380

381 Although the above protocols are presented to formulate a purified canine adenovirus vector
382 with O-206, they are also convenient for the formulation with O-201 adjuvant. For the 1 mL
383 process, only adapt the volume of aqueous phase to 450 µL (instead of 440 µL with O-206) and
384 of O-201 to 550 µL (instead of 560 µL for O-206). O-201 was developed as the improved version
385 of O-206 to strengthen the cellular responses. Both adjuvants are safe, efficient, and
386 commercially available. However, O-201 has shown superiority over O-206 across several
387 vaccination protocols in pigs[11,12], cattle[13,14], and goats[12]. It was recently observed that the O-201
388 adjuvant also improved the efficacy of CAV-2 vaccines in mice and piglets (data available upon
389 request).
390

391 The formulation of purified vectors is well-adapted to vaccination trials, as it removes the most
392 part of production leftovers while increasing infectious titers[10]. However, the protocols were also
393 tested with clarified lysates of infected cells, which contain cellular debris and culture medium.
394 Then, the biological activity of the vector and the physical properties of the resulting emulsion
395 were preserved.

396
397 Although this protocol is based on CAV-2-vectored vaccines, other types of adenovirus could be
398 successfully formulated. Moreover, it can be extended to other viral vectors if their biological
399 activity is well conserved. Special attention is required for enveloped viruses, as the surfactant
400 may alter their envelope and weaken their biological activity. Some components in the viral
401 particles could also interact with the adjuvant and impair its physical properties.
402
403 In addition, these protocols are convenient for the formulation of inactivated vaccines and non-
404 reactive antigens such as purified proteins or synthetic peptides, with appropriate tests. Indeed,
405 antigenic media may contain proteins with polar and non-polar groups, which have properties
406 similar to surfactants. Some enzyme residues, such as esterases, could also weaken the stability
407 of W/O/W emulsion. In this case, the composition of the antigenic medium must be adapted to
408 improve its stability.
409
410 It should be noted that this protocol covers only the formulation of O-201 and O-206 adjuvants
411 and their laboratory applications. In addition, it is not intended to be scalable outside the volume
412 range described here.
413
414 The formulation can only be stored for a limited time presumably due to a stability deficit in the
415 CAV2 preparation. It is well known that the activity of adenoviral vectors is maintained at -80 °C
416 for years in a medium containing glycerol (10%, v/v), which is not the case at 4 °C. Each vaccine
417 requires extemporaneous preparation and should be used within 10 days, but once formulated
418 the vaccine can be transported in a refrigerated packaging.
419
420 Once properly formulated at the laboratory scale with minimal verifications, the resulting vaccine
421 formulations will be useful in evaluating the benefits of W/O/W adjuvantation across a wide
422 range of vaccine models, antigens, and animal species. The first protocol is particularly suitable
423 for rodent vaccination, whereas the second one is well-adapted for the vaccination of large
424 animals such as pigs, small ruminants, and cattle.
425
426 Free of components of animal origin, O-201 and O-206 adjuvants are composed of a combination
427 of a mineral oil with a well-balanced surfactant. The research on innovative formulations will
428 guide further vaccine development in fruitful directions.
429
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435
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437 Manon Broutin, Matthieu Bricaud, Jennifer Maye, Jérémie Bornères, Juliette Ben Arous and
438 Nicolas Versillé were employed by SEPPIC, part of Air Liquid Healthcare, when the work was
439 performed; Fleur Costa and Bernard Klonjkowski declare no other competing interests.

440
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473

Figure 1

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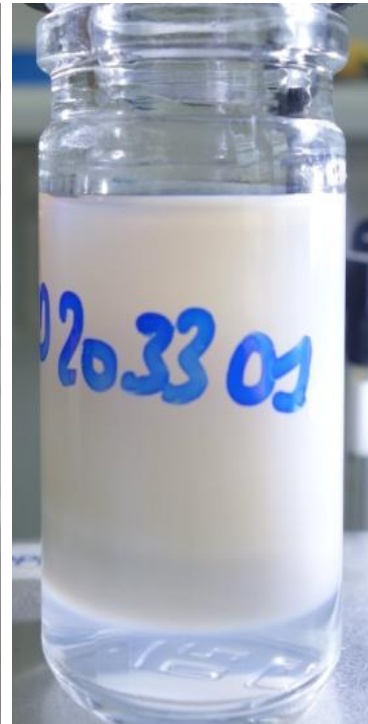
Stable emulsion

A.

Unstable emulsions

B.

C.



D.

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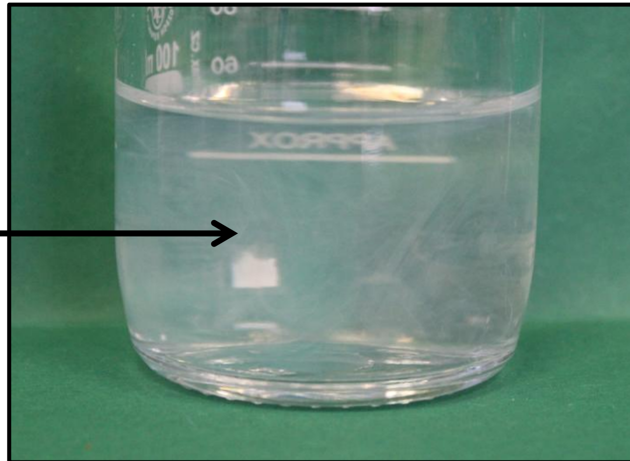
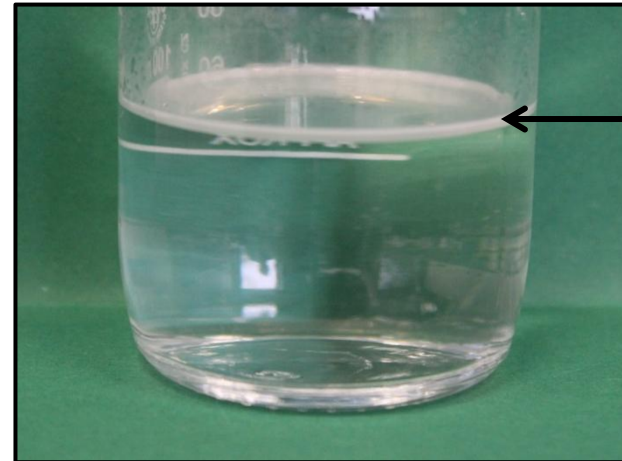
A. Oil-in-water emulsion



B. Water-in-oil emulsion



C. Water-in-oil-in-water emulsion

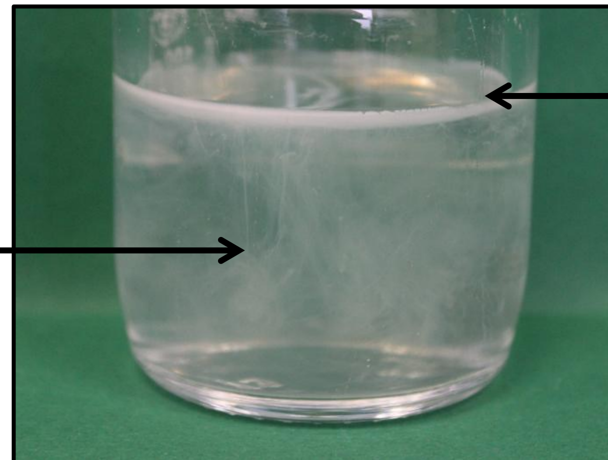


Figure 3

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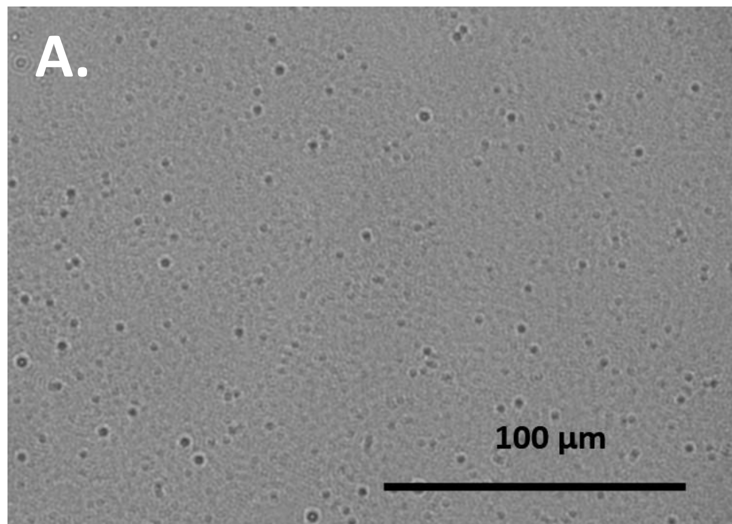

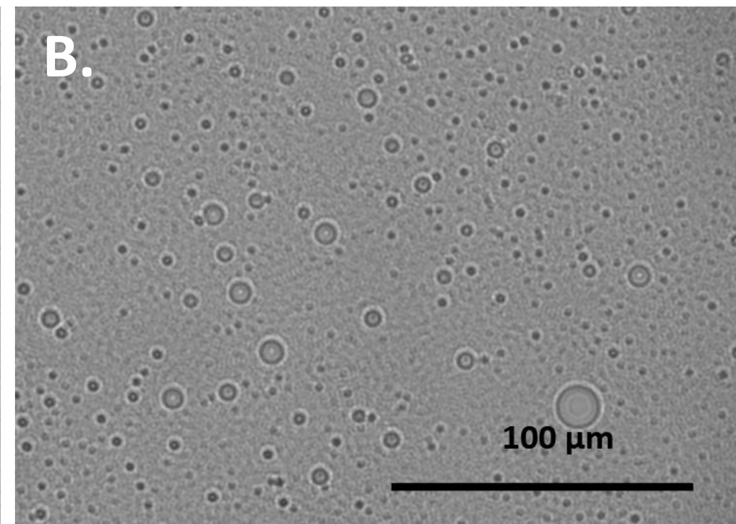

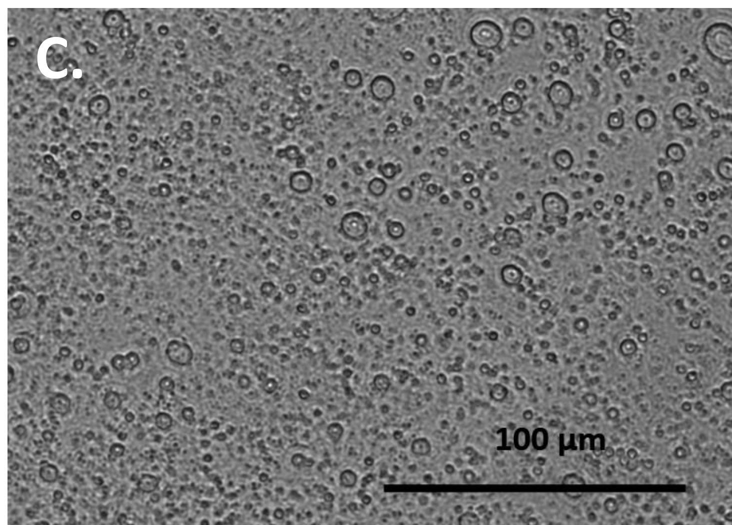

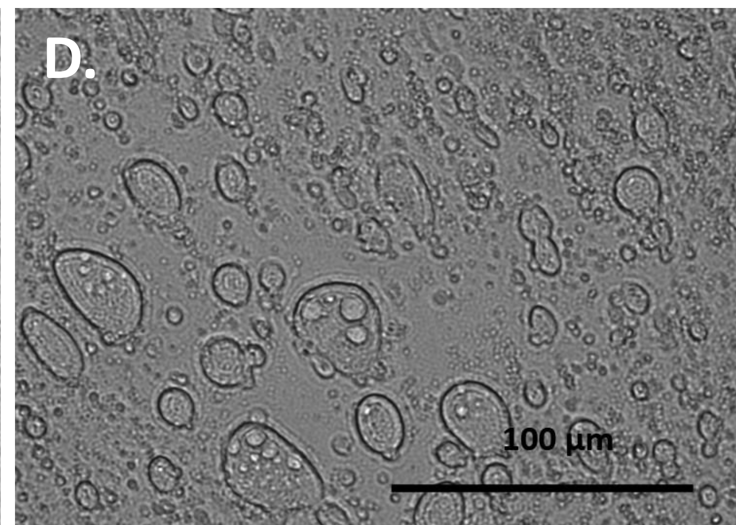

Figure 4

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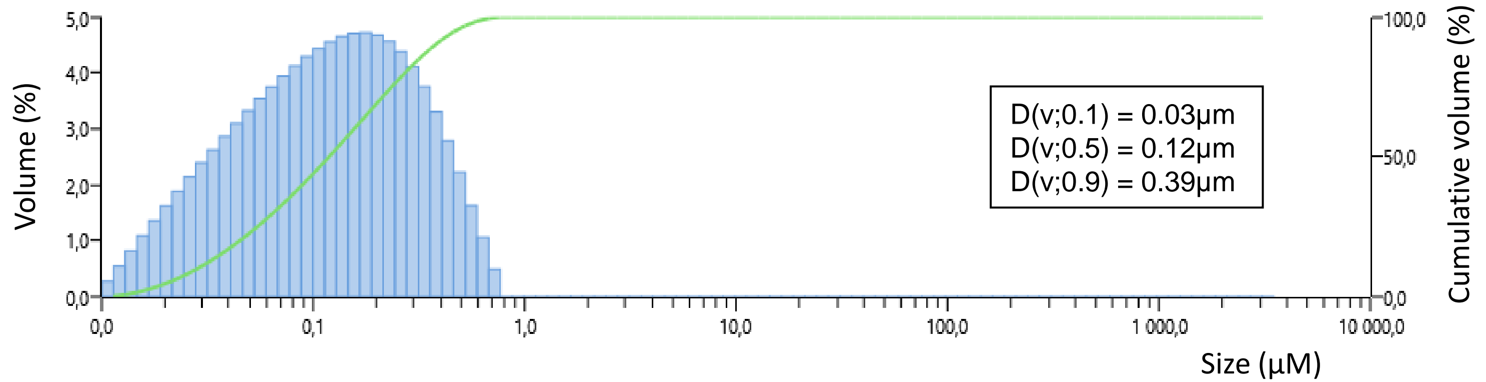
A. Well-formulated emulsion



B. Unstable emulsion

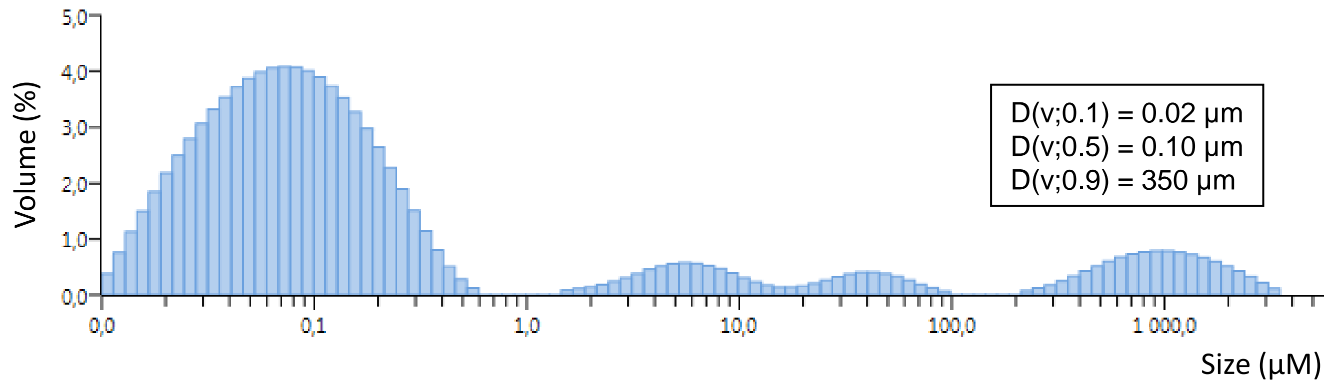


Figure 5

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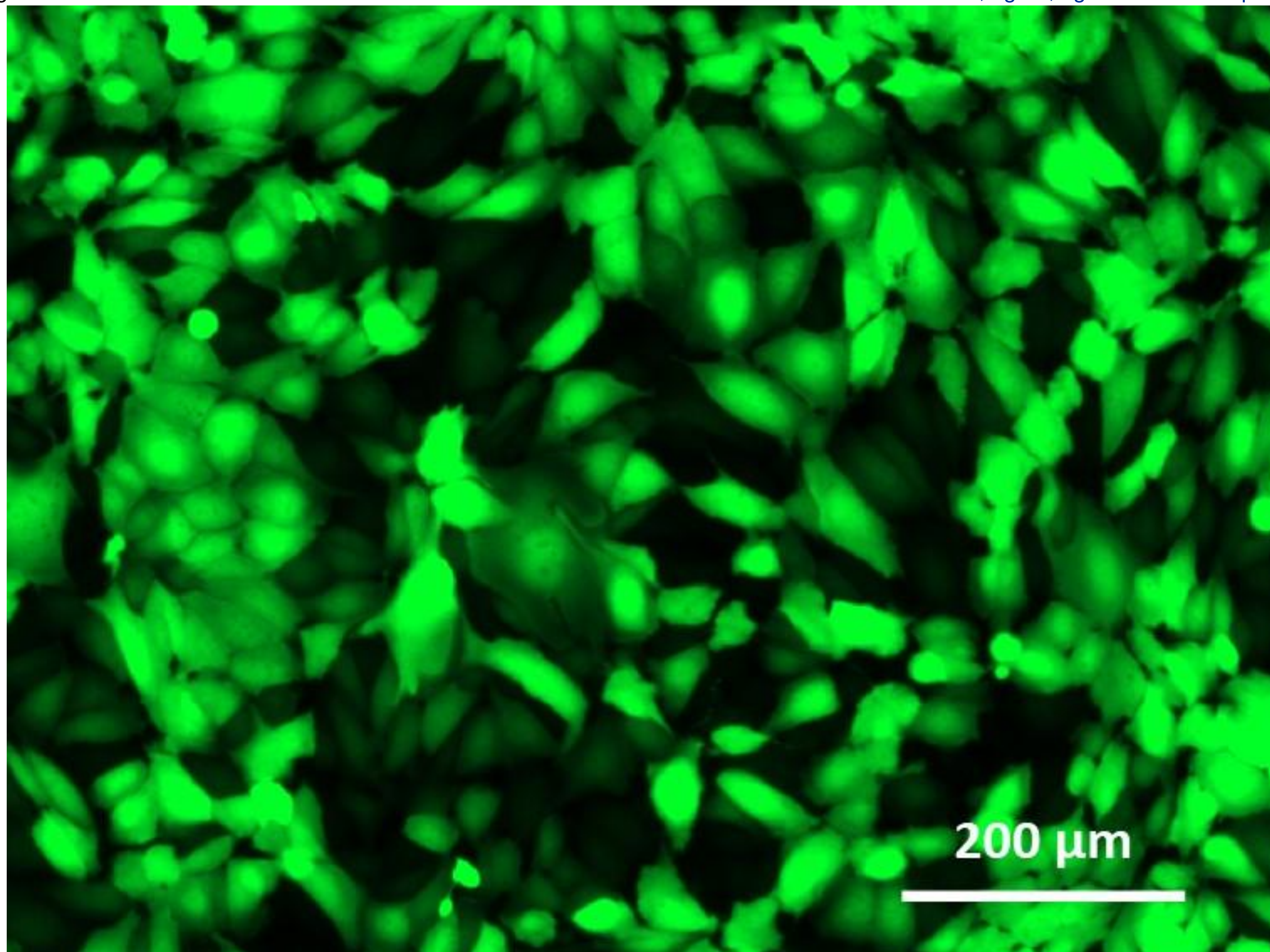

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
15 mL conical tube	FALCON	352096	
20 mL dispersing tube	IKA	3700600	Tube with rotor-stator element. Sterile model, with pierceable membrane
50 mL dispersing tube	IKA	3701600	Tube with rotor-stator element. Sterile model, with pierceable membrane
DMEM (1x) + GlutaMAX-I	GIBCO	61965-026	
Fetal Calf Serum	EUROBIO	CVFSVF00-01	
Homogenizer	IKA	3646000	
Laser diffraction particle size analyzer	Malvern Panalytical	Mastersizer 3000	
MDCK (NBL-2)	ATCC	CCL-34	
Microtube 2 mL	EPPENDORF	30120094	
O-201	SEPPIC	MONTANIDE ISA 201 VG	
O-206	SEPPIC	MONTANIDE ISA 206 VG	
Penicillin (10,000 U/mL) Streptomycin (10,000 µg/mL)	GIBCO	15140-212	
Pipette Tips C POSD 1000 µL S 180/3	RAININ	17008609	100 µL – 1000 µL
Positive-Displacement Pipette MR-1000	RAININ	17008580	100 µL – 1000 µL
Sodium Chloride 0.9% injectable	BBRAUN		
Sodium pyruvate 100 mM (100x)	GIBCO	11360-039	
Sterile glass vial	WEST PHARMACEUTICAL SERVICES	8072035	Optional
Syringe 1 mL	BBRAUN	9166017V	Syringe without rubber
Syringe 10 mL	BBRAUN	4606728V	Syringe without rubber
Syringe 2 mL	BBRAUN	4606701V	Syringe without rubber
Syringe 5 mL	BBRAUN	4606710V	Syringe without rubber

Vial adapter	WEST PHARMACEUTICAL SERVICES	8072035	Optional
Vortex mixer	SCIENTIFIC INDUSTRIES	SI-0256	Vortex-Genie 2

Dear Editor,

We are pleased to send you a revised version of the previous formatted manuscript file. In the revised manuscript, we have carefully considered the editorial comments.

Sincerely

Bernard Klonjkowski