Protocol: Fabrication of PDMS devices for in vitro skeletal muscle tissue template

Gilbert Lab – U of T – Chávez-Madero C. – 2023

Materials

Form3 – 3D printer SLA laser-based (FormLabs)
Preform software (Free)
Resin clear V4 (RS-F2-GPCL-04) – Printing parameters set to Adaptive Layer Thickness.
Trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (**FOTS**) (Millipore Sigma)
Plasma chamber (Harrick Plasma);
Isopropanol.
Ecoflex 00–30 (Smooth-On Inc., PA)
Polydimethylsiloxane (PDMS)
Polystyrene weighing dishes
Lab spatula (Polystyrene optional)
Vacuum chamber (non-chemical)
FOTS Vacuum chamber (Only for FOTS chemical coating)
Vacuum pump
Air in a Can (www.uline.ca or compressed air)
Analytical Balance (Capacity for >150g)
Mold release agent for use for silicones, urethanes, and resins (Ease release 200)
Chemical hood
Benchtop oven (=>100°C)
Sharp Tip Tweezer.
96 well plate non-sterile.
Leveller tool (App phone)
Tape
bladeless scalpel

Safety

Several of the reagents pose a potential risk to human health. Ensure that you read the safety documentation for all reagents in this protocol, don appropriate personal protective equipment, and make use of a chemical safety hood when required.

Method

All of the steps are critical and proposed as techniques to use in a standard laboratory to fabricate molds for microdevices normally practiced in specialized *clean rooms*. Specifically, clean rooms have robust protocols that consider nano/microparticles in the air-space or in the equipment used that can impede the successful casting of designs. As such, failures associated with executing this protocol may be related to unsuccessful chemical reactions at any number of the following steps. As such, avoid deviating from the protocol below if you will cast devices in a standard laboratory environment (i.e. outside of a clean room).

Part A – 3D design and printing

1. Design the 3D positive piece using Solidworks or any other CAD software. The Gilbert Lab 96-well MyoTACTIC plate design file is found within our online repository: https://github.com/gilbertlabcode/MyoTACTIC-SolidWork-CAD-file. We provide the *.STL Positive design*.
2. Use the software of preference to translate the .STL design to *gcode*. For example, we use "PreForm" for the FormLabs Form3 3D printer with the following settings:
    a. Adaptative layers (w/ most updated parameters)
    b. With **no** internal support.
    c. We use clear resin. In general, we recommend using a long-lasting non-degradable high-resolution material, which is crucial for reproducing the micropost morphology.
3. After printing the part, clean the printed part without touching or disturbing the microposts by following cleaning protocols from the 3D printing company.
    a. Remark: cleaning strategy is resin specific.

Part A – Casting of the 3D original positive mold (Positive to negative)

1. By visual inspection, make sure that the 3D-printed positive model is completely dry and dust free. Failure to do so will impede the casting by capturing alien particles.
    a. Remark: if the 3D-printed piece is minimally sticky on the surface, the negative cast will not recreate the positive shape successfully and a new 3D piece will be required.
2. Position the 3D printed model with the myoTACTIC designs facing upward inside of a hand-made aluminum foil container that is at least 2 cm higher than the 3D printed model, allowing room from the MyoTACTIC 3D-print of 1 cm per side (see Appendix E1). Use any tape that can bond to the 3D piece as a means of securing the position of the plate inside of the casting foil container. We will refer to this 3D-printed piece as the *positive mold* and it will be used in section B5.

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3. Using an analytical balance, measure the EcoFlex 00-30 silicone, which is a 2-part polymer mixture. In-house we find that the MyoTACTIC plate holds 100 mL of EcoFlex in volume. Therefore, to generate the negative mold, we mix 80 mL each of parts A:B with a tolerance of -/+ 1 mL. If a mixture of the two parts has a variance that is larger than 1 mL in volume, the elastic properties of the resulting piece change, and the casting may fail.
    a. To achieve +/- 1 mL variance, it is crucial that the two parts are weighed together within the same inert polystyrene weighing dish.
    b. If the Ecoflex is > 6 months old, or was not stored correctly in the sealed containers, the product might not be functional and this will impede casting.
    c. Individually mix bottles A and B vigorously by vertical shaking before pouring.
4. Using a spatula, mix the 160 mL of parts A and B rigorously. A homogenous dispersion of the polymers is strongly required. The estimated time of mixture should not be < 5 minutes, and

bubbles in very small sizes and high density that are visible to the eye are expected (see Appendix E2).

a. Scratching the polystyrene plate will introduce particles to your mixture that may impede polymer crosslinking.
b. Remark: Once mixed, A+B will begin to polymerize into a solid phase (at 45 minutes post-mixing). The next steps must be completed in less than 40 min.

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5. Immediately after mixing the EcoFlex polymer solution, transfer the *positive mold,* which is within the aluminum container, to a chemical hood. Shake the bottle of Ease Release 200 for 1 minute, and then use the Ease Release 200 to spray the positive mold uniformly from a 20-25 cm distance. The result should be that a mild gloss is left on the surface of the positive mold. At this point, let the ease release incubate on the positive mold for a minimum of 10 minutes and up to a maximum of 20 minutes.
   a. Spraying more than the required amount of ease release on the positive mold may contribute to improper EcoFlex crosslinking such that diffusion of phases may occur. If you are using a brand other than Ease Release 200, or if you read the instructions on the can and they differ from what is listed in this protocol, then default to following the manufacturer's spraying recommendations.
   b. Spraying less than the required amount of ease release on the positive mold may contribute to EcoFlex biding in the 3D printed part, which will cause defects in the casting and failure to continue the following steps of this protocol.

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6. Use a leveller to make sure that the vacuum chamber dedicated to non-chemical use is levelled.
7. Place the polystyrene weighing dish containing the A:B mixture inside the levelled non-chemical vacuum chamber and associated vacuum pump.
8. Securely connect to the air-in hose and turn on the vacuum to degas the mixture of A:B. Apply ~4 cycles of vacuum-air ON/OFF, and in each cycle, you are expecting bubbles to form that look bigger than 0.5 cm in diameter during the ON stage. At this point, you turn it OFF to allow all the bubbles to escape from the copolymer (i.e. degas). This total process should not exceed 20 minutes (Appendix E2).
   a. This step should be conducted in active observation as the crosslinking of A:B occurs within 40 minutes.
   b. We call this the **Ecoflex pre-degas step**.
   c. When degassing, be cautious with the change of pressure, as your polystyrene dish might flip over and the content of A:B spread over the chamber surface.
9. After pre-degassing A:B, bring the ease release coated *positive mold* from part B5 and place it in the vacuum chamber. Pour the copolymer A:B into a single corner of the device from a low height, allowing it to spread across the entire surface. These steps are critical to avoid bubbles. You should stop pouring when the A:B polymer liquid is at least ~1 cm in height above the 3D printed model.
   a. Securely connect to the air-in hose and turn on the vacuum to degas the EcoFlex on top of the positive mold.

b. Apply ~4 cycles of vacuum-air ON/OFF, and in each cycle, you are expecting bubbles to form that look bigger than 0.5 cm in diameter during the ON stage, at which point you turn it OFF to allow all the bubbles to escape from the copolymer.
c. The migration of air bubbles to the surface from within the 96 wells will be visible, and a successful mold degassing will occur **within 10 minutes**. Turn off the pump and the gas valve slowly to avoid liquid A:B material displacement.
d. The Ecoflex-coated positive mold is referred to as the positive-to-negative part.

10. Ideally, allow the positive-to-negative to be undisturbed inside the chamber. If the vacuum chamber is to be occupied by other lab projects, then right after degassing, place the positive-to-negative part on a levelled counter. It should not be disturbed for a minimum of 12 hrs (it can sit for up to 48 hours). The determination of incubation time can be assessed by touching the corner of the positive-to-negative with tweezers as the density change from liquid-gel to solid-gel is mechanically evident.

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11. Detach the Ecoflex negative from the positive mold. To do this step, we recommend putting the positive-negative part in the oven at a temperature of 37 °C for ~15 minutes, allowing a more successful separation of the molds.
    a. Try to pull the Ecoflex from only two corners in a straight direction without ripping any of the material; only stretch Ecoflex up to four-fold its original size as this will result in tearing the Ecoflex.
    b. If your 3D printed piece was produced with a low thermosensitivity resin, then **DO NOT** use the 37 °C oven in this step.
        i. Remark: Check the resin datasheet for thermosensitivity information.
    c. Store the 3D printed model in a dry, light-free, low-traffic space (i.e., a drawer) for future casting. Evaluate resin degradation over time, as a new fresh print might be required for better casting.
12. After Ecoflex is successfully detached, post-curing is required, as recommended by the manufacturer. Place the Ecoflex negative mold in the oven at a temperature of 100 °C for one hour. Then remove it from the oven and let it cool down to room temperature.
13. Use a spray bottle containing isopropanol, set the sprayer to medium dispersion, and completely soak the negative-side surface of the EcoFlex.
14. Dry the isopropanol-washed Ecoflex using Air in a Can, or any other compressed air, to fully dry the isopropanol.
    a. The EcoFlex negative mold should be completely dry and without stickiness. If wet or sticky, the FOTS coating step will fail.
15. Repeat steps 13 and 14 one more time.

<u>Part B – Casting of Negative mold for PDMS production (Negative to positive)</u>

1. Using a vacuum chamber dedicated to FOTS, place an empty and clean 96-well plate in the chamber's centre and place both inside the chemical hood.
    a. Use the leveller tool to ensure the vacuum chamber is balanced.
2. Next, a plasma cleaner with air (or nitrogen) is used to eliminate reactive particles from the surface of the Ecoflex negative mold. A 60-second exposure starting from the evident purple plasma is sufficient. At that point, turn off the plasma equipment and release pressure slowly.
3. Rapidly transfer the negative mold to the FOTS chamber and place it on top of the 96-well plate perpendicularly, creating a 90° cross-like shape with the two plates.
4. Place 100 µL of FOTS at each end of the 96-well plate within the centre well (see Appendix E4). Make sure that the FOTS-containing wells are not blocked by the Ecoflex mold and apply vacuum for a window of 3 – 10 minutes.
    a. The vacuum time depends on the pressure pump; we recommend testing to start with only 3 minutes period and iterate upward if the coating fails.
    b. Extending the vacuum of the chemical deposition can produce chemical leakage from the vacuum pump, resulting in failure to coat the negative mold.
    c. Turn off the pump and ensure that the chamber is in negative pressure when removing the hose; this usually is done with the side switch of the chamber. Be sure to check the switch orientation before starting the degassing process.
    d. Let the system sit undisturbed overnight (~12 hrs).
5. The next day, slowly degas the FOTS chamber inside the chemical hood. A light opaque layer should be visible to the eye if the coating is successful. It is also sometimes possible to see a change in the colour of the Ecoflex negative mold.
6. Grab the negative mold from the bottom without touching the molding upper part and place it in a 70°C oven for one hour. Then, remove it from the oven and let it cool down to room temperature.
7. Once cooled to room temperature, the FOTS-treated Ecoflex negative should be washed with isopropanol wash as described in Step B.13-15. The EcoFlex negative mold should be completely dry and without stickiness. The Ecoflex negative is now ready for generating PDMS final parts.
    a. We recommend using this Ecoflex negative right after treatment; in practice, the successful casting of PDMS can be produced even six months after. We recommend storing at room temperature and with little light exposure.

Part C – PDMS: final models (Negative to positive)

1. Make 128 mL of PDMS in a clean inert polystyrene weighing dish, adding 120 mL of monomer and 8 mL of curing agent (i.e., ratio is 15:1).
    a. Prepare the two reactive polymers using the same procedural steps listed above for successful mixing and curing.
        i. Using a spatula, mix the monomer and curing agent rigorously. Homogenous dispersion of the polymers is required. The estimated time of the mixture should be at least 10 minutes, and bubbles in very small sizes and high density must be observable (see Appendix E2). Scratching the polystyrene will produce particles that may impede polymer cross-linking.
    b. Use a leveller to make sure that the vacuum chamber dedicated to non-chemical use is levelled.
    c. Using the levelled non-chemical vacuum chamber and a vacuum pump, put the mixed liquid PDMS plate inside the chamber, securely connect to the air-in hose and turn on the vacuum to degas the mixture (see Appendix E2).
    d. Apply ~4 cycles of vacuum-air ON/OFF, and in each cycle, you are expecting bubbles to form that look bigger than 0.5 cm in diameter during the ON stage, at which point you turn it OFF to allow all the bubbles to escape from the copolymer.
    e. After, let the system **ON** to eliminate the bubbles for 30 minutes or, as fully degassed, the estimated time is ~1 hour (see Appendix E2).
    f. We call this the **PDMS pre-degas step.**

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2. While the PDMS is degassing in the polystyrene dish, transfer and place the negative Ecoflex mold in a chemical hood.
3. Take the Ease Release 200 and shake the bottle of Ease Release 200 for 1 minute, and then use the Ease Release 200 to spray the Ecoflex negative mold uniformly from a 20-25 cm distance. The result should be that a mild gloss is left on the surface of the mold. At this point, let the ease release incubate on the mold for a minimum of 10 minutes and up to a maximum of 20 minutes.
    c. Spraying more than the required amount of ease release on the positive mold may contribute to improper PDMS crosslinking such that diffusion of phases may occur.
    b. Spraying less than the required amount of ease release on the positive mold may contribute to EcoFlex biding in the PDMS, which will cause defects in the casting or ripping of critical parts of the microstructures (i.e., hooks of the MyoTACTIC).
    c. If you are using a brand other than Ease Release 200 or read the instructions on the product that differ from what is listed in this protocol, then default to following the manufacturer's spraying recommendations.

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4. After the wait time, transfer the negative Ecoflex mold to an analytical balance and tare. As the liquid PDMS is fully degassed, pour 100 mL of liquid PDMS into a single corner of the device from a low height and allow it to spread across the entire surface. These steps are

critical to avoid bubbles. You should stop pouring when the PDMS liquid reaches **100 g** (equivalent to 100 mL) in volume.

   d. Remark: As the pouring is occurring while the weight is being measured, be observant not to over-pour the **100 mL of volume**, as the plate has been designed with specific thicknesses that are volume dependent. If over-pouring occurs, a spatula can be used to remove the extra volume; however, this fix is undesired.

5. Transfer the negative Ecoflex with the liquid PDMS to a levelled non-chemical vacuum chamber, and apply the ~4 cycles of vacuum-air ON/OFF. In each cycle, you are expecting bubbles to form that look bigger than 0.5 cm in diameter during the ON stage, at which point you turn it OFF to allow all the bubbles to escape from the copolymer.
6. After, let the system **ON** to eliminate the bubbles for 1 to 1.5 hours or, as fully degassed, if bubbles are visible to the eye in the hook region, allow the degassing to occur for longer.
   a. TIP: If bubbles persist, we recommend rotating the Ecoflex positive with the liquid PDMS 90° from its original position, the change in direction may allow the pressure to escape more efficiently.
7. Move the negative mold with the liquid PDMS to the levelled 70°C oven overnight with minimal disturbance.
8. The day after, take the mold and now solid PDMS from the oven and let it reach an approximate temperature of 30°C.
   a. Remark: Do not wait longer than 24 hours to detach the plate, as the ease release might dry, or the two materials could absorb it, or when separating, the plates may break by the higher shear force in the two surfaces.
9. To detach the PDMS, use a bladeless scalpel holder by running the handle between the PDMS and the EcoFlex interface. The separation of the two polymers is visible in the walls; continue the scalpel movement from the upper edge of the EcoFlex to separate all four sides.
   a. Remark: The EcoFlex and PDMS have similar mechanical properties and require separation with slow, gentle movements, as the hooks of the PDMS design might suffer defects if not separated with caution; the total time of EcoFlex PDMS separation steps can take up to 20 minutes.
10. From one corner, use the bladeless scalpel to lift the PDMS along with slowly pushing your fingers underneath the PDMS, which will release the junction of the PDMS and EcoFlex; following, slowly pull from the corner in a straight upward direction, and the PDMS should start to detach from the EcoFlex, keep lifting slowly until fully separated.
    a. Avoid rapid pulling or overstretching.
11. Remark: After obtaining the first cast of the PDMS MyoTACTIC plate, we recommend going back to Part A of this protocol following the <u>*positive mold*</u> steps to generate a polyurethane (PU) long-term usage plate, in which case the PDMS will be considered as the 3D printed positive part in the description of the steps; we make the recommendation as the Ecoflex has only worked for casting up to 8 plates. After using the EcoFlex as mold more than eight times, the entire process in this protocol needs to start again from the well-stored 3D printed model.
    a. To do a 2-phase PU polymer, follow the manufacturer's protocol; the steps are in high-similarity to the EcoFlex mix.

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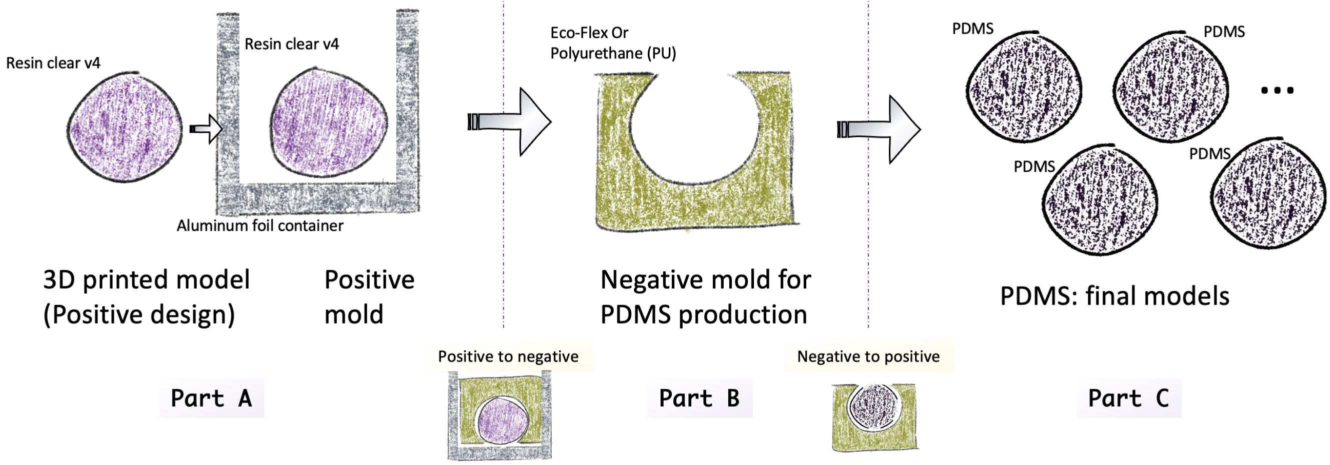
12. Before happy tissue seeding, use a single-edge razor blade to cut the PDMS plate into groups of 6 ± 2 MyoTACTIC wells for tissue seeding purposes.
13. PDMS requires a full isopropanol wash followed by two cycles of water washes with vigorous shaking.
    a. Remark: Do not wait more than one day before proceeding to this step, as the PDMS could have absorbed undesired molecules (i.e., ease release).
    b. It is required to eliminate any ease release agents that may still be in the PDMS.
    c. If sonication equipment is available, we recommend a 15-minute water bath exposure to the sonicating chamber.
14. PDMS wash is done the following way, fill a 50 mL conical tube with isopropanol (IPA; you may need more than one tube depending on how many tissues you are seeding).
15. Submerge wells in the IPA-containing tube, tightly secure the lid and use a vortex mixer in medium to high intensity (7/10) for six cycles of 30 seconds (Appendix E5).
16. Use a different 50 mL conical tube to process with water wash, position the PDMS in the tube with water, tightly secure the lid, and use a vortex mixer in medium to high intensity (7/10) for six cycles of 30 seconds.
17. Followed the washes, thoroughly drying the PDMS with compressed air.
18. Autoclave sterilize the PDMS device within an autoclave bag for 20 minutes, vacuum or gravity.
19. Happy tissue culture seeding.

Appendix

E0: Schematic of protocol



Purple circle – The desired 3D part, in this case, MyoTACTIC.
Gray rectangular block – The aluminum foil container.
Green box – Negative mold, in this case, EcoFlex.
Black circles – the final PDMS parts, MyoTACTIC platform ready to use.

E1: Aluminium foil container for positioning 3D printed model

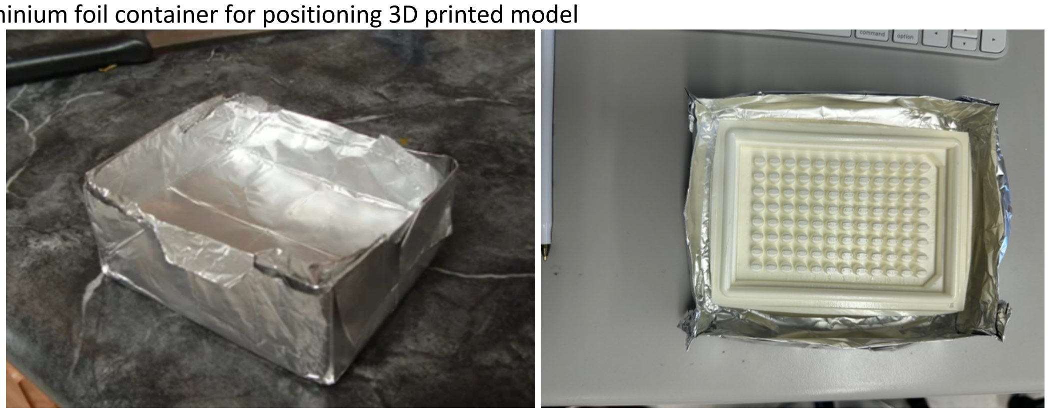
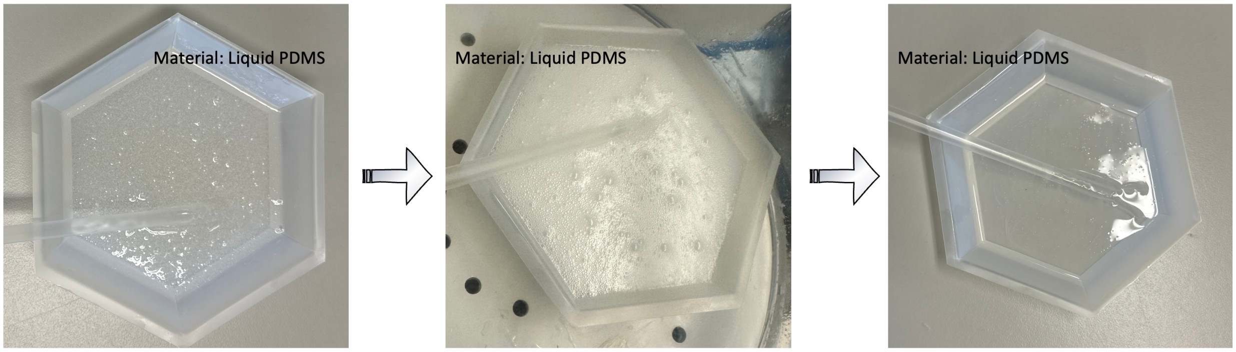


Image from: https://www.instructables.com/Cool-Freeze-Foil-Box/

E2: Expected bubble density for a homogenous mixture of polymers.



Mixing

Degassing

Ready to pour,
Bubbles-free

E4: Negative plate positioning over the 96-well plate for FOTS surface coating.

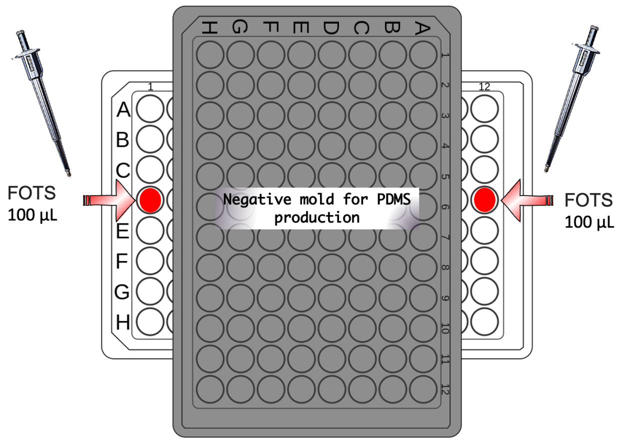


Image modified from: https://www.thomassci.com/Equipment/Single-Channel-Pipettors/_/ADJUSTABLE-VOLUME-PIPETTORS
https://commons.wikimedia.org/wiki/File:96-Well_plate.svg

E5: PDMS MyoTACTIC wells in isopropanol/water wash.