Journal of Visualized Experiments

High-Throughput DNA Plasmid Multiplexing and Transfection Using Acoustic Nanodispensing Technology

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59570R2
Full Title:	High-Throughput DNA Plasmid Multiplexing and Transfection Using Acoustic Nanodispensing Technology
Keywords:	Acoustic Droplet Ejection, High-throughput, Transfection, DNA plasmid, mammalian cells, liquid handling, fluorescent protein
Corresponding Author:	Cyril Couturier, ph.D INSERM U1177 Drugs and Molecules for Living Systems Lille, FRANCE
Corresponding Author's Institution:	INSERM U1177 Drugs and Molecules for Living Systems
Corresponding Author E-Mail:	cyril.couturier@univ-lille.fr
Order of Authors:	Cyril Couturier, ph.D
	Béatrice Colin
	Benoit Deprez
	Nicolas Rocq
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lille, France

TITLE:

High-Throughput DNA Plasmid Multiplexing and Transfection Using Acoustic Nanodispensing Technology

AUTHORS AND AFFILIATIONS:

Béatrice Colin[1], Nicolas Rocq[2], Benoit Deprez[1], Cyril Couturier[1]

[1]Université de Lille, Institut Pasteur de Lille, Institut National de la Santé et de la Recherche Médicale (Inserm) U1177, Drugs and Molecules for Living Systems, Lille, France

[2]L'atelier du Développeur, Marquette lez Lille, France

Corresponding author:

Cyril Couturier (cyril.couturier@univ-lille.fr)

E-mail addresses of co-authors:

Beatrice Colin (beatrice.colin@univ-lr.fr)

Nicolas Rocq (nicolas.rocq@gmail.com)

Benoit Deprez (benoit.deprez@univ-lille.fr)

KEYWORDS:

acoustic droplet ejection, high-throughput, transfection, DNA plasmid, mammalian cells, liquid handling, fluorescent protein

SUMMARY:

This protocol describes high-throughput plasmid transfection of mammalian cells in a 384-well plate using acoustic droplet ejection technology. The time-consuming, error-prone DNA dispensing and multiplexing, but also the transfection reagent dispensing, are software-driven and performed by a nanodispenser device. The cells are then seeded in these prefilled wells.

ABSTRACT:

Cell transfection, indispensable for many biological studies, requires controlling many parameters for an accurate and successful achievement. Most often performed at low throughput, it is moreover time-consuming and error-prone, even more so when multiplexing several plasmids. We developed an easy, fast, and accurate method to perform cell transfection in a 384-well plate layout using acoustic droplet ejection (ADE) technology. The nanodispenser device used in this study is based on this technology and allows precise nanovolume delivery at high speed from a source well plate to a destination one. It can dispense and multiplex DNA and transfection reagent according to a predesigned spreadsheet. Here we present an optimal protocol to perform ADE-based high-throughput plasmid transfection which makes it possible to reach an efficiency of up to 90% and a nearly 100% cotransfection in cotransfection experiments. We extend initial work by proposing a user-friendly spreadsheet-based macro, able to manage up to four plasmids/wells from a library containing up to 1,536 different plasmids, and a tablet-based pipetting guide application. The macro designs the necessary template(s) of the source plate(s) and generates the ready-to-use files for the nanodispenser and tablet-based application.

The four-steps transfection protocol involves i) a diluent dispense with a classical liquid handler, ii) plasmid distribution and multiplexing, iii) a transfection reagent dispense by the nanodispenser, and iv) cell plating on the prefilled wells. The described software-based control of ADE plasmid multiplexing and transfection allows even nonspecialists in the field to perform a reliable cell transfection in a fast and safe way. This method enables rapid identification of optimal settings for a given cell type and can be transposed to higher-scale and manual approaches. The protocol eases applications, such as human ORFeome protein (set of open reading frames [ORFs] in a genome) expression or CRISPR-Cas9-based gene function validation, in nonpooled screening strategies.

INTRODUCTION:

The method presented here describes in detail how to perform DNA plasmid multiplexing and transfection in mammalian cells at high throughput using an acoustic-based liquid nanodispenser in a 384-well plate, even for nonspecialists in the field. This recently published method[1] allows performing as much as 384 independent plasmid DNA multiplexing and transfection conditions in one experiment, in less than 1 h. Single or cotransfection experiments were successful, reaching a near 100% cotransfection within the transfected cells population. This protocol makes transfection easier because most of the tedious, time-consuming, and error-prone steps are now software driven (see **Figure 1** for a general overview). Further efforts have been made to develop dedicated tools to enhance the ease of use while avoiding human errors during the overall process and to promote successful transfection even for nonspecialists in the field. The described protocol includes a "user-friendly" macro spreadsheet that we developed in order to manage 384 independent transfection conditions with multiplexing possibilities of up to four plasmids in each well. The macro automatically generates templates of the source plate(s) to load the expected DNA plasmid volume from starting stock solutions and the files required to drive the nanodispenser software upon the experimental design that has been inputted. As the manual dispensing of DNA in a 384-well source plate is tedious and error-prone, we also developed a dedicated tablet-based application to guide the user while dispensing DNA solution according to the template.

[Place **Figure 1** here.]

Many cell-based experiments start with plasmid DNA transfection, and even if many dedicated reagents have been and are still being developed to enhance transfection efficiency and/or ease the procedure, much remains to be done[2–4]. DNA plasmid cell transfection involves several steps to reach high efficiency, such as an initial complex uptake, endosomal escape, and cytoplasmic transport to the nucleus[5,6]. In addition to calcium precipitation or physical techniques such as electroporation or microinjection using dedicated devices[7], modern chemical methods have focused on enhancing DNA cell delivery while lowering the cell cytoxicity[8,9]. The use of lipids or cationic polymers forming liposome-like complexes and, more recently, nonliposomal polymeric chemistry systems has made transfection easier and more efficient[10]. Despite these developments, cell transfection still requires specific skills to be accurately performed as most of these physical or chemical transfection protocols require scientists to manually prepare each DNA transfection reaction condition, thus impairing the throughput. To circumvent this problem,

reverse transfection protocols have been developed using chemical transfection reagents[11–13], enabling the user to test or combine several plasmids in a faster way. In these protocols, nucleic acid complexes with transfection reagents are formed before seeding the cells on the complexes. However, these reverse protocols are still limited by the manual handling of DNA solutions and by the combination of each of the independent conditions. Although it is feasible to perform them in a 96-well plate format, the DNA preparation and dispenses will be tedious, and there likely will be mistakes. When different amounts of several DNA plasmids are required and multiplexed with each other, cell transfection becomes even harder to achieve and more time-consuming, and human errors become quite inevitable. Scaling up to the 384-well plate format in a reverse transfection approach, in spite of few multiplexed DNA transfection conditions, becomes an impossible challenge due to the following reasons. i) The DNA amounts, transfection reagent, or reaction mixture volumes to manage are lower than 1 µL for each well. ii) The multiplexing of plasmids for 384 independent conditions becomes extremely complicated. The delivery in each of the 384 wells is also iii) highly time-consuming and iv) error-prone. Indeed, dispensing the right solution in the expected wells is hard to manage because the low volumes already dispensed do not allow visual monitoring between the empty and already filled wells. v) Finally, there is a high risk of drying the mixture by evaporation before the cells are added due to the time needed to perform the necessary dispensing steps. In summary, the limiting factor to set up high-throughput DNA plasmid transfection assays appears to be the miniaturization of the assay, which implies low-volume multiplexing and managing that cannot be handled manually anymore but are also hardly achievable in a reliable way by classical peristatic liquid handlers.

As a proof of difficulty to automatize such assays and gain high-throughput, only a few attempts to automate transfection have been published so far: a 96-well plate format using a commercial liquid handling device and calcium phosphate precipitation[14] and, more recently, a lipoplex reagent, and a microfluidic chip enabling 280 independent transfections[15] but requiring specialized skills in this field. Another method, acoustophoresis, allowing liquid levitation and leading to fluid manipulation and mixing, was used to perform DNA transfection in 24- to 96-well plate formats[16]. Although feasible, this approach suffers from an extremely low throughput as the mixing of cells with DNA transfection mixture requires a 60 s incubation for every single point before seeding. This implies a duration of at least 96 min for a complete 96-well plate. Furthermore, this protocol is far away from being amenable to the overall biologists' audience as this work was done with an in-house designed and manufactured device which is currently not available on the market. On the contrary, in the last few years, an easy to use software-driven acoustic-based dispensing technology has emerged with nanovolume dispenser devices. Using focused acoustic energy, these devices allow the tightly controlled ejection of small liquid volumes from 2.5 nL to 500 nL from a source plate to a destination one[17]. This technology, called (ADE), has numerous advantages: it is fully automated, contactless, tipless, accurate, precise, and highly reproducible, and it has a high throughput[18]. First devoted to delivering dimethyl sulfoxide (DMSO) solutions, settings have been enhanced to dispense aqueous buffers[19]. Acoustic nanodispensers, then, seem suitable for reverse cell transfection protocols and could circumvent most of the above-mentioned manual limitations. As no plasmid transfection attempts were previously described using this technology, we recently evaluated the suitability of an acoustic-based dispensing system to perform reverse cell transfection.

Taking advantage of the nanodispenser throughput and ease of use, we optimized a reverse transfection protocol for HeLa cells by cross-testing several parameters that can influence DNA transfection on a 384-well, single plate, namely, the total DNA amount and source DNA starting concentration, diluent volume, transfection reagent, and number of spread cells. The developed protocol circumvents the above-described manual limitations of cell transfection and presents several advantages over other automated transfection attempts. First, it is miniaturized, thus allowing for cost-effective transfection reagent by saving DNA plasmid preparations and transfection reagent. Secondly, it is much more high-throughput and reproducible than the manual protocol (even for beginners), as transfection of an entire 384-well plate can be achieved in less than 1 h. Finally, it is software-driven, allowing the control of the dispensed DNA amount and the multiplexing of several plasmids. Indeed, thanks to the nanodispenser software (**Table of Materials**), the user can elaborate a study plan to control the volumes to be dispensed from a defined source well plate to a destination one.

The protocol presented here is mainly intended for those who have access to a nanodispenser and would like to set up transfection experiments at high throughput, but also for those who want to rapidly optimize their transfection parameters for a given cell type by applying this protocol to cross-test several parameters at high throughput. Indeed, we have shown that optimized parameters identified with this nanoscale protocol can be transposed to larger-scale and manual transfection experiments. Finally, as the transfection reagent used in the present protocol allows DNA or siRNA transfection according to the manufacturer, the protocol is also of interest to those aiming at performing array approaches for gene overexpression or knockdown. The destination plates prefilled with DNA can be conserved up to 7 days before use in a transfection assay without loss of efficacy, which is another advantage of the following protocol for this kind of application.

PROTOCOL:

1. Advance preparations

1.1. Preparation of the peristaltic liquid handler programs

NOTE: For the diluent and cell dispensing steps of the protocol, a dedicated program must be prepared, taking into account the height of the dispensing head to the plate used and the step intent.

1.1.1. For the 1 µL diluent dispense step, mount a 1 µL cassette and prepare a program with the settings as described in steps 1.1.1.1 and 1.1.1.2.

1.1.1.1. Adjust the flow rate parameter to **High** for the best throughput as no biological material damage is expected in this step. Adjust the dispense height to 0.6 mm (according to the cell culture plate used, **Supplemental Figure 1**) to allow the 1 µL drop to touch the bottom of the wells during dispensation.

NOTE: This step is crucial to avoid the retention of the droplets on the dispensing head until reaching a sufficient volume to drop.

1.1.1.2. Adjust the plate clear height to 5.5 mm to allow a free displacement of the dispensing head over the plate after dispensing each row. Visually control the proper settings of the peristaltic liquid handler head height: make sure no drops are retained on the dispensing tips while dispensing and verify that the head is high enough to allow displacement of the head after dispensing each row.

NOTE: Avoiding drop retention is a crucial parameter as it will impair the accuracy of the volume of the dispensation.

1.1.2. For dispensing the 40 µL cell suspension, mount a 40 µL cassette and prepare a program with the settings as described in steps 1.1.2.1–1.1.2.3.

1.1.2.1. Adjust the flow rate parameter to **Low** to dispense cells with a low speed to avoid promoting potential damages to the cells by shear stress and high impact on the bottom of the wells.

1.1.2.2. Adjust the dispensation height to 11.4 mm (according to the cell culture plate used), high enough to lower the cell impact on the bottom of the wells during the dispensing process but low enough to avoid retention of the droplets on the dispensing head. Adjust the plate clear height to 16 mm to allow free displacement of the dispensing head over the plate after dispensing each row.

1.1.2.3. Visually control the proper settings of the peristaltic liquid handler head height: make sure no drops are retained on the dispensing tips while dispensing and verify that the head is high enough to allow displacement of the head after dispensing each row.

NOTE: Avoiding drop retention is a crucial parameter as it will lead to dispensing an unreliable cell number.

1.2. DNA plasmid preparation (classical miniprep extraction protocol)

1.2.1. Grow a transformed DH5α bacteria strain in LB medium supplemented with 125 µg/mL ampicillin selection antibiotic (**Table of Materials**) overnight at 37 °C and under gentle agitation (200 rpm) on an orbital shaker (**Table of Materials**).

1.2.2. Harvest 2 mL of the culture, pellet the cells by centrifuging for 5 min at 6,000 x *g*, and discard the supernatant.

1.2.3. Resuspend the cell pellet with 250 µL of resuspension buffer containing RNase A (**Table of Materials**). Add 250 µL of lysis buffer and incubate for 5 min at room temperature, according to

the manufacturer's instructions.

1.2.4. Stop the lysis reaction by adding 300 µL of neutralization buffer (**Table of Materials**). Centrifuge the tubes for 5 min at 11,000 x *g*.

1.2.5. Place a new plasmid minicolumn (**Table of Materials**) in a 2 mL collection tube and decant the supernatant in the column by centrifuging for 1 min at 11,000 x *g*.

1.2.6. Discard the flow-through and place the minicolumn back in the collection tube.

1.2.7. Wash the plasmid minicolumn with 500 µL of optional washing buffer (**Table of Materials**) and centrifuge for 1 min at 11,000 x *g*, according to the manufacturer's instructions.

1.2.8. Discard the flow-through and place the plasmid minicolumn back in the collection tube.

1.2.9. Add 700 µL of washing buffer (**Table of Materials**) supplemented with ethanol and centrifuge for 1 min at 11,000 x *g*, according to the manufacturer's instructions.

1.2.10. Discard the flow-through and centrifuge the plasmid minicolumn and its collection tube 1x more for 2 min at 11,000 x *g* to dry the silica membrane.

1.2.11. Place the dried plasmid minicolumn in a new 1.5 mL tube and add 30 µL of distilled water prewarmed at 60 °C, incubate it for 2 min at room temperature, and then, centrifuge it for 1 min at 11,000 x *g*.

1.2.12. Discard the plasmid minicolumn and keep the eluate containing the purified DNA plasmid.

1.2.13. Measure the DNA concentration of the eluted DNA using a microvolume spectrophotometer (**Table of Materials**).

1.2.13.1. Turn on the spectrophotometer and choose the **DNA measurement** settings.

1.2.13.2. Raise the sampling arm of the spectrophotometer and pipette 1 µL of water onto the measurement pedestal to perform a blank calibration.

1.2.13.3. Lower the sampling arm, start the blank measurement, and wait for completion.

1.2.13.4. Raise the sampling arm and wipe the sample from the upper and lower pedestals.

1.2.13.5. Pipette 1 µL of the DNA solution onto the lower pedestal to measure it.

1.2.13.6 Lower the sampling arm, start the DNA concentration measurement, and wait for completion.

1.2.13.7. Raise the sampling arm and wipe the sample from the upper and lower pedestals.

1.2.13.8. For further DNA concentration measurements, repeat steps 1.2.13.5–1.2.13.7.

1.2.14. Once the measurements are finished, store the DNA solutions at 4 °C until use.

2. Experimental design and generation of the picklists to drive the ADE-based dispenses

NOTE: A dedicated "user-friendly" spreadsheet macro was developed to manage DNA amounts and mix up to four plasmids in a 384-well plate format. Based on the entered experimental design, this macro generates the necessary files to drive the ADE-based DNA transfection protocol by nanodispenser. In order to generate these files, several fields have to be filled in the **Template Sheet** as shown in **Figure 2**.

[Place **Figure 2** here]

2.1. Enter the nanodispenser protocol parameters in the pink fields. Set the transfection reagent (TR) mixture value to 500 nL. Set the minimal volume value in the source plate wells to 4 µL. Set the maximal volume in the source plate wells to 11.5 µL.

NOTE: The nanodispenser used here can only transfer a maximum of 500 nL in one run of ADE. These pink fields are prefilled with the recommended values but can be modified according to the user needs.

2.2. Enter 100 ng/µL DNA starting concentrations in the blue fields corresponding to the underlying DNA.

NOTE: This value is the optimal concentration previously defined but can, however, be modified for different user needs.

2.3. Enter the desired DNA amount in the grey/green fields. Enter the amounts and plasmid names for the 384 wells, ensuring the same spelling if the same plasmid is used in several wells.

2.4. Generate the source plate design, the picklists file, and 384-well pipetting guide files. Click on **Generate Picklists** to allow the macro to generate the 384-well dispensing guide, the DNA-picklist file, and the TR-picklists file from data collected on the corresponding sheets. If requested, correct the orange-filled cell values as it indicates errors or volumes that cannot be handled by the nanodispenser.

2.5. Print the template(s) from the **Source Plate** sheet. Plasmid names and minimal volume to fill in the wells are indicated. Likewise, transfection reagent mixture volumes that will next have to be filled in the following wells are indicated as **TR** and highlighted in green.

3. DNA source plate preparation using the 384-well pipetting guide application

3.1. Dilute the stored DNA plasmid from step 1.2.14 to 100 ng/µL using distilled water.

3.2. Calibrate the 384-well grid to the plate dimensions: open the 384-well pipetting guide application on a tablet (**Figure 3**). Place the source plate on the grid on the lower screen, and in the upper left calibration menu, click **+** or **-** (or use the red cursor) to enhance or reduce the size of the grid and wells in order to adjust the green wells to the four corner wells of the plate.

[Place **Figure 3** here]

3.3. Using double-sided tape, mount the 3D-printed plate adapter on the screen to avoid source plate movements while dispensing. If needed, move the calibrated grid using the rotation arrows and **Up**/**Down**/**Right**/**Left** buttons to adjust the grid on the screen to the plate position. Once the grid and well sizes are properly calibrated and located, tick the **Lock calibration** box.

3.4. Click on **FILE** and open the **384 wells pipetting guide.csv** file. Follow the screen instructions to manually dispense the indicated volume of the indicated plasmid at the indicated concentration in the white highlighted well corresponding to the proper target destination of the expected plate. Use **-** or **+** arrows to go back or further in the DNA dispensing process. Stop dispensing when reaching the first **Transfection reagent** solution to load.

3.5. Once the DNA dispensations are finished, remove the source plate from the adapter. If several source plates have to be filled, then place a new source plate on the adapter and follow the dispensing instructions. Once the DNA dispensation has finished, centrifuge the DNA-filled source plate(s) (at 1,500 x *g* for 2 min) to ensure proper liquid leveling and to remove bubbles leading to inaccuracy in the ADE-based transfers.

4. Peristaltic liquid handler-based 1 µL diluent dispensation in the destination plate

NOTE: Perform steps 4.1–4.5 in a biological safety cabinet.

4.1. Disinfect the 1 µL cassette head by spraying it with a spray disinfectant (**Table of Materials**), and allow it to enter the tip holder. Absorb the remnant disinfectant on absorbing paper. Mount the 1 µL cassette on the peristaltic liquid handler device. Turn on the device and make sure the cassette type setting is correct (1 µL), as well as the plate format (384 wells).

4.2. Disinfect the entire lumen of the tubing: insert the tube organizer (holding the eight tubes together) in a sterile vessel and fill it with 5 mL of 70% alcohol. Using the priming function of the peristaltic liquid handler, first flush the alcohol in the tubing and then rinse it by passing 5 mL of distilled water and 5 mL of serum-free medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 100 U/mL penicillin-streptomycin; **Table of Material**), successively filling in the same vessel.

4.3. Prime the tubing with serum-free medium by filling a new sterile vessel with 10 mL of

prewarmed serum-free medium and diving the tube organizer in it. Press the prime button of the peristaltic liquid handler for about 10 s. Make sure the tip is not clogged by visually inspecting the flow from the dispensing head.

4.4. Fill the plate with 1 µL of diluent. Place a sterile 384-well culture plate (destination) on the peristaltic liquid handler plate carrier and remove its lid.

4.5. Run the precalibrated program to dispense 1 µL in each well of the 384-well plate. The dispensing time is approximately 8 s. Then replace the lid of the 384-well plate.

NOTE: Alternatively, this step can be handled manually, in a safety cabinet, using a multichannel micropipette.

5. Performance of a survey to control the manually dispensed volumes

NOTE: For details, see **Figure 4**.

5.1. Run the nanodispenser program, go to the diagnostic tab, tick the source plate **Out** box, load the source plate on the plate holder, and tick **In** to enter the plate.

5.2. Select **Survey** in the **Miscellaneous** menu and click on **Launch**. Set the nanodispenser to the aqueous buffer dispensing mode by selecting **384LDV_AQ_B2**, and press **Ok**. Select the prefilled wells to analyze and click on the **Go** button. Verify that the measured volumes match the expected ones and ensure no wells have been loaded with volumes of more than 12 µL as this will avoid transfers.

[Place **Figure 4** here]

6. ADE-driven DNA dispensation into the destination plate

6.1. Run the picklist software, set the 384-well source and destination plate types to 384_LDV and Greiner 384PS_781096, respectively (**Figure 5**). Set the device to aqueous buffer dispensing mode by selecting **384LDV_AQ_B2**.

[Place **Figure 5** here]

6.2. Click on **Import**, select the DNA-Picklist.csv file, and save the protocol. Click on **Simulate** to perform a simulation of the programmed dispensations to make sure that the picklist matches the expected experimental design. Click on the **Run** button to start the dispensing program: when asked, insert the requested source plate (DNA solutions manually filled) and the destination plate (diluent-filled) in the nanodispenser.

NOTE: The dispensing time is approximately 5–20 min for a complete 384-well plate, depending on the selected volumes and the total number of dispensations in the experimental design.

6.3. Alternatively, pause the protocol here as the diluent- and DNA-filled plates can handle dry or frozen storage for up to 7 days. For dry storage, let the plates dry on the bench at room temperature and then store them the same way. Thaw and centrifuge (at 1,500 x *g* for 2 min) frozen and stored plates before use in a transfection step (section 7).

7. ADE-driven transfection reagent dispensation

7.1. In a biosafety cabinet, extemporaneously dilute lipopolyplex transfection reagent in serum-free medium to a 1x final concentration. Vortex and immediately dispense this transfection reagent mix according to the predefined source plate(s) designed by the macro and using the precalibrated 384-well pipetting guide application as described in step 3.4.

NOTE: Do not centrifuge the source plate once it is loaded with the reagent as no transfection is noticed after centrifugation.

7.2. Run the nanodispenser program to perform a "survey" as described in section 5, in order to control the volumes of all the manually filled **TR** wells of the source plate(s) to avoid dispensing errors due to volumes exceeding 12 µL.

7.3. Perform a reset of the DNA picklist in the picklist software, and verify that the device parameters are still set to aqueous buffers and to the source and destination plate types used, as in step 6.1.

7.4. Click on **Import** and choose the TR-Picklist.csv file. Save the protocol when prompted, and (this is optionally but strongly recommended) perform a simulation of the programmed transfection reagent mixture dispensations to ensure proper design of the dispensations by clicking on the **Simulate** button.

7.5. Click on the **Run** button to start the dispensing program: as requested, place the source plate(s) (TR-mixture-filled) and the destination plate (diluent- and DNA-filled) in the nanodispenser.

NOTE: The dispensing time is approximately 15–20 min for a complete 384-well plate when dispensing 500 nL of TR mixture.

7.6. Incubate 15–30 min at room temperature after adding the TR to the DNA as indicated by the manufacturer's protocol.

8. Peristaltic liquid handler-based cell dispensation

8.1. Prepare the peristaltic liquid handler for dispensing cells. Disinfect a 10 µL cassette head by spraying it with Aniospray Surf 29 Disinfectant and absorbing the remnant on paper. Mount the cassette on the peristaltic liquid handler device, change the cassette type setting to 10 µL, and

make sure the plate format is set to 384 wells.

8.2. Disinfect the 10 µL cassette tubing as previously described in step 4.2. Dive the tube organizer in a sterile vessel and flush the tubing with 5 mL of 70% alcohol, then with 5 mL of distilled water, and finally, with 5 mL of serum-free medium, successively filling in the same vessel and until each tube is empty.

8.3. Prepare the cell suspension to dispense. From a confluent HeLa cell B10-culture dish, wash the cells 1x with 1x phosphate-buffered saline (PBS) solution, and then dissociate the cells with trypsin/EDTA for 5 min at 37 °C.

8.4. Verify the cell dissociation under a microscope and stop the trypsin/EDTA action by adding 10 mL of complete medium (DMEM supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin; see the **Table of Material**) in the culture dish. Harvest cells in a 50 mL tube and count the cells under the microscope, using a Malassez cell or an automatic cell counter.

8.5. Prepare at least 25 mL of HeLa cell suspension at a concentration of 37,500 cells/mL in complete medium (i.e., 1,500 cells/40 µL) for a complete 384-well plate, to ensure tube priming and 40 µL/well dispensation.

8.6. To dispense the cells, fill a new sterile vessel with the prepared cell suspension and stir it to avoid sedimentation leading to inaccuracy in the cell density of the dispensation. Insert the tube organizer in this solution and press the **Prime** button until the cell suspension is starting to flush from the dispensing head. Make sure the tip is not clogged by visually inspecting the flow from the dispensing head while flushing, and ensure each tube is loaded with cell suspension.

8.7. Load the DNA and TR-filled 384-well destination plate on the peristaltic liquid handler plate carrier and remove its lid. Run the precalibrated program to dispense 40 µL of the cell suspension on the complete 384-well plate (i.e., 1,500 cells/well). The dispensing time is about 8 s. Replace the lid of the 384-well plate.

NOTE: Alternatively, this 40 µL can be manually dispensed using a multichannel micropipette.

9. Custom biological assay (cell transfection efficiency monitoring)

NOTE: Following the experimental settings and intent of the experiment, use the required methods for luminescence, fluorescence, high-content screening, and reverse transcription quantitative polymerase chain reaction (RT-qPCR). In this section of the protocol, cell transfection efficiency is evaluated by automated fluorescence microscopy and image analysis.

9.1. Incubate the plate at 37 °C with 5% CO_2 in a water-saturated atmosphere and until proper protein expression.

NOTE: Here, a 48 h incubation time is used for HeLa cells to monitor the transfection efficiency,

using tdTomato- and mVenus-expressing plasmids.

9.2. Remove the culture medium 48 h posttransfection by inverting the plate, add 30 µL/well of 10% formalin using the peristaltic liquid handler (10 µL cassette), and incubate for 15 min at room temperature.

9.3. Remove the formalin by inverting the plate; then, incubate the cells for 15 min at room temperature with 0.1 ng/mL Hoechst diluted in 1x PBS solution.

9.4. Wash the cells 3x for 15 min with 80 µL of 1x PBS adjusted to pH = 8 in order to recover the high fluorescence signal lost by the 6.9 pH of the formalin solution incubation step.

9.5. Using an automated fluorescent microscope, acquire images of two or three fluorescent channels (Hoechst, tdTomato, and mVenus) sequentially with 10x objectives and a proper emission filter set (4',6-diamidino-2-phenylindole [DAPI], dsRed, and fluorescein isothiocyanate [FITC], respectively).

9.6. To evaluate transfection efficiencies, use image analysis software to determine the transfection efficiencies using script analysis based on nuclei staining.

REPRESENTATIVE RESULTS:

In order to determine if the ADE technology could be used for an automated reverse transfection protocol, we monitored cell transfection efficiency by fluorescence microscopy, using a red fluorescent tdTomato expressing plasmid. First aiming at determining the best transfection parameters, different diluent volumes and total amounts of DNA were cross-tested. Diluent volume was used to allow the DNA droplets, once dispensed, to spread all over the wells to circumvent inhomogeneous transfection observed in preliminary experiments (i.e., only in the center of the wells). As shown in **Figure 6A**, the transfection of HeLa cells using lipopolyplex reagent[20] was successful. Interestingly, by using a 1 µL diluent volume, DNA amounts ranging from 5 to 30 ng showed the same efficiency and up to 90% cell transfection compared to higher amounts, such as 50 and 100 ng, for which an abrupt decrease was observed. We tried various diluent volumes ranging from 15 nL to 4 µL and identified 1 µL to be the best condition, as significantly exemplified here using 30 ng of DNA.

[Place **Figure 6** here].

In order to further enhance the throughput of this protocol, we next examined if a source plate storage prefilled with DNA and diluent solutions could be stored and used at a later stage. Two ways of efficiently storing DNA were tested, namely dry storing the plate by letting it dry on the bench or frozen storage (at -20 °C). Both storage methods did not lead to significantly different results than freshly dispensed DNA solution stored for up to 7 days (**Figure 6B**), and both methods made it possible to perform transfection from stored DNA prefilled plates, such as a bank of plasmids.

Finally, as plasmid transfection most often occurs using at least two different plasmids, we next examined the DNA multiplexing ability of the protocol presented here using the best identified conditions (1 µL of diluent and 30 ng of DNA). The previously used tdTomato red-fluorescent-protein-expressing plasmid was modified to express mVenus, a bright yellow fluorescent protein, and both were then used in cotransfection attempts. Red- or green-fluorescent-positive cell analysis (**Figure 6C**) showed the transfection efficiency to be about 80%; however, in the red population, nearly 100% of the cells were also cotransfected with the mVenus-expressing plasmid as can be seen in the representative software-based image analysis of **Figure 6D**.

FIGURE AND TABLE LEGENDS:

Figure 1: Experimental workflow. Schematic representation of the optimal automated high-throughput reverse transfection protocol (from experimental design to custom biological assay). Manual steps are indicated by the hand symbol and the approximate time for each step is written in a red box.

Figure 2: Generation of the picklists to drive the ADE dispensation using the spreadsheet macro. Several parameters have to be filled, namely (**1**) the transfection reagent (TR) and the minimal/maximal volumes to be used in the source plate, (**2**) the initial plasmid concentrations to be dispensed in the source plate, and (**3**) the whole-plate design, including the expected plasmid amounts and multiplexing in each of the 384-wells. (**4**) **Generate Picklists** activation allows the different fields to be verified and, once properly filled, picklists for DNA and TR dispensation and the necessary source plate template are automatically generated.

Figure 3: Use of the 384-well pipetting guide application. (**1**) Calibration of the 384-well grid to the plate size; (**2**) mount of a universal 3D-printed plate adapter used to attach to the tablet using double-sided tape; (**3**) placement of the plate on the adapter; (**4**) displacement of the grid to center it to the mounted plate. (**5**) Lock of the calibration step. (**6**) Opening of the 384 wells pipetting guide.csv file. (**7**) Given the file list, the application will indicate the expected source plate name, reagent (DNA or transfection reagent), the concentration, and the volume to dispense into the target wells, which will be illuminated one by one. (**8**) Left and right arrow buttons allow the user to follow the pipetting guide to easily dispense the reagents according to the spreadsheet macro source plate template(s).

Figure 4: Defining the survey software parameters. (**1**) Start the nanodispenser program. (**2**) Open the **Diagnostics** tab. (**3**) Insert the source plate by checking **Out** for the source plate and, then, **In**. (4) In the Miscellaneous box, select **Survey** in the drop-down menu. (**5**) Launch the survey program by clicking on **Launch**. (**6**) Define the source plate type in the menu. (**7**) Select the prefilled wells to measure. (**8**) Start the analysis by clicking on **Go**. (**9**) Once the survey is performed, the measured volumes are written in the corresponding selected wells.

Figure 5: Performance of the picklist-based dispensations. (**1**) Start the nanodispenser software. In the Protocol tab, select (**2**) the sample plate format and (**3**) the destination plate type. (**4**) Select the **Pick List** tab. (**5**) Click on **Import** and select the proper *.csv file (DNA-PickList or TR-Picklist).

(**6**) Once selected, click on **Import**. (**7**) Perform a dispensation simulation by clicking on **Simulate**. (**8**) Start the programmed dispensation by clicking on **Run**.

Figure 6: Representative results. (**A**) Impact of the DNA amount and diluent volume on the transfection efficiency. HeLa cells were reverse transfected using the nanodispenser device and lipopolyplex, using a 1x concentration as recommended by the manufacturer. Of the recommended diluent (serum-free medium), 15–4,000 nL was used with 10–100 ng amounts of red-fluorescent-expressing plasmid (tdTomato). Transfection efficiencies were determined 48 h posttransfection using image-based analysis software. The results are expressed as a percentage of transfected cells for the increasing DNA amount, and the diluent volume shows the optimal conditions: 30 ng of total DNA with an increasing diluent volume and 1 µL of diluent with increasing DNA amounts. The error bars represent the SEM with $n \geq 4$. Two-way ANOVA and Bonferroni post-test were used for statistical analysis. *$p < 0.05$ compared to other dots. (**B**) Stability of the prepared DNA plates. Diluent (1 µL) was dispensed using the peristaltic liquid handler, and 30 ng of DNA was dispensed and immediately transfected using lipopolyplex reagent dispensed by ADE (control) or either stored at room temperature once dry or frozen at -20 °C. At days 0, 2, or 7, dry DNA was rehydrated with 1 µL of diluent dispensed using the peristaltic liquid handler, and frozen plates were thawed at room temperature. Cells were then seeded using the peristaltic liquid handler according to the described protocol. The error bars represent the SEM with $n \geq 3$. Two-way ANOVA and Bonferroni post-test were used for statistical analysis. ns = nonsignificantly different. (**C**) Plasmid DNA cotransfection efficiency. HeLa cells were transfected with 30 ng of mVenus- and tdTomato-expressing plasmid loaded in two separate source wells (using a 1.7 ratio of mVenus over tdTomato in order to level their relative fluorescence output). Transfection efficiencies were compared 48 h posttransfection using image-based analysis software and were expressed as a percentage of transfected cells and a percentage of cotransfected cells within the transfected population. The percentage of cotransfected cells was determined by calculating the green-fluorescence-expressing cell number in the red fluorescent population cells. The error bars represent the SEM with $n \geq 3$. Two-way ANOVA and Bonferroni post-test were used for statistical analysis. ns = nonsignificantly different. (**D**) Representative fields of fluorescence microscopy from the image acquisition shown in panel **C** using three fluorescent channels (Hoechst, tdTomato, and mVenus) sequentially acquired by an imaging platform (**Table of Materials**), using 10x objectives and a proper emission filter set (DAPI, dsRed, and FITC, respectively). This figure has been modified from Colin et al.[1].

Supplemental Figure 1: Diagram showing a suitable dispense height for the drop to touch the bottom of the well to avoid its retention on the dispensing tip. On the left, proper settings allow the drop to spread on the well surface avoiding its retention on the dispensing tips. On the right, bad settings lead to droplet retention that can be observed during the head movement to the next raw.

DISCUSSION:

The establishment and optimization of an accurate high-throughput transfection method for a given cell line require scientists to follow some key parameters described in this section. We strongly encourage starting with the recommended values throughout the protocol as these

settings optimized for HeLa cells also proved to be efficient for HEK cells. However, as the best parameters may depend on the cell lines and transfection reagents, optimal conditions can be defined by the varying cell number, diluent volume, total DNA amount, and transfection reagent nature, concentration, or even volume used as was the case during the optimization of this protocol for HeLa cells[1].

The overall protocol presented here has been developed and further optimized to allow cell transfection even by novices in the field. In order to reach this goal, key tools to render the protocol as simple as possible and avoid human errors have been developed: a user-friendly spreadsheet macro to easily design the experiment and a tablet application to guide the user to properly fill the source plate(s).

Thus, to ensure the reliability of the protocol, only a few critical steps have to be controlled: i) a proper experimental design; ii) the proper peristaltic dispensations from the classical peristaltic liquid handler; iii) the proper DNA and transfection reagent acoustic-based dispensations; iv) avoiding the centrifugation of the source plate before the transfection reagent dispensation as that seemed to impair transfection. Following these few recommendations would ensure the efficient transfection of the cells.

Proper experimental design

The experimental setup has been rendered user-friendly by the development of the macro spreadsheet which just has to be filled with the expected DNA plasmids and some key parameter values. Once filled, the macro first analyzes the entered parameters to detect potential errors, such as suitable minimal and maximal volumes of the content of the source wells and the transfection reagent dispensation volume. Furthermore, based on the DNA concentrations entered in each of the four possible rows and the plasmid quantity entered in the underlying fields, the macro verifies if the expected volumes to dispense are multiples of 2.5 nL (volume of the drops dispensed by the nanodispenser). Once a check for any errors has been performed, the macro calculates the total amount of each DNA sample that will have to be dispensed and, then, designs the source plate template (by sorting the DNA names in alphabetical order). The volumes indicated in the source plate(s) take into account the working volumes expected in a source well (calculated from the minimal and maximal volume values filled in the template sheet). All the expected DNA dispensations in each of the wells are then written on the DNA-picklist sheet. The list of DNA-transfected wells is then used to write the TR-picklist sheet using the transfection reagent volume indicated on the template sheet. The volumes calculated on the source plate sheet are then transferred to the 384-well pipetting guide sheet. Data from the DNA-picklist, TR-picklist, and 384-well pipetting guide are then used to generate the corresponding files in the *.csv format.

Proper DNA and TR dispensation on the source plate

As dispensing on a 384-well source plate and, more specifically, locating the target well can promote errors and are furthermore time-consuming, we have developed a dedicated tablet-based application similar to iPipet[21]. Unlike iPipet, the one described here can be used with Android (only Android version 4.4 and up is supported). Based on the 384-Wells-Pipetting-

Guide.csv file generated by the macro spreadsheet, it helps the user in the overall dispensing process. Whereas its use for a few dispensations is not worth it, it can be interesting to save time and avoid errors if a large number of DNA and TR dispensations are expected. The .csv file must contain well, plate, name, concentration, and volume information. This application could then be used for other applications, such as dispensing solutions (reagents, dye, compound, etc.) in the target well, according to a user dedicated csv file. Furthermore, it allows the user to illuminate an entire row or line by entering the relevant information into the target well column using this expected format: Row_1 (to 24) or Line_A (to P).

Troubleshooting poor cell transfection efficiency

Several parameters described below may impair cell transfection in the described ADE-based protocol and would have to be individually checked and circumvented in case of efficiency problems.

One of the first important parameters for transfection is the quality of the cells and the density used during sowing. Although each cell type will require different parameters, some of them must be respected to ensure a successful transfection. First of all, the cell suspension must be prepared extemporaneously from a subconfluent plate to avoid cell stress before transfection, and they should not be left lying on the bench for too long (2 h maximum). Second, the seeding density of the cells must be low enough for two reasons: to avoid cellular contacts and promote a high cell surface once spread but also because actively dividing cells better take up foreign nucleic acid[22,23]. Unfortunately, the optimal cell density for transfection varies depending on cell types and transfection technology and has to be determined for each cell line. These are crucial parameters for ensuring effective transfection.

Another important parameter that can modulate transfection efficiency is the cell passage number[24]. Indeed, cells in culture are continually subjected to evolution due to competition and natural selection. It is well known that differential gene expression between low and high cell passage numbers is expected in most cell lines due to dedifferentiation as the passage number increases. Following this phenomenon, transfection efficiency may also be affected. However, passage-related effects have been demonstrated to be heavily dependent on the cell line and the culture conditions. On top of that, what is considered a “high” passage level varies from one cell line to another. This means that the passage number range under which a set of experiments can be reliably performed has to be determined for each given cell line.

Another parameter which enhances the difficulty in determining the best conditions for transfection is that culture medium composition also plays a crucial role since the presence of serum and/or antibiotics modulate transfection efficiency. Indeed, most commercial protocols recommend the use of serum-free medium during the transfection step to enhance efficiency or circumvent problems of poor efficiency[3,25–27]. However, this parameter is indeed more complex to apprehend as it has been shown that, for a given cell line, early versus late passages may enhance or lower efficiency depending on serum presence or absence in the culture medium[28]. Other researchers preconize the use of antibiotic-free medium for the passage before the transfection when culturing cells, in order to obtain high-quality cells for transfection[29]. To

conclude, when optimizing transfection conditions for a given cell type, each of these parameters should be tested: early or late passage cells and the use of medium with or without serum during the last passage before harvesting the cells and/or during the transfection step itself.

During the optimization of the protocol presented here, two kinds of reagent were used: a liposomal reagent forming liposome-like complexes and lipopolyplex reagent, a nonliposomal polymeric compound[1]. Whereas we had success for years manually transfecting HeLa cells with the first one, poor transfection efficiency was observed in the current automated protocol. This was probably due to a required vortexing step when mixing DNA with transfection reagent that cannot be performed in the 384-well plate format. The lipopolyplex does not need such a step and, therefore, led to higher transfection efficiencies in all the settings tested. Although this has not been confirmed in the current study, avoiding transfection reagents that require a physical mixing step such has pipetting or vortexing will probably lead to better results.

We also recommend the use of a transfection reagent compatible with reverse transfection as the presented protocol is based on a reverse transfection. Some cells are known to be difficult to transfect and some dedicated chemical compounds are developed to promote higher transfection efficiency[30,31]. If aiming at transfecting hard-to-transfect cells, we recommend testing the given cell-type or cell-line-dedicated transfection reagents, assuming reverse transfection is feasible with these ones.

Several parameters detailed in the underlying paragraphs may have an impact and somewhat impair the ADE dispensing process, possibly ruining the final experiment.

The nanodispenser was developed to dispense 2.5 nL droplets from source wells filled with 3–12 µL of aqueous buffer (i.e., 9 µL working volume). The nanodispenser device used in this study integrates a dynamic fluid analysis technology for the determination of the fluid composition and liquid height in the source plate in order to control the power needed to eject 2.5 nL droplets[19]. While filling the source plate manually or by the classical peristaltic liquid handler, the volumes are most often not so accurate than those determined by the dynamic fluid analysis. This is a crucial point to take into account as the device is not able to dispense from source wells loaded with more than 12 µL. Thus, their presence would compromise the experiment. Of course, a list of the performed dispensations can be generated at the end of the program but this requires the user to adjust volumes and run a program to recover the missed dispensations only. To avoid these disagreements, it is recommended to perform a “survey” once the DNA plasmids have been loaded to verify the expected volume in each concerned well.

A crucial parameter for droplet ejection is the variation in liquid surface tension[17,32]. Water DNA solutions are known for their viscosity; this might impair the dispensing process by ADE. Whereas the physical parameters were not determined in our previous study[1], higher DNA solution concentrations in the source plate resulted in lower transfection efficiency, even for the same total amount of DNA dispensed, probably because of this phenomenon. Thus, we recommend using a plasmid dilution of 100 ng/µL for best results, although other concentrations might be tested for user convenience. To ensure proper ADE with the user-needed DNA concentration, a

colorant can be added to the solution to monitor the droplet ejection in the wells or, even better, on the plate lid. As the presented protocol's first goal was to gain high throughput, cheap minicolumn-based plasmid DNA minipreparation kits compatible with plate-based high-throughput plasmid purification protocols were used. Whereas it worked properly during the performance of the experiment, in case of low efficiency and poor cell viability after transfection, it is recommended to use higher-grade DNA purification methods such as midi- or maxi-preparations or even endotoxin-free commercially available kits that would ensure a better DNA purity and lower toxicity for the cells[33].

Troubleshooting inhomogeneous cell transfection over the well surface

First attempts when setting up the protocol led to cell transfection in the center of the wells, where the drops were sent by ADE. Indeed, we noticed that the DNA and transfection mixture was drying before cell addition due to the low volumes dispensed (barely 500 nL). To circumvent this problem, a diluent dispensing step was added to allow the DNA/TR mixture to spread all over the wells before cell addition. This resulted in a homogeneous cell transfection in the well. Thus, when reproducing the experiment and the DNA/TR mixture does not spread all over the wells, the diluent volume may be adjusted according to the user's needs.

As the acoustic liquid handler can distribute volumes in the nanoliter range, the described method potentially does not have any technical limitations. However, we noticed that aqueous-solution-filled wells are subject to evaporation, which could represent a limitation. If the exact volumes to be dispensed are lowered by this evaporation, the nanodispensing will not be performed to the expected end. In this case, an error report is generated by the nanodispenser. To circumvent this problem, use higher volumes than expected while staying in the acceptable upper range (indeed, lower than 12 µL). However, if evaporation leads to the impairment of some dispensations, an error report is generated by the nanodispenser. This file can be used to fill the source plate with new reagent to be dispensed on the concerned destination wells. Doing this in a short time interval did not seem to impair the transfection efficiency.

The protocol described here is the first one to obtain such throughput for independent DNA plasmid transfections. Best rates reached before were for 288 different conditions that required highly specialized skills to be performed simultaneously[15]. This, apart from its high throughput, the current protocol has other significant advantages as the overall process has been optimized to allow its use by nonspecialists and tools have been developed to avoid errors, namely i) a dedicated spreadsheet macro allowing the easy design of the experimental template, ii) the automatic generation of the corresponding DNA and transfection reagent source plate(s) template by this macro, iii) the generation of two ready-to-use files to control the software-driven dispensations of DNA and transfection reagent by the nanodispenser device, and iv) the export of a "384-well pipetting guide" file corresponding to the source plate(s) designed, to be used by a dedicated tablet-based application also developed in order to avoid human errors while dispensing in the 384-well source plate(s).

Future improvements of the protocol

In order to enhance throughput and reproducibility, the source plate filled with plasmids could

be stored at 4 °C or frozen as usual for DNA stock solutions. Furthermore, we showed that the preloaded destination plate prefilled with DNA can also be stored dry or frozen for at least 7 days before adding the transfection reagent and cells, leading to the enhancement of the overall throughput and ease of the protocol. DNA conservation for more than 4 years has previously been reported using optimized media[34] and should, therefore, be tested in the context of this protocol as it will push it a step further, enabling the long-term storage of plates prefilled with banks of plasmids and ready-to-use in transfection experiments.

We previously showed that the identified optimal transfection conditions could be transferred to higher-scale experiments, from 96-well to 10 cm culture dishes[1], by calculating the DNA amount, transfection reagent volume, and cell density that should be used based on the optimized 384-well plate protocol. As 1536-well plate dispensation is manageable by the nanodispenser too, the protocol can also be performed at a lower scale, thus enhancing its throughput. However, a major limitation to reach this format is the ability to dispense cells and manage the final read-out in this format. Cell dispensing by ADE has already been successfully performed in the 1536-well plate format[35] using a solution of neutral density that prevented cell seeding and ensured equal cell density in time. Based on the cell number used here and the surface ratio of 384 (0.056 cm^2) versus 1536 wells (0.025 cm^2), 500–650 cells would be dispensed in this last format. Such a cell dispensation number range has been shown to be highly reliable if 14%–18% concentrated solution of neutral density is used. Under these settings, 100 nL of cell suspension would be distributed over 1536 wells. With a working solution of 5–8 µL in such wells, adding 5–8 µL of culture medium using classical peristaltic liquid handlers would dilute the antiseeding solution to less than 0.3%, thus allowing proper cell seeding. Transfecting cells on such a low scale thus seems technically possible; however, the residual concentration effect on transfection efficiency remains to be determined by further work.

Using source plate types bearing higher working volumes to dispense cells, such as 384-well polypropylene source plates having a working volume of 45 µL, would allow 100 nL cell solution dispensations from only four source wells for an overall 1536-well plate. Furthermore, new nanodispensers are able to dispense drops of 25 nL instead of the 2.5 nL that was used in this protocol. This drop size, then, divides 10-fold the dispensing time but imply to use multiple of 25 nL volumes which, however, stay compatible with the different volumes dispensed in the protocol presented here.

Based on these latest works and technological improvements, a further ADE cell dispensing step to achieve a 1536-well plate transfection could be easily added to the current protocol. However, reaching such miniaturizations is only worth it if the bioassay is concomitantly feasible in such a miniaturized format.

In conclusion, we developed an easy, high-throughput and accurate transfection method bearing several advantages due to miniaturization: (1) lowering the costs of transfection reagent; (2) reducing the waste of DNA preparations; (3) ensuring that even beginners can successfully perform cell transfection. Indeed, it requires only a few easy manual steps, namely diluting DNA to 100 ng/µL, dispensing it on a source plate (one plasmid/well) according to the spreadsheet-

generated template and using the tablet-based pipetting guide, and preparing the cell suspension before seeding. The ADE-based nanodispenser is in charge of the time-consuming and error-prone dose delivery and multiplexing of the plasmids, according to the given template of the experiment.

Furthermore, whereas this protocol could ensure most of the basic biological intents of classical transfection experiments, it could also open up new ways for array-based experiments. For example, expressing or knocking down each human protein-coding gene from the human ORFeome collection[36] or CRISPR-Cas9 library-based approaches[37], respectively, would require less than 24 h on a dedicated automated platform (53 x 384-well plates), rather than 2–3 days of human work, assuming the use of a DNA-preloaded plate bank. Due to its high efficiency and high-throughput performance, the protocol presented here might even be able to achieve new nonpooled approaches for CRISPR-Cas9 based-studies with gRNA libraries/CRISPR-Cas9-expressing plasmids. Indeed, mild cellular phenotype changes, which currently cannot allow the required cell-sorting step, would finally be manageable, as one well would represent one knocked gene.

ACKNOWLEDGMENTS:

The authors disclosed a receipt of the following financial support for the research, authorship, and/or publication of this article: Inserm, Lille University, Lille Pasteur Institute, Conseil Régional Nord, and PRIM (Pôle de Recherche Interdisciplinaire pour le Médicament). The authors wish to thank Dr. S. Moureu, Dr. B. Villemagne, Dr. R. Ferru-Clément, and Dr. H. Groult for their critical review and corrections of the manuscript.

DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Colin, B., Deprez, B., Couturier, C. High-Throughput DNA Plasmid Transfection Using Acoustic Droplet Ejection Technology. *SLAS Discovery: Advancing Life Sciences R & D*. 2472555218803064, doi: 10.1177/2472555218803064 (2018).

2. Bio, M. Optimising Transfection Performance. 3.

3. Thermo Fisher Scientific. Factors Influencing Transfection Efficiency | Thermo Fisher Scientific - FR. https://www.thermofisher.com/fr/fr/home/references/gibco-cell-culture-basics/transfection-basics/factors-influencing-transfection-efficiency.html.

4. Boussif, O. et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America*. **92** (16), 7297–7301 (1995).

5. Figueroa, E. et al. A mechanistic investigation exploring the differential transfection efficiencies between the easy-to-transfect SK-BR3 and difficult-to-transfect CT26 cell lines. *Journal of Nanobiotechnology*. **15** (1), 36, doi: 10.1186/s12951-017-0271-8 (2017).

6. Kirchenbuechler, I., Kirchenbuechler, D., Elbaum, M. Correlation between cationic lipid-based transfection and cell division. *Experimental Cell Research*. **345** (1), 1–5, doi: 10.1016/j.yexcr.2014.11.019 (2016).

7. Zhang, Z., Qiu, S., Zhang, X., Chen, W. Optimized DNA electroporation for primary human T cell engineering. *BMC Biotechnology*. **18** (1), 4, doi: 10.1186/s12896-018-0419-0 (2018).
8. Cao, D. et al. Transfection activity and the mechanism of pDNA-complexes based on the hybrid of low-generation PAMAM and branched PEI-1.8k. *Molecular bioSystems*. **9** (12), 3175–3186, doi: 10.1039/c3mb70261h (2013).
9. Bos, A.B. et al. Development of a semi-automated high throughput transient transfection system. *Journal of Biotechnology*. **180**, 10–16, doi: 10.1016/j.jbiotec.2014.03.027 (2014).
10. Colosimo, A. et al. Transfer and expression of foreign genes in mammalian cells. *BioTechniques*. **29** (2), 314–318, 320–322, 324 passim (2000).
11. Villa-Diaz, L.G., Garcia-Perez, J.L., Krebsbach, P.H. Enhanced transfection efficiency of human embryonic stem cells by the incorporation of DNA liposomes in extracellular matrix. *Stem Cells and Development*. **19** (12), 1949–1957, doi: 10.1089/scd.2009.0505 (2010).
12. Sabatini, D.M. Reverse transfection method. WO2001020015A1 (2001).
13. Raymond, C. et al. A simplified polyethylenimine-mediated transfection process for large-scale and high-throughput applications. *Methods* (San Diego, CA). **55** (1), 44–51, doi: 10.1016/j.ymeth.2011.04.002 (2011).
14. Junquera, E., Aicart, E. Recent progress in gene therapy to deliver nucleic acids with multivalent cationic vectors. *Advances in Colloid and Interface Science*. **233**, 161–175, doi: 10.1016/j.cis.2015.07.003 (2016).
15. Woodruff, K., Maerkl, S.J. A High-Throughput Microfluidic Platform for Mammalian Cell Transfection and Culturing. *Scientific Reports*. **6**, 23937, doi: 10.1038/srep23937 (2016).
16. Vasileiou, T., Foresti, D., Bayram, A., Poulikakos, D., Ferrari, A. Toward Contactless Biology: Acoustophoretic DNA Transfection. *Scientific Reports*. **6**, 20023, doi: 10.1038/srep20023 (2016).
17. Hadimioglu, B., Stearns, R., Ellson, R. Moving Liquids with Sound: The Physics of Acoustic Droplet Ejection for Robust Laboratory Automation in Life Sciences. *Journal of Laboratory Automation*. **21** (1), 4–18, doi: 10.1177/2211068215615096 (2016).
18. Grant, R.J. et al. Achieving accurate compound concentration in cell-based screening: validation of acoustic droplet ejection technology. *Journal of Biomolecular Screening*. **14** (5), 452–459, doi: 10.1177/1087057109336588 (2009).
19. Sackmann, E.K. et al. Technologies That Enable Accurate and Precise Nano- to Milliliter-Scale Liquid Dispensing of Aqueous Reagents Using Acoustic Droplet Ejection. *Journal of Laboratory Automation*. **21** (1), 166–177, doi: 10.1177/2211068215602191 (2016).
20. Day, R.N., Davidson, M.W. The fluorescent protein palette: tools for cellular imaging. *Chemical Society Reviews*. **38** (10), 2887–2921, doi: 10.1039/b901966a (2009).
21. Zielinski, D., Gordon, A., Zaks, B.L., Erlich, Y. iPipet: sample handling using a tablet. *Nature Methods*. **11** (8), 784–785, doi: 10.1038/nmeth.3028 (2014).
22. Brunner, S. et al. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Therapy*. **7** (5), 401–407, doi: 10.1038/sj.gt.3301102 (2000).
23. Nii, T. et al. Single-Cell-State Culture of Human Pluripotent Stem Cells Increases Transfection Efficiency. *BioResearch Open Access*. **5** (1), 127–136, doi: 10.1089/biores.2016.0009 (2016).
24. Noonan, D.J., Henry, K., Twaroski, M.L. A High-Throughput Mammalian Cell-Based Transient Transfection Assay. *Signal Transduction Protocols*. **284**, 051–066, doi: 10.1385/1-59259-816-1:051 (2004).
25. Transfection | TransIT Transfection Reagents | Mirus Bio.

https://www.mirusbio.com/products/transfection.
26. American Type Culture Collection. General protocol for transfection of stem cells, primary cells, and continuous cell lines with ATCC TransfeX Transfection Reagent. https://www.atcc.org/~/media/Transfection%20protocols/TransfeX/TransfeX%20General%20Protocol%20on%20letterhead.ashx (2015).
27. American Type Culture Collection. Transfection Reagents for Nucleic Acid Transfer into ATCC Cells. http://www.lgcstandards-atcc.org/~/media/PDFs/Marketing%20Material/Cell%20Biology/Transfection%20reagents%20for%20nucleic%20acid.ashx (2017).
28. de Los Milagros Bassani Molinas, M., Beer, C., Hesse, F., Wirth, M., Wagner, R. Optimizing the transient transfection process of HEK-293 suspension cells for protein production by nucleotide ratio monitoring. *Cytotechnology*. **66** (3), 493–514, doi: 10.1007/s10616-013-9601-3 (2014).
29. Promega. FuGENE® 6 Transfection Reagent. https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/fugene-6-transfection-reagent-protocol.pdf (2011).
30. Olden, B.R., Cheng, Y., Yu, J.L., Pun, S.H. Cationic polymers for non-viral gene delivery to human T cells. *Journal of Controlled Release: Official Journal of the Controlled Release Society*. **282**, 140–147, doi: 10.1016/j.jconrel.2018.02.043 (2018).
31. Park, E., Cho, H.-B., Takimoto, K. Effective gene delivery into adipose-derived stem cells: transfection of cells in suspension with the use of a nuclear localization signal peptide-conjugated polyethylenimine. *Cytotherapy*. **17** (5), 536–542, doi: 10.1016/j.jcyt.2014.11.008 (2015).
32. Wood, R.W., Loomis, A.L. XXXVIII. The physical and biological effects of high-frequency sound-waves of great intensity. *The London, Edinburgh, and Dublin Philosophical Magazine and Journal of Science*. **4** (22), 417–436, doi: 10.1080/14786440908564348 (1927).
33. Mamat, U. et al. Eliminating Endotoxin at the Source – A Novel Competent Cell Line with Modified Lipopolysaccharide for Low-Endotoxin Plasmid Production. *28th Annual Symposium of the Protein Society.* San Diego, CA (2014).
34. Ivanova, N.V., Kuzmina, M.L. Protocols for dry DNA storage and shipment at room temperature. *Molecular Ecology Resources*. **13** (5), 890–898, doi: 10.1111/1755-0998.12134 (2013).
35. Lesnick, J., Lejeune-Dodge, A., Ruppert, N., Jarman, C. High-Precision Cell Dispensing with the Labcyte Echo® Liquid Handler. https://www.labcyte.com/content/applications/high-precision-cell-dispensing-with-the-labcyte-echo-liquid-handler (2017).
36. Yang, X. et al. A public genome-scale lentiviral expression library of human ORFs. *Nature Methods*. **8** (8), 659–661, doi: 10.1038/nmeth.1638 (2011).
37. Peng, J., Zhou, Y., Zhu, S., Wei, W. High-throughput screens in mammalian cells using the CRISPR-Cas9 system. *The FEBS journal*. **282** (11), 2089–2096, doi: 10.1111/febs.13251 (2015).

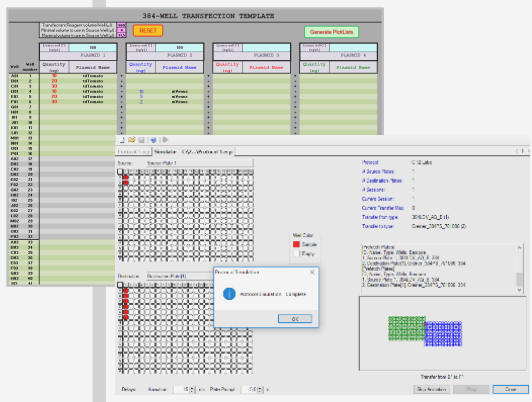
Figure1

Click here to access/download;Figure;Colin-Couturier-

EXPERIMENTAL DESIGN

❶ PICKLISTS GENERATION

Spreadsheet Macro



Verification
Cherry Pick software

PLATES PREPARATION & STORAGE

❷ SOURCE PLATE PREPARATION

384 wells
pipetting guide

❸ DILUENT DISPENSE

Peristaltic
liquid handler
1 µL

8 s

❹ DNA DISPENSE

Acoustic
nanodispenser
30 ng

≈ 5 min

NO STORAGE

STORAGE
(up to 7 days)

Thaw

TRANSFECTION

❺ TRANSFECTION REAGENT DISPENSE

Acoustic
nanodispenser
500 nL

≈ 20 min

❻ CELLS PREPARATION

Cell dilution
37500 cells/mL

❼ CELLS SEEDING

Peristaltic
liquid handler
1500 cells/40 µL

45 s

CUSTOM BIOLOGICAL ASSAY

Luminescence, Fluorescence,
High Content screening, RT-qPCR...

Figure2

384-WELL TRANSFECTION TEMPLATE

Step 2.1

Transfectant Reagent volume/Well (µl) :	500
Minimal volume to use in Source Well (µl) :	4
Maximal volume ti use in Source Well (µl) :	11.5

① RESET

④ Generate PickLists

Step 2.2

		[source well C°] (ng/µL)	100		[source well C°] (ng/µL)	100		[source well C°] (ng/µL)		[source well C°] (ng/µL)	
		PLASMID 1			PLASMID 2			PLASMID 3		PLASMID 4	
Well	Well number	Quantity (ng)	Plasmid Name		Quantity (ng)	Plasmid Name		Quantity (ng)	Plasmid Name	Quantity (ng)	Plasmid Name
A01	1	2.5	tdTomato	+	27.5	mVenus	+				
B01	2	5	tdTomato	+	25	mVenus	+				
C01	3	7.5	tdTomato	+	22.5	mVenus	+				
D01	4	10	tdTomato	+	20	mVenus	+				
E01	5	12.5	tdTomato	+		mVenu					
F01	6	15	tdTomato	+		mVenu					
G01	7	17.5	tdTomato	+	12.5	mVenu					
H01	8	20	tdTomato	+	10	mVenu					
I01	9	22.5	tdTomato	+	7.5	mVenu					
J01	10	25	tdTomato	+	5	mVenu					
K01	11	27.5	tdTomato	+	2.5	mVenu					
L01	12	30	tdTomato	+							
M01	13			+							
N01	14			+							
O01	15			+							
P01	16			+							
A02	17	2.5	mVenus	+	27.5	tdToma					
B02	18	5	mVenus	+	25	tdToma					
C02	19	7.5	mVenus	+	22.5	tdToma					
D02	20	10	mVenus	+	20	tdToma					
E02	21	12.5	mVenus	+	17.5	tdToma					
F02	22	15	mVenus	+	15	tdToma					

Step 2.3 ③

Microsoft Excel

DNA-Pickist.csv, T.R.-Picklist.csv and 384-Wells-Transfer-Guide.csv files were generated in folder :
C:\JOVE

OK

Step 2.4

Source Plate n°1

(minimal volume to fill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
A	mVenus 7.60µl															
B	tdTomato 7.60µl															
C	TR 11.50µl															
D	TR 8.50µl															
E																
F																
G																
H																
I																
J																
K																

Step 2.5

DNA-Pickist

	A	B	C	D	E
1	Destination Well	Source Well	Transfer Volume	Source Plate Name	
2	A02	A01	25	Source Plate 1	
3	B02	A01	50	Source Plate 1	
4	C02	A01	75	Source Plate 1	
5	D02	A01	100	Source Plate 1	
6	E02	A01	125	Source Plate 1	
7	F02	A01	150	Source Plate 1	
8	G02	A01	175	Source Plate 1	
9	H02	A01	200	Source Plate 1	
10	I02	A01	225	Source Plate 1	
11	J02	A01	250	Source Plate 1	
12	K02	A01	275	Source Plate 1	

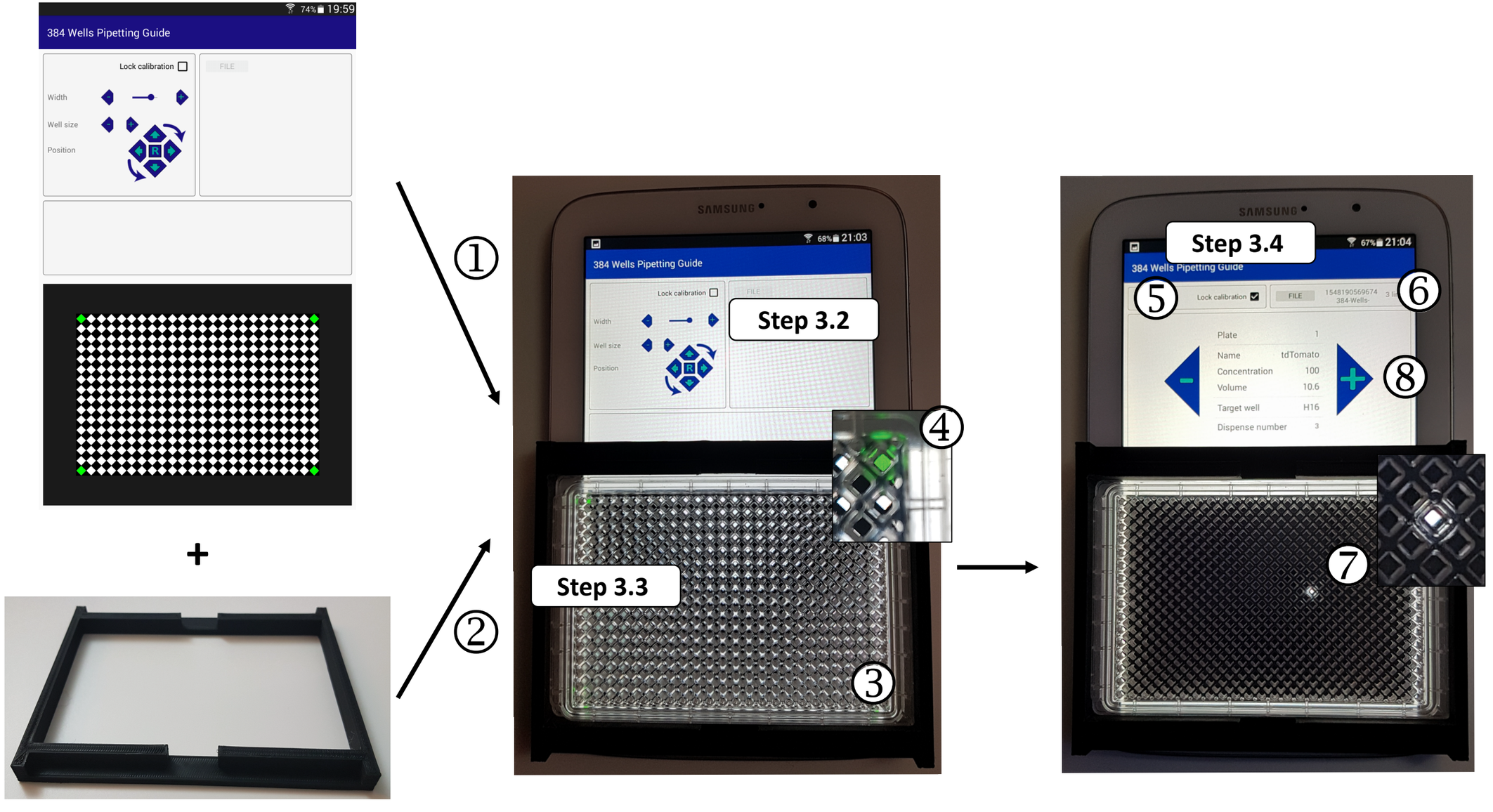
T.R.-Picklist

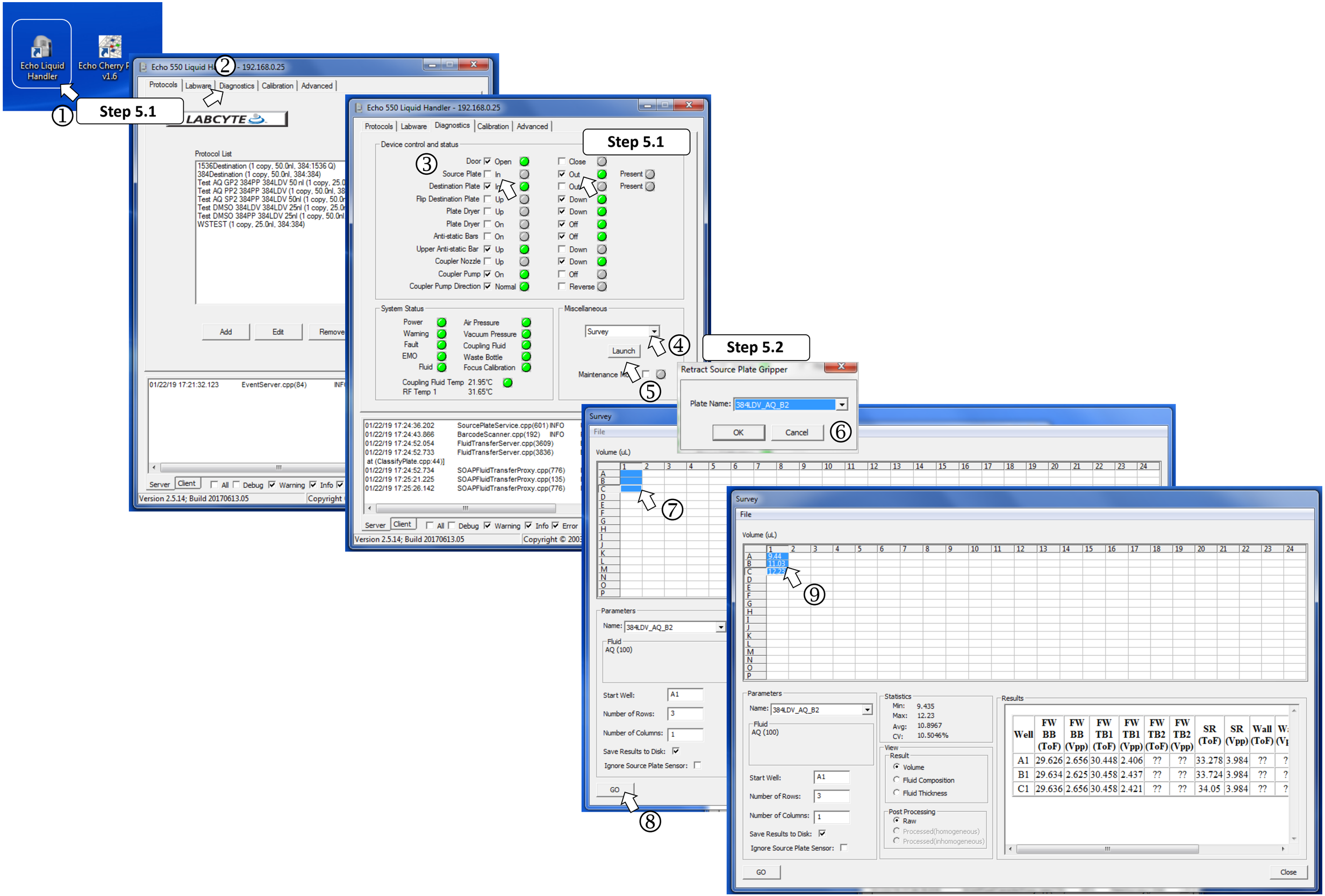
	A	B	C	D	E
1	Destination Well	Source Well	Transfer Volume	Source Plate Name	
2	A02	C01	500	Source Plate 1	
3	B02	C01	500	Source Plate 1	
4	C02	C01	500	Source Plate 1	
5	D02	C01	500	Source Plate 1	
6	E02	C01	500	Source Plate 1	
7	F02	C01	500	Source Plate 1	
8	G02	C01	500	Source Plate 1	
9	H02	C01	500	Source Plate 1	
10	I02	C01	500	Source Plate 1	
11	J02	C01	500	Source Plate 1	

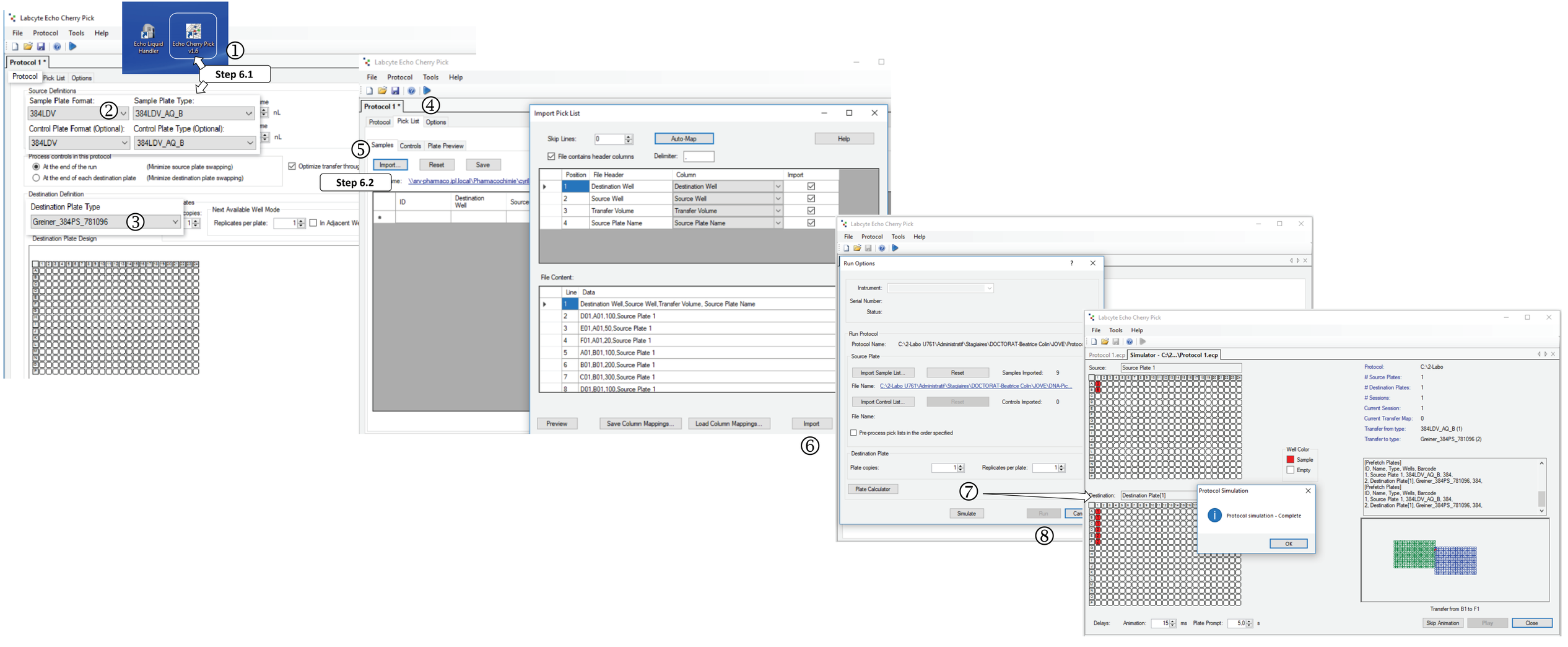
384-Wells-Pipetting-Guide

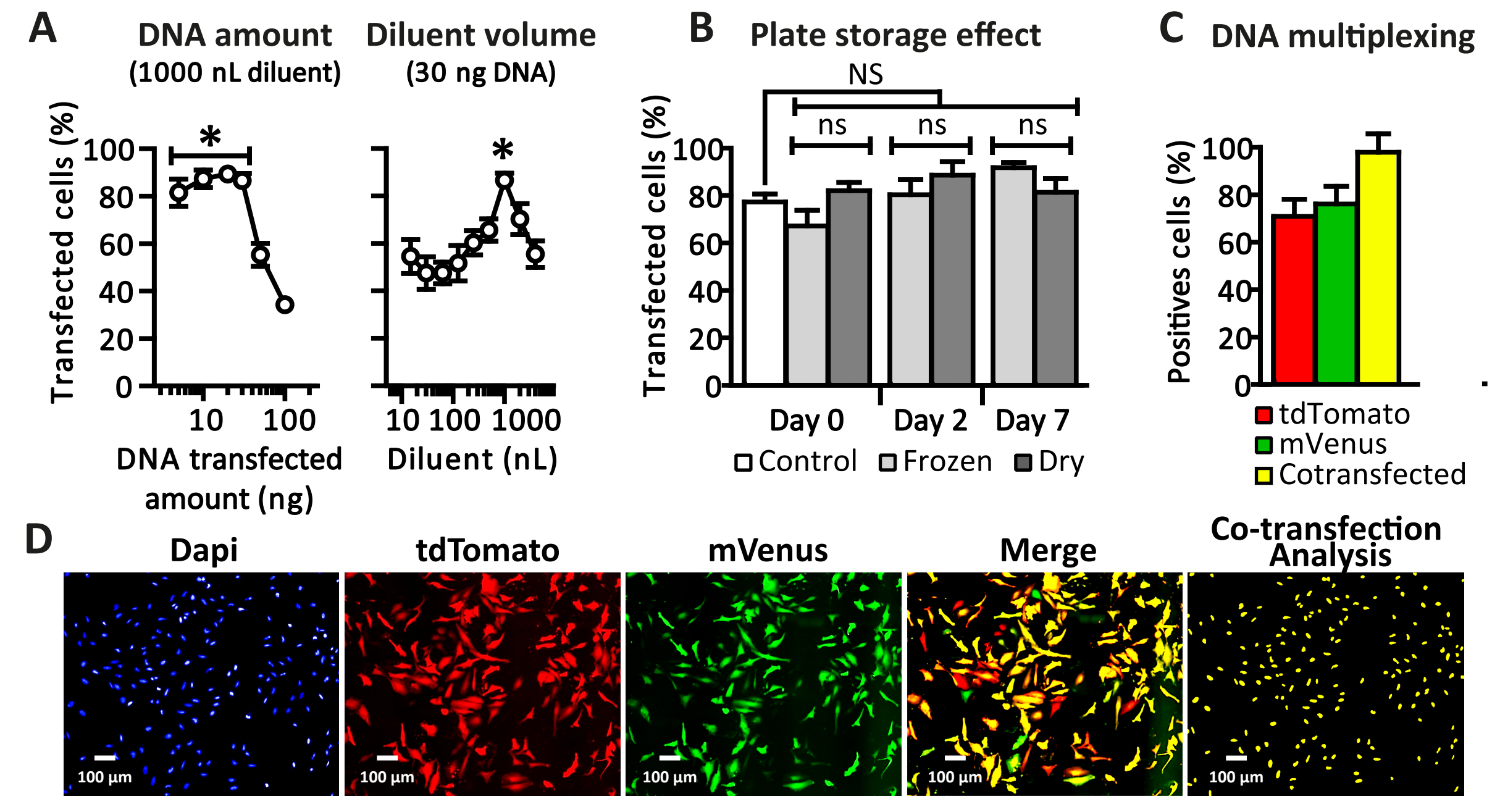
	A	B	C
1	Well	Plate	Name	Concentration	Volume
2	A01	Source Plate 1	mVenus	100	7.60
3	B01	Source Plate 1	tdTomato	100	7.60
4	C01	Source Plate 1	TR	100	11.50
5	D01	Source Plate 1	TR	100	8.50
6					

Figure3









Name of Material/ Equipment	Company
384LDV Microplate	Labcyte
384-well Microplate µClear Black	Greiner
Ampicilin	Sigma
Android Tablet	Samsung
Aniospray Surf 29	Anios
Columbus software	Perkin Elmer
Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX Supplement, pyruvate	Thermo Fisher Scientific
Echo Cherry Pick 1.5.3 software	Labcyte
Echo550	Labcyte
Fetal Bovine Serum	Thermo Fisher Scientific
Formalin solution, neutral buffered, 10%	Sigma-Aldrich
HeLa cells	ATCC
Hoechst 33342, Trihydrochloride, Trihydrate	Thermo Fisher Scientific
INCell Analyzer 6000	GE Healthcare
LB medium	Thermoischer Scientific LB Broth Base (Lennox L Broth Base)®, powder
Lysis Buffer (A2)	Macherey-Nagel
MicroFlo 10µL cassette	Biotek Instruments Inc
MicroFlo 1µL cassette	Biotek Instruments Inc
MicroFlo Dispenser	Biotek Instruments Inc
Microvolume spectrophotometer	Denovix

mVenus plasmid	mVenus cDNA was cloned by enzymatic restriction digestion and ligation in Age1/BsrG1 sites of the tdTomato-N1 plasmid
Neutralization Buffer (A3)	Macherey-Nagel
NucleoSpin Plasmid kit	Macherey-Nagel
Optimal-Modified Eagle Medium (Opti-MEM) Medium	Thermo Fisher Scientific
optional Wash bufferWash Buffer (A4)	Macherey-Nagel
orbital shaker	incubated large capacity shaker
Penicillin-Streptomycin	Thermo Fisher Scientific
Phosphate-Buffered Saline	Thermo Fisher Scientific
Plasmid mini-columns	Macherey-Nagel
Resuspension Buffer (A1)	Macherey-Nagel
RNAse A	Macherey-Nagel
tdTomato-N1 plasmid	Addgene
TransIT-X2 Dynamic Delivery System	Mirus Bio
Wash Buffer (AW)	Macherey-Nagel
3D printer	Creality

Blender Software	https://www.blender.org/ Free software under GNU General Public License (GPL).

Catalog Number	Comments/Description
LP-0200	
781906	
A9393-5G	Selection antibiotic for bacteria transformed with ampicilin expressing vector
Galaxy Note 8	used to guide the user while the source plate manual dispense
2421073	disinfectant to clean the MicroFlo head
	image analysis software
10566032	cell culture medium
	Software enabling ADE-based dispenses by the Echo550 device from a *.csv file; nanodispenser software
	ADE-based dispenser
16000044	to add in cell culture medium
HT501128-4L	to fix cell
HeLa (ATCC® CCL-2™)	
H3570	10 mg/mL Solution in Water
29043323	automated laser-based confocal imaging platform
12780052	culture medium for bacteria growth
740912.1	Buffer from the NucleoSpin Plasmid kit used to prepare plasmid from bacterial culture
7170013	to use with the Microflo Dispenser
7170012	to use with the Microflo Dispenser
7171000	peristaltic pump-based liquid handler device
DS-11 Spectrophotometer	Measure the DNA concentration of samples

	Vector type: Mammalian Expression, Fusion Protein: mVenus
740913.1	Buffer from the NucleoSpin Plasmid kit used to prepare plasmid from bacterial culture
740588.50	used to prepare plasmid from bacterial culture
31985070	
740914.1	Buffer from the NucleoSpin Plasmid kit used to prepare plasmid from bacterial culture
444-7084	Used to grow bacteria under gentle agitation and 37°C
15140122	10,000 U/mL
10010001	
740499.250	Silica membrane mini-column to prepare plasmid from bacterial culture
740911.1	Buffer from the NucleoSpin Plasmid kit used to prepare plasmid from bacterial culture
740505	Enzyme from the NucleoSpin Plasmid kit used to prepare plasmid from bacterial culture
Plasmid #54642	Vector type: Mammalian Expression, Fusion Protein: tdTomato
MIR 6000	
740916.1	Buffer from the NucleoSpin Plasmid kit used to prepare plasmid from bacterial culture
CR10S	used to print the plate adapter

version 2.79b used to design the plate adapter

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	High-Throughput DNA Plasmid Transfection Using Acoustic Droplet Ejection Technology
Author(s):	Beatrice Colin, Benoit Deprez, Cyril Couturier

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

■ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

■ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

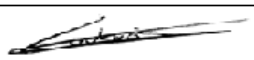
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Cyril Couturier
Department:	INSERM U1177 Drugs and Molecules for Living Systems
Institution:	Institut Pasteur de Lille, 1 rue Pr. Calmette, 59000 Lille, France
Title:	MCU (Maitre de Conference Universitaire)
Signature:	
Date:	Sept. 17, 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear JoVE editorial Board,
Please find here all the answers to reviewer's comments.
Kinds regards.
C. Couturier

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a well written manuscript. As transfections are routinely utilised in biological research, the protocol described in the manuscript will appeal to many readers.

Major Concerns:
None.

Minor Concerns:
Line 33: replace cells transfection with cell transfection.
Line 34: replace multiplexes DNA with multiplex DNA.
Line 358: replace (T-R.) with (T.R.).

All these minor changes have been done in the text.

Reviewer #2:

Manuscript Summary:
The use of dispensing by ADE to streamline the assembly of transfection reactions is very exciting. Its ability to increase throughput, reduce reagent consumption, and speed condition optimization should have a broad and significant impact on the conduct of experiments requiring transfection. The use of a spreadsheet macro to elicit the picklist generation makes the experimental setup more straightforward and reduces the potential for manual error. The introduction of this spreadhsheet tool extends the work beyond that described by the authors in their SLAS Discovery 2018 publication.

Minor Concerns:
I support proceeding with publication of this work in the form of a video demonstrating the methods. Please see below for some comments, questions, and minor recommended modifications.

Will the macro be made available to the journal audience?
The macro has been developed to further enhance the ease of conducting high throughput transfection experiments and was of course intended to be made available to the readers. Since first submsission to JOVE, a new tool has been developed to facilitate our DNA dispenses in the source plate and would also be made available to the readers.

As written, the manuscript would benefit from editing of the grammar. Although I could figure out the authors' intentions in most cases, there were some instances in which it was not so clear. For example, the second sentence in lines

412-414 is not readily understandable. Additionally, there are many cases in which subtle word changes may improve clarity.
This paragraphe has been modified and we hope it became clearer for the reader.

For example on line 57, would the words easy, straightforward, or simple better convey the authors' message than does obvious?
The sentence has been modified to " *Plasmid DNA transfection is the starting point of many cell-based experiments but whereas many dedicated reagents have been and are nowadays still developed to enhance transfection efficiency and ease, it is still not so simple*"

on line 330 suitability in place of ability
This change has been performed, and also line 80 dealing with the same thing.

on line 450 recommend in place of preconize may be more readily comprehensible.
This has been modified

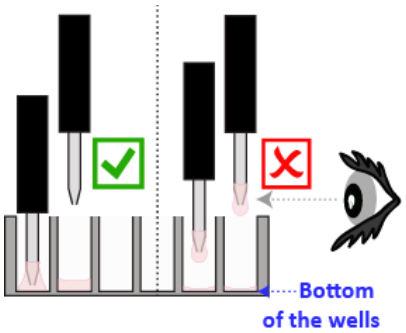
The authors should cite their own publication (reference 8) in the introduction. In fact, citing it at the end of line 98 would provide the reader/viewer with a reference for parameter optimization using the approach described in this work.
The introduction has been modified and the way to optimize parameters using our recently published work has been implemented in the new version of the introduction.

Line 81: it would be helpful for the authors to delineate what they see as the limitations of manual cell transfection.
The introduction has been drastically modified and these points were more detailed.

Line 108: the suggested adjustments for dispense speed and height are vague. It would be helpful to

provide examples of suitable values for the conditions and destination plates described in the method.
We agree with you and whereas it can only be applied to the Microflo liquid handler, the head dispense and displacements heights have been added in the corresponding paragraphs that have furthermore been rewritten in a more detailed way (1.1 to 1.2.5).

Line 109: a picture or diagram showing a suitable dispense height and configuration required for the drop to touch the bottom of the well would be helpful. The right embedded figure has been added in supplemental figure 1 and we propose to film this crucial setting point in the video.



Lines 113-114: similar examples of suitable dispense speed and dispense height (with a picture or diagram) would be useful.
In this step of diluent dispense, the setting to higher speeds is just intended to gain higher throughput as no biological impact is expected. The sentence has then been modified to clarify this point : "adjust the speed to higher values to enhance the throughput, and the height of the dispense to the cell culture plate used, in order to allow the 1 µL drop touch the bottom of the well during the dispensing process.

"Lines 115-116: it is not clear how dispense height should be adjusted to avoid drop retention.
The right above embedded figure should be added but instead, we propose to film this crucial setting point in the video. Jove editorial board members may require both of them for enhanced clarity.

Line 157: Please provide specific examples of input values and the corresponding DNA-Picklist.csv, T.R.-picklist.csv, and source plate design. Such examples would provide transparency to the calculations and operations being conducted through the spreadsheet macro. Additionally, it would highlight the information that is required in the picklists.
We have filled the fields with current value we use as an example of experimental design using the macro spreadsheet.

Lines 205-206: It is helpful to provide the manual alternative to the MicroFlo.
We agree, as we performed it manually without any loss of efficiency in our hands. That is the reason why we added this alternative in the protocol for users that do not have access or do not want to use such liquid handlers.

Lines 209 and 220-221: The recommendations to run a source plate survey and a simulation prior to the actual experiment are indeed valuable precautions that will help prevent experimental errors!
We agree, and furthermore highlighted this point in 2.4.1.2 step.

Line 231: are there particular conditions that are used to dry the DNA?
No particular conditions have been used to dry DNA, as we let it dry on the bench at room temperature. We added this precision in the paragraph 2.4.5. : "*For dry storage, let the plates dry on the bench at room temperature and then store them the same way. Thaw and centrifuge (1500 g, 2 min) frozen stored plates before use in transfection step 2.5.*"

If opting for dry storage, is it still beneficial to add the initial 1 uL diluent?
As explained in the "**Troubleshooting inhomogeneous cell transfection over the well surface" section of the discussion, t**he 1 µl diluent is indeed used to spread DNA and transfection mixture on the overall surface of the well to allow homogeneous transfection all over the well instead of the center of the wells as we noticed while develloping the assay.

Lines 237-239: Is there a specified timeframe that the T.R. is usable for after vortexing?
According to the manufacturer protocol no information is provided except *"Prepare co-transfection complexes (Immediately before transfection) "*and *". Warm TransIT-X2 to room temperature and vortex gently before using*". Once added to the DNA mixture, an incubation of 15 to 30 minutes is recommended before cell addition. However as the dispense time is about 20 minutes, we did not let more incubation time except that needed to prepare cell dispense that in turn comply to the recommended range. Agreeing with you, we modified the manuscript to add this point in 2.5.7 step: "*Respect a 15 to 30 minutes incubation after transfection reagent addition on DNAs, as indicated in the manufacturer protocol*".

Line 242: Perhaps "Do not centrifuge" should be highlighted, because the results of this operation are fatal to the experiment.
We agree and used bold letter for this sentence, however, Jove Editorial board members may have another point of view.

Line 278: complete medium should be defined either here or in the Table of Materials.
This has been added in the table of materials.

Lines 316-317: What is the volume of Hoechst in PBS per well?

This missing information has been corrected : **"**…, then incubate cell for 15 min with 40 µL of 0,1 ng/mL Hoechst diluted in 1X phosphate-buffered saline (PBS) solution."

Lines 319-320: It would be helpful to understand how pH 8 leads to recovery of lost fluorescence signal.

Whereas fluorescent proteins were readily observable in live cells, we chose a 4% paraformaldehyde cell fixation protocol as it is widely used in many applications such as screening assays in which our transfection protocol would be benefit. However, while controlling each steps of our experiments, after cell fixation, a clear decrease in fluorescence intensity was noticed using fluorescence microscopy. Trying to understand and circumvent this problem, we found that GFP and YFP chromophore could be protonated in resin medium due to acidic pH (*Gang, Y. et al. 2017 doi: 10.3389/fnins.2017.00121*): Embedding and Chemical Reactivation of Green Fluorescent Protein in the Whole Mouse Brain for Optical Micro-Imaging). The authors were able to circumvent this problem by chemical reactivation of GFP using alkaline buffer incubation. As the commercially available paraformaldehyde solution has a 6.9 pH (lower than most fluorescent protein pKa) we then tried to use alkaline PBS (ph8 to 8.5) to rinse the fixative medium. Although this was not quantified, as this was not the topic here, it led to recovery of fluorescence when compared to the use of native PBS. This notion has now been added in the manuscript in 3.4.s ection: "*Wash the cells 3 times for 15 min with 80 µL 1X PBS adjusted to pH8 in order to recover high fluorescence signal lost by the 6.9 pH of the formalin solution incubation step*".

Page 8, Table of Materials: NucleoSpin Plasmid does not occur elsewhere in the manuscript.
It has now been implemented in the new version of the text in section

Additionally, it would be helpful to specify in the comments column that the role of Aniospray Surf 29 as is a disinfectant for cleaning the MicroFill cartridge(thus you do not need it if adding diluent and cells manually).
This has been done in the requested xls table of material.

Line 383: I believe that the T.R. addition step is missing after addition of diluent and before addition of cells. This is presented in the **2.5. Step5: ADE driven Transfection reagent dispense** and more precisely in paragraph 2.5.6.

Lines 497-498: Although ADE has been used to dispense cell solutions, this is from a solution of neutral density (Histodenz™) that allows the cells to remain suspended. Would such a solution be compatible with transfection? Additionally, would the cell volume to be dispensed fall below the 500 nL that the authors describe as the upper limit of the Echo's dispense capability.
The corresponding paragraph has been modified to be more precise and answer yours questions.

Line 504: The methods described in this manuscript ensure successful transfection only if the conditions are pre-optimized such has been the case with the described plasmids and HeLa or HEK cell lines. Success in transfection can vary with DNAs and cell line.
The overall manuscript has been rewrite and the necessity to optimize several parameters to succeed in transfecting given cell types/lines has been much more highlighted in the new version of the text.

Figure 4 C: Please clarify how % of co-transfected cells is determined.This has now been added in the manuscript: “Percent co-transfected cells was determined by calculating Green fluorescence expressing cell number in the red fluorescent population cells”.

Reviewer #3:

Manuscript Summary:

The manuscript by Couturier et al. describes a high-throughput method for transfecting cells using a acoustic droplet ejection technology. The method uses low volumes of reagents/DNA and can multiplex several plasmids.

Major Concerns:

NONE

Minor Concerns:

- In the troubleshooting section, the authors may also want to include the following parameters that could influence transfection: passage number, serum in media, and antibiotics in media.

As requested, these parameters have been added.

- The manuscript should be proofread for small syntax errors.

This has been done and we hope the new version of the manuscript satisfy your expects.

Reviewer #4:

Manuscript Summary:

The manuscript submitted by Colin and colleagues, titled „ High-Throughput DNA Plasmid Transfection Using Acoustic Droplet Ejection Technology" introduces a protocol for the expression of recombinant proteins in eukaryotic cells, based on contactless, acoustic reagent handling.

The work proposes the following major results:

* Multiplex transfection and co-transfection (two plasmids) on model eukaryotic cell line.
* Fully automated processing of biological reagents
*High expression efficiency for the chosen fluorescent proteins.

Altogether, the paper reads too much as a commercial validation for an available device (the LabCyte ECHO550) and does not bring sufficient novelty on the general topic of contactless molecular biology or DNA transfection. For the following major reasons:

1. Acoustic-based DNA transfection in eukaryotic cells has been already reported. This implies that the basic concept reported here is not novel, but mostly an improved version with higher efficiency. Which is however appreciable in terms of commercial upscaling, yet lacking any breakthrough novelty. In addition, the authors do not cite the previous reports on Acoustophoretic-based DNA transfection.

We agree with you: the aim of our protocol was to develop high throughput transfection using available device on the market and not to develop a new method for liquid handling. Our method is new as it is the first to use the ADE technology for cell DNA transfection and furthermore to reach the 384 wells plate format and in such a high throughput. It is furthermore promising for the 1536 upper scale level.
On the other hand, accoustophoresis or liquid levitation is not a ADE method. It allows fluid manipulation and mixing and was used to perform DNA transfection in up to 96 wells plate formats. However, this method can not be compared to the ADE-based nanodispenser whose usability is far away from that of a in house developed device still unavailable to the scientist community, and furthermore suffering from a very low throughput. Such as nanodispenser, this method of DNA transfection might be interesting in the future due to its tip-less approach and low volume management (in the range of µL in this transfection application), but will have to be improved. In its current state, it requires a 60 seconds incubation of cells and DNA mixture for each single point before seeding, implying at minima 1.6 hours for a complete 96 well plate and 6.4 h even if not amenable yet for a 384 wells plate format.

This method being however promising it has been added in the new version of the introduction.

2. Based on point 1 above, the work strength should be on the flexibility and efficiency of the new method. However, one relatively easy case is reported, i.e. the transfection of HeLa cells with fluorescent proteins.

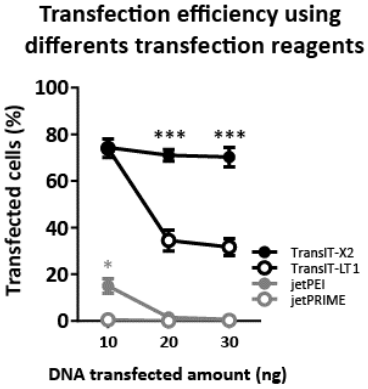
We agree Hela cells are generally easy to transfect, and that is the reason why we made this choice for our proof of concept instead of choosing hard to transfect cells. As transfection is dependent of cells type, one would optimize conditions for its given cell type of interest as we did for HeLa cells in our initial work. Using our fast and reliable 384 wells plate format transfection protocol, one could easily compare efficiencies of given cell-type or cell-line dedicated transfection reagents, DNA amount, diluent nature and volume, and the proper incubation time to detect the reporter used. This was not

our goal to be exhaustive and test all commercially available kits or reagents for all given cell types but rather prove that nanodispenser and its high throughput would be benefit to transfection of DNA plasmids too.

We used Fluorescent proteins expressing plasmid in order to quantify the positive cells easily, but whereas it worked properly, fluorescence is not a strong readout as no amplification of the signal is expected (one fluorophore lead to one amount of fluorescence whose strength is relative to its brightness and quantum yield). That mean that protein expression has to raise the detection limit level to be monitored by the imaging system. In most transfection reagent developer companies, bioluminescent protein expressing plasmids are used in their demonstrations as the luciferase catalytic activity lead to signal amplification and allow to raise huge signal to noise ratios and lower the transfected DNA amount. In our lab, using our protocol with bioluminescent plasmids forced us to lower DNA amounts (and complete with mock plasmid to reach the 30 ng total amount) due to huge signals. However using bioluminescent approach, we would not have been able to distinguish between a low amount of cells highly transfected and expressing high amount of luciferase or a high amount of cells transfected and each expressing a low amount of the reporter. Using fluorescent proteins was the simplest way to quantify positives transfected cells in the wells but is definitively not the best option as reporter.

No comparison to other, standard methods is given, and in my experience, I expect that very similar results could be obtained with other commercial kits.

What do you mean by commercial kits? Other device to peform nanodispensing or other transfection reagent? If this last, of course, several transfection reagents were tested in preliminary experiments when developing our protocol, but focusing on reagent efficient in reverse transfection protocol: JetPEI and JetPrime from polyplus, and TransITX2 and LT1 from Mirus. As you can see in the embedded figure, they led to different transfection efficiencies. JetPEI and the TransIT-X2 reagents were then chosen as best to represent two kind of transfection reagents (respectively liposomal and nonliposomal) and then further used. TransIT reagent leading to best transfection efficiency was then chosen for this optimized protocol. We agree that testing other reagents and other cell types would gain to other results (with better or lower efficiencies). However, our goal in the present protocol was not to find the best way to transfect all cell types and testing all the transfection reagents available on the market, but rather prove the concept that ADE could be used to perform DNA multiplexing and transfection at high speed and furthermore easily even for non specialists.



Transfection efficiency using different transfection reagents. HeLa cells were reverse transfected using the here described protocol. Non-liposomal (jetPEI; jetPRIME, Polyplus) and lipopolyplex (TransIT-LT1; TransIT-X2, Mirus) 1X concentration, were used with 10, 20 and 30 ng of the green fluorescent expressing plasmid (mVenus) for a 1 µL diluent volume. Transfection efficiencies were determined 48h later using image-based analysis software as described. Results are expressed as percent of transfected cells according to the DNA amounts used. The error bars represent the SEM with n 3. Two-way ANOVA and Bonferroni post-test were used for statistical analysis. ***p < 0.001 in black, for TransIT-X2 compare to TransIT-LT1 and in grey for jetPEI compared to jetPRIME.

The authors should challenge their new protocol with cells and/or plasmids which are transfected at low efficiency with state-of-the-art approaches and show a significant improvement. What do you mean by a plasmid transfected to low efficiency? Is it a plasmid prepared using non optimal protocol and of poor quality including some bacterial debris? is it a large plasmid that hinders its management by the transfection reagent and impair the DNA passage into the cell? Or is it a plasmid bearing a reporter with low signal readout or low efficiency promoter?.

Indeed, low transfection efficiency is rather a matter of cell line biology rather than of plasmid that transfect at low efficiency. In a work dedicated to understand which steps were involved in the difference of transfection efficiency, thus using a hard versus an easy to transfect cell lines, it has been shown that the discrepancy mainly involve initial complex uptake and cytoplasmic trafficking to the nucleus (*Figueroa, E. et al. 2017)*.

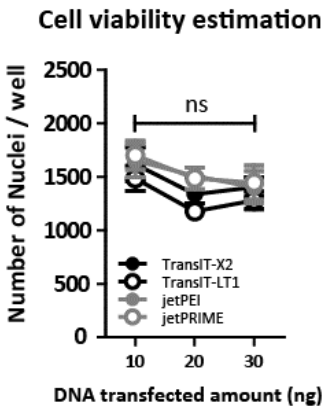
Here we used plasmids prepared from cheap silica mini-columns mostly used for molecular biology rather than transfection experiments. One can be sure that using midi- or maxi-preparation and even endotoxins free DNA preparation kits would ensure better results than we observed. This has now been more precised in the manuscript.

As most biologist who experienced it knows, transfection efficiency is generally inversely proportional to the DNA size and in order to succeed in our proof of concept, we effectively used quite small plasmids (5443bp for tdTomato and 4823bp for mVenus expressing plasmids). However, this protocol is now routinely used in our lab and CrisprCas9 expressing plasmids with higher size (more than 10,2kb) have been also transfected successfully. However, a mild decrease in efficiency has been noticed for these one but as they were also prepared using cheap minicolumns based purification, one could expect better results using higher DNA purification quality grade. Furthermore, such bigger plasmids would benefit to test other commercially available reagents more specifically developed for this purpose.

What do you mean by state-of-the-art approaches? We focused on reagent enabling reverse transfection protocol as we developed a high throughput reverse transfection-based method. Whereas it was not fully described in the present protocol whose first goal is to present the optimized protocol, in our initial work, results gained with our ADE protocol was compared with manual handling and gained same results in 384 wells plate format. As an improvement, the nanodispenser throughput is far away what can be achieved by hand which is furthermore more error prone than a nanodispenser performing not only the tedious work of dispensing but most of all the multiplexing according to the generated pickilist. To show our protocol could be used to set up conditions that would be used in larger scale, the optimized conditions worked successfully in higher well sizes and in cell culture dishes in transfection experiments handled manually.

3. Cell viability is not reported.

We did not study cell viability more precisely as every transfection method is a balance between cell transfection and cell mortality. Indeed we did not notice drastic cytotoxic effect and cell stress in the experiments we performed, as can be seen on the acquired images 48h post-transfection. Data analysis from our previous SLAS published work did not showed significant differences in cells number at 48h comparing 4 different transfection reagents leading to low or high transfection efficiencies. However, as 1500 cells were seeded during the transfection process, and the growth rate of Hela being more than 32h (*Stangegaard M et al 2006*), higher cell number was expected at 48h. That then means that our transfection protocol led to lowering growth rate or some cell mortality indeed happened. This important parameter was then added in the present version of the manuscript.



Effect of transfection on cell viability 48h post-transfection. 1500 HeLa cells/wells were reverse transfected with 10, 20 or 30 ng of green fluorescent protein expressing plasmid (mVenus) according to the here decribed protocol (1 µL diluent volume) and using non-liposomal (jetPEI ; jetPRIME, Polyplus) and lipopolyplex (TransIT-LT1 ; TransIT-X2, Mirus) 1X concentration transfection reagent. 48h later cell nucleus were stained using Hoechst incubation as described in the protocol and cell nucleus numbering was determined using image-based analysis software. Results expressed as number of cells for the indicated transfected DNA amount. The error bars represent the SEM with n=3. Two-way ANOVA and Bonferroni post-test were used for statistical analysis.

Dear editor,
Find below the answers to your editorial comments. Please note that all changes made in the manuscript are highlighted in blue in the current revised version.
I added a comment as I don't know how to manage one request without commercial terms.
Kinds regards.
C. Couturier

Editorial comments:

1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested. We worked from this version.

2. Please obtain explicit copyright permission to reuse Figure 6 from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
We obtain the SLAS explicit copyright permission to reuse data form our previous figures and the file has been loaded a .docx file by copying these informations from their RightLinks website as their official document was sent in pdf format (we loaded it too on your submission website).
The figure legend has been modified: "*Figure 6: This figure has been modified from [Colin, et al. 20181].*"

3. Please revise lines 789-791 to avoid previously published text.
The text has been change to : "*In conclusion, we developed an easy, high throughput and accurate transfection method bearing several advantages due to miniaturization: (1) lowering the costs of transfection reagent; (2) reducing the waste of DNA preparations; (3) ensuring even beginners to successfully perform cell transfection.*"

4. Please address specific comments marked in the attached manuscript.
All these comments have been addressed.

5. For culture media and buffer, please spell out at first use and provide composition. If they are purchased, please cite the Table of Materials.
Complete and serum-free media compositions, and reference to table of material have been added.

6. As we can only film 2.75 pages of the protocol, please review and shorten the highlighted portion to 2.75 pages. For example, some of the shorter Protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Note that the highlighted content should be continuous and contain essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Most of the important steps have been combined in bigger step with a maximum of 4 sentences as requested.

7. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
This has now been done this way.

8. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
The highlighted parts have been changed to faithful your requests.

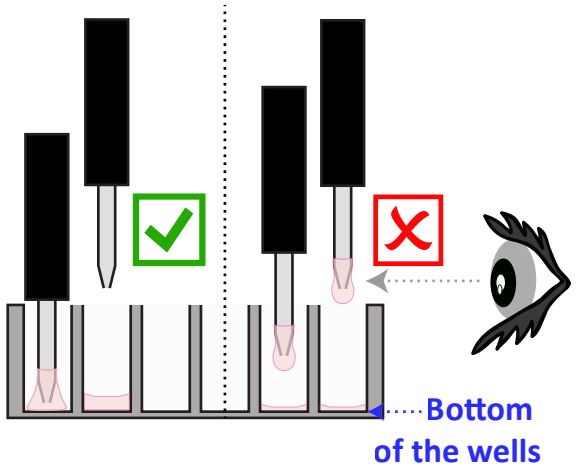
9. Please note that the numbering of protocol steps has been updated according to JoVE guidelines. Please review for accuracy and update the specific step numbers mentioned in the protocol or labeled in figures (Figure 2-5).
We kept your numbering style but as several steps have been combined and then rewritten, numbering has changed. We then updated the correct numbering in all the figures.

10. Figure 1: Please change the time unit “sec” to “s”. Is “MicroFlo” a commercial product? If so, please replace it with a generic term.
This has been changed to “s” and microflo to “peristaltic liquid handler”.

11. Figures 2-5: Please update the specific step numbers labeled in these figures.
The steps numbers have been updated in the figures.

12. Please upload supplemental Figure 1.
Supplemental figure 1 has been loaded.

13. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.
All relevant supplies/equipments have been added to the Table of Material, and furthermore sorted in alphabetical order as requested.



Supplemental Figure 1 : Diagram showing a suitable dispense height for the drop to touch the bottom of the well to avoid its retention on the dispensing tip: On the left, proper settings allow the drop to spread on the well surface avoiding its retention on the dispensing tips. On the right, bad settings lead to droplet retention that can be observed during the head movement to the next raw.

Transfectant Reagent volume/Well (µl) :	500
Minimal volume to use in Source Well (µl) :	4
Maximal volume ti use in Source Well (µl) :	11,5

Well	Well number	[source well C°] (ng/µL)	PLASMID 1	
		Quantity (ng)	Plasmid Name	
A01	1			+
B01	2			+
C01	3			+
D01	4			+
E01	5			+
F01	6			+
G01	7			+
H01	8			+
I01	9			+
J01	10			+
K01	11			+
L01	12			+
M01	13			+
N01	14			+
O01	15			+
P01	16			+
A02	17			+
B02	18			+
C02	19			+
D02	20			+
E02	21			+
F02	22			+
G02	23			+
H02	24			+
I02	25			+
J02	26			+
K02	27			+
L02	28			+
M02	29			+
N02	30			+
O02	31			+
P02	32			+
A03	33			+

B03	34		+
C03	35		+
D03	36		+
E03	37		+
F03	38		+
G03	39		+
H03	40		+
I03	41		+
J03	42		+
K03	43		+
L03	44		+
M03	45		+
N03	46		+
O03	47		+
P03	48		+
A04	49		+
B04	50		+
C04	51		+
D04	52		+
E04	53		+
F04	54		+
G04	55		+
H04	56		+
I04	57		+
J04	58		+
K04	59		+
L04	60		+
M04	61		+
N04	62		+
O04	63		+
P04	64		+
A05	65		+
B05	66		+
C05	67		+
D05	68		+
E05	69		+
F05	70		+
G05	71		+
H05	72		+
I05	73		+
J05	74		+
K05	75		+
L05	76		+
M05	77		+
N05	78		+

O05	79		+
P05	80		+
A06	81		+
B06	82		+
C06	83		+
D06	84		+
E06	85		+
F06	86		+
G06	87		+
H06	88		+
I06	89		+
J06	90		+
K06	91		+
L06	92		+
M06	93		+
N06	94		+
O06	95		+
P06	96		+
A07	97		+
B07	98		+
C07	99		+
D07	100		+
E07	101		+
F07	102		+
G07	103		+
H07	104		+
I07	105		+
J07	106		+
K07	107		+
L07	108		+
M07	109		+
N07	110		+
O07	111		+
P07	112		+
A08	113		+
B08	114		+
C08	115		+
D08	116		+
E08	117		+
F08	118		+
G08	119		+
H08	120		+
I08	121		+
J08	122		+
K08	123		+

L08	124		+
M08	125		+
N08	126		+
O08	127		+
P08	128		+
A09	129		+
B09	130		+
C09	131		+
D09	132		+
E09	133		+
F09	134		+
G09	135		+
H09	136		+
I09	137		+
J09	138		+
K09	139		+
L09	140		+
M09	141		+
N09	142		+
O09	143		+
P09	144		+
A10	145		+
B10	146		+
C10	147		+
D10	148		+
E10	149		+
F10	150		+
G10	151		+
H10	152		+
I10	153		+
J10	154		+
K10	155		+
L10	156		+
M10	157		+
N10	158		+
O10	159		+
P10	160		+
A11	161		+
B11	162		+
C11	163		+
D11	164		+
E11	165		+
F11	166		+
G11	167		+
H11	168		+

I11	169		+
J11	170		+
K11	171		+
L11	172		+
M11	173		+
N11	174		+
O11	175		+
P11	176		+
A12	177		+
B12	178		+
C12	179		+
D12	180		+
E12	181		+
F12	182		+
G12	183		+
H12	184		+
I12	185		+
J12	186		+
K12	187		+
L12	188		+
M12	189		+
N12	190		+
O12	191		+
P12	192		+
A13	193		+
B13	194		+
C13	195		+
D13	196		+
E13	197		+
F13	198		+
G13	199		+
H13	200		+
I13	201		+
J13	202		+
K13	203		+
L13	204		+
M13	205		+
N13	206		+
O13	207		+
P13	208		+
A14	209		+
B14	210		+
C14	211		+
D14	212		+
E14	213		+

F14	214		+
G14	215		+
H14	216		+
I14	217		+
J14	218		+
K14	219		+
L14	220		+
M14	221		+
N14	222		+
O14	223		+
P14	224		+
A15	225		+
B15	226		+
C15	227		+
D15	228		+
E15	229		+
F15	230		+
G15	231		+
H15	232		+
I15	233		+
J15	234		+
K15	235		+
L15	236		+
M15	237		+
N15	238		+
O15	239		+
P15	240		+
A16	241		+
B16	242		+
C16	243		+
D16	244		+
E16	245		+
F16	246		+
G16	247		+
H16	248		+
I16	249		+
J16	250		+
K16	251		+
L16	252		+
M16	253		+
N16	254		+
O16	255		+
P16	256		+
A17	257		+
B17	258		+

C17	259		+
D17	260		+
E17	261		+
F17	262		+
G17	263		+
H17	264		+
I17	265		+
J17	266		+
K17	267		+
L17	268		+
M17	269		+
N17	270		+
O17	271		+
P17	272		+
A18	273		+
B18	274		+
C18	275		+
D18	276		+
E18	277		+
F18	278		+
G18	279		+
H18	280		+
I18	281		+
J18	282		+
K18	283		+
L18	284		+
M18	285		+
N18	286		+
O18	287		+
P18	288		+
A19	289		+
B19	290		+
C19	291		+
D19	292		+
E19	293		+
F19	294		+
G19	295		+
H19	296		+
I19	297		+
J19	298		+
K19	299		+
L19	300		+
M19	301		+
N19	302		+
O19	303		+

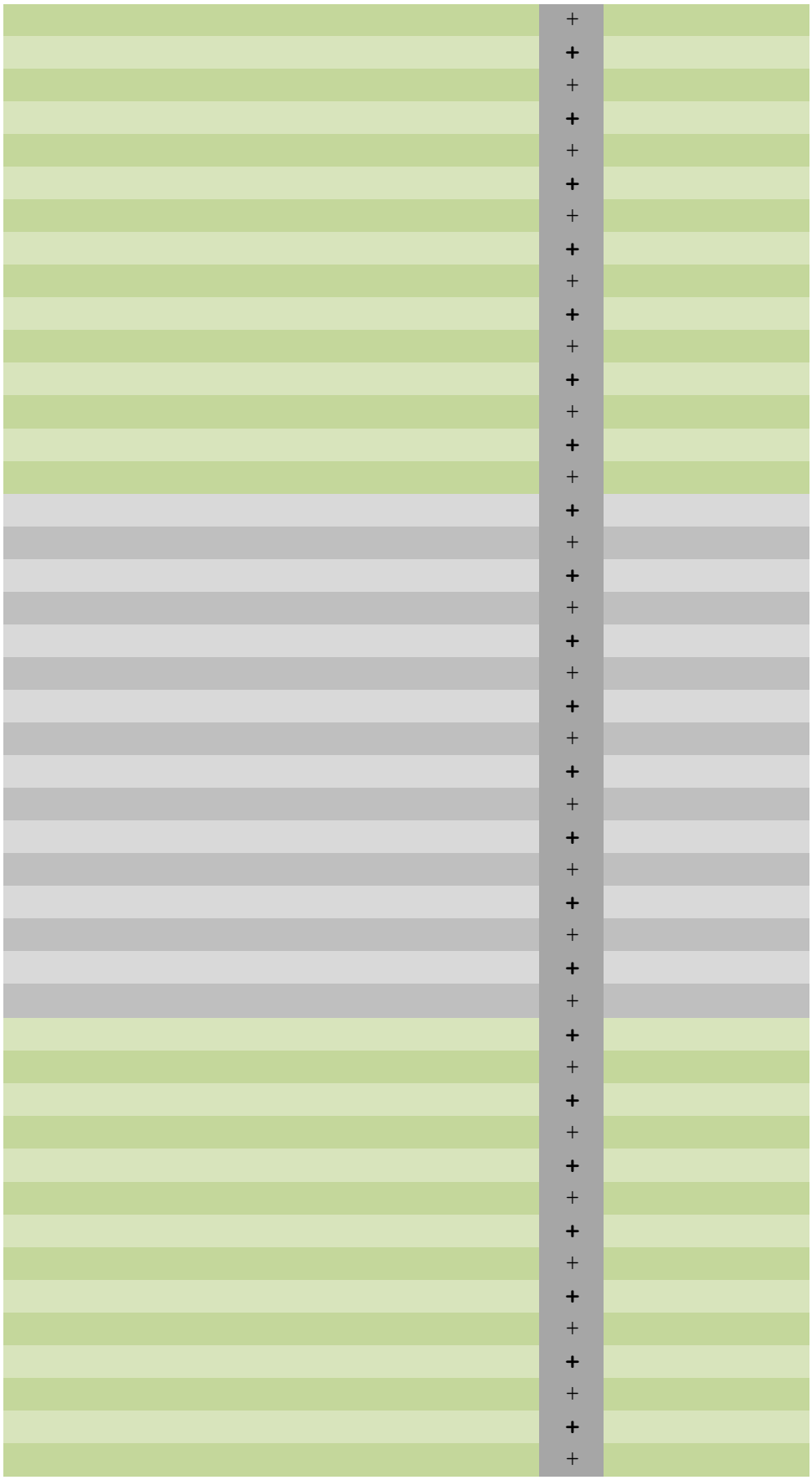
P19	304		+
A20	305		+
B20	306		+
C20	307		+
D20	308		+
E20	309		+
F20	310		+
G20	311		+
H20	312		+
I20	313		+
J20	314		+
K20	315		+
L20	316		+
M20	317		+
N20	318		+
O20	319		+
P20	320		+
A21	321		+
B21	322		+
C21	323		+
D21	324		+
E21	325		+
F21	326		+
G21	327		+
H21	328		+
I21	329		+
J21	330		+
K21	331		+
L21	332		+
M21	333		+
N21	334		+
O21	335		+
P21	336		+
A22	337		+
B22	338		+
C22	339		+
D22	340		+
E22	341		+
F22	342		+
G22	343		+
H22	344		+
I22	345		+
J22	346		+
K22	347		+
L22	348		+

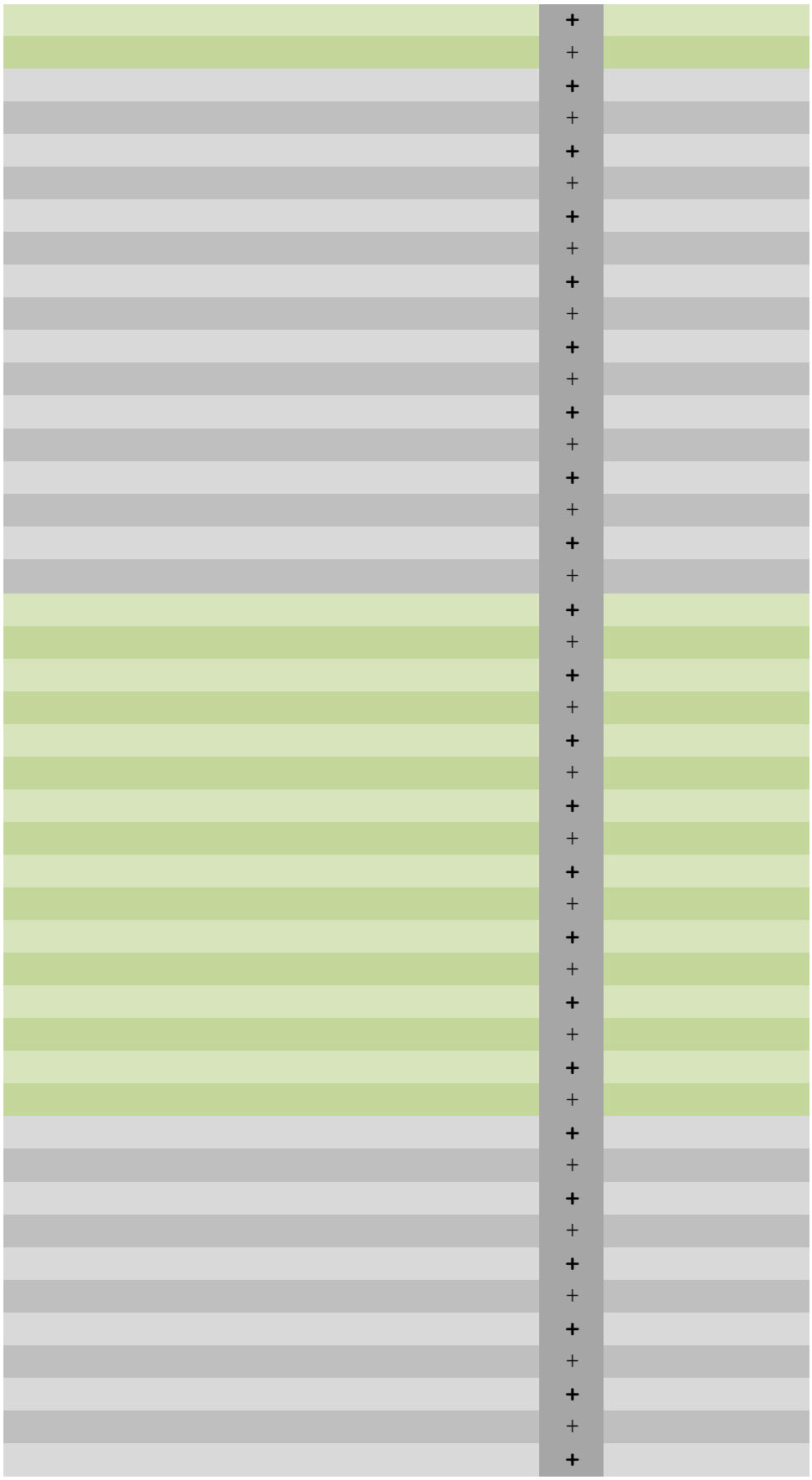
M22	349		+
N22	350		+
O22	351		+
P22	352		+
A23	353		+
B23	354		+
C23	355		+
D23	356		+
E23	357		+
F23	358		+
G23	359		+
H23	360		+
I23	361		+
J23	362		+
K23	363		+
L23	364		+
M23	365		+
N23	366		+
O23	367		+
P23	368		+
A24	369		+
B24	370		+
C24	371		+
D24	372		+
E24	373		+
F24	374		+
G24	375		+
H24	376		+
I24	377		+
J24	378		+
K24	379		+
L24	380		+
M24	381		+
N24	382		+
O24	383		+
P24	384		+

384-WELL TRANSFECTION TE

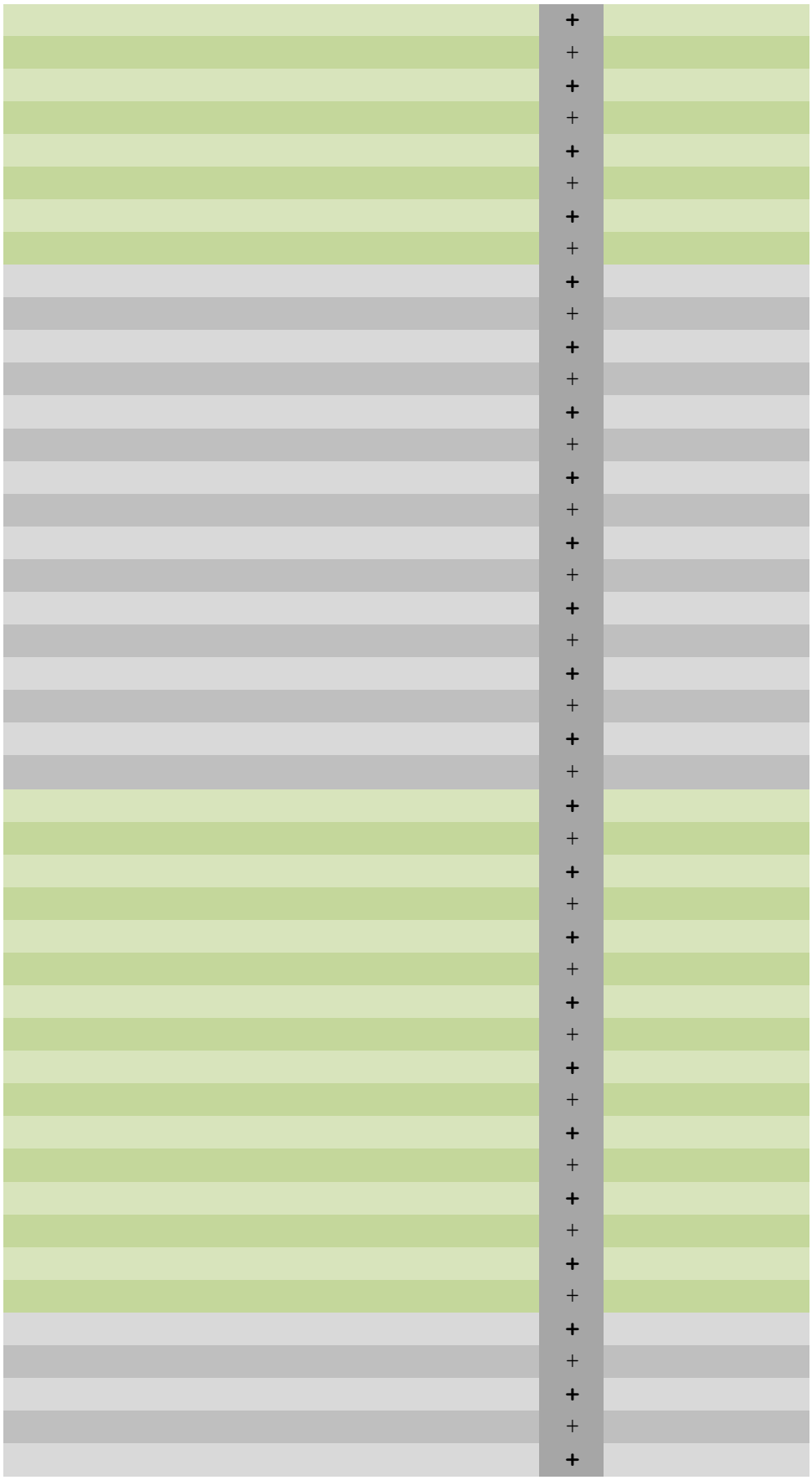
RESET

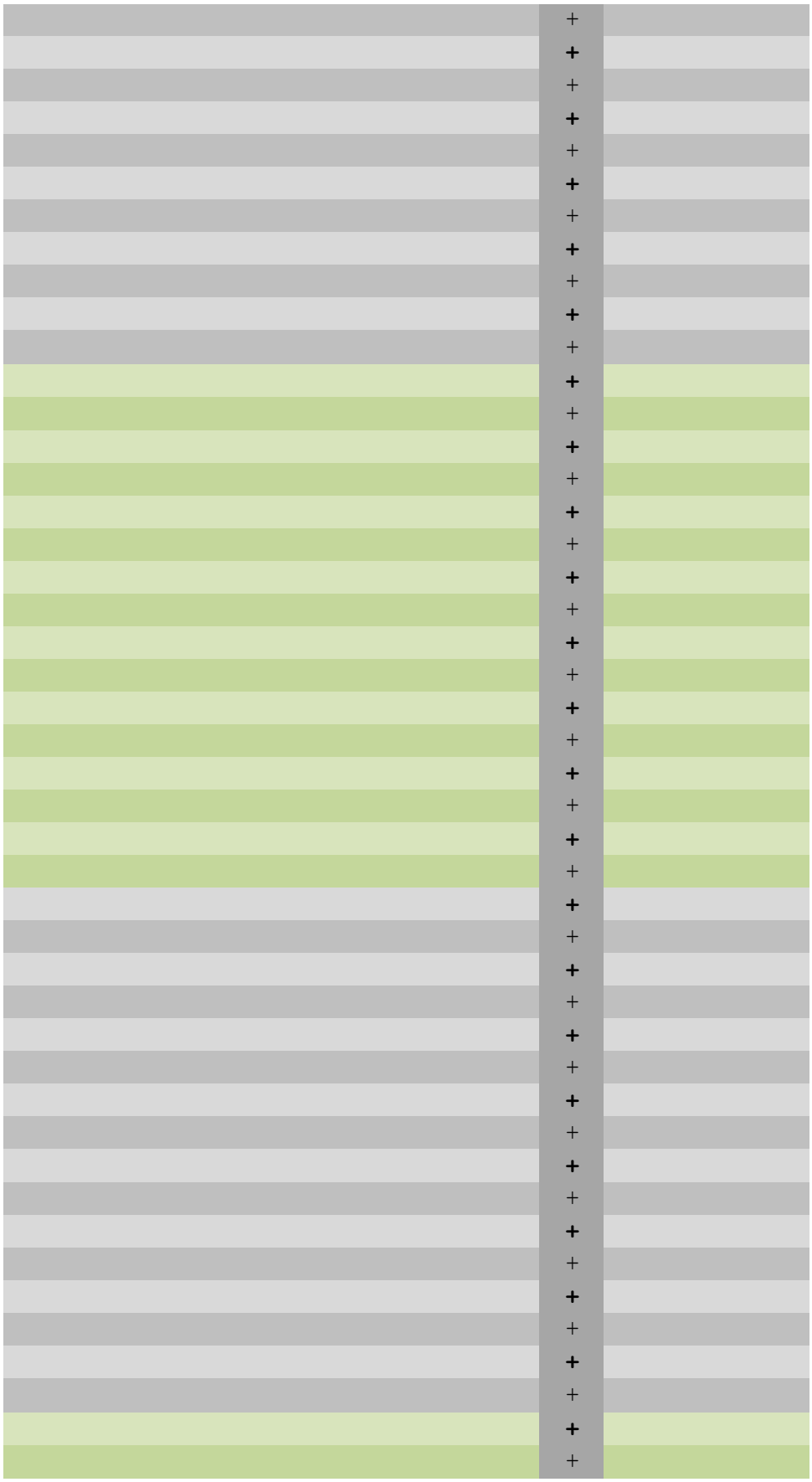
[source well C°] (ng/µL)			[source well C°] (ng/µL)
PLASMID 2			
Quantity (ng)	Plasmid Name		Quantity (ng)
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	
		+	

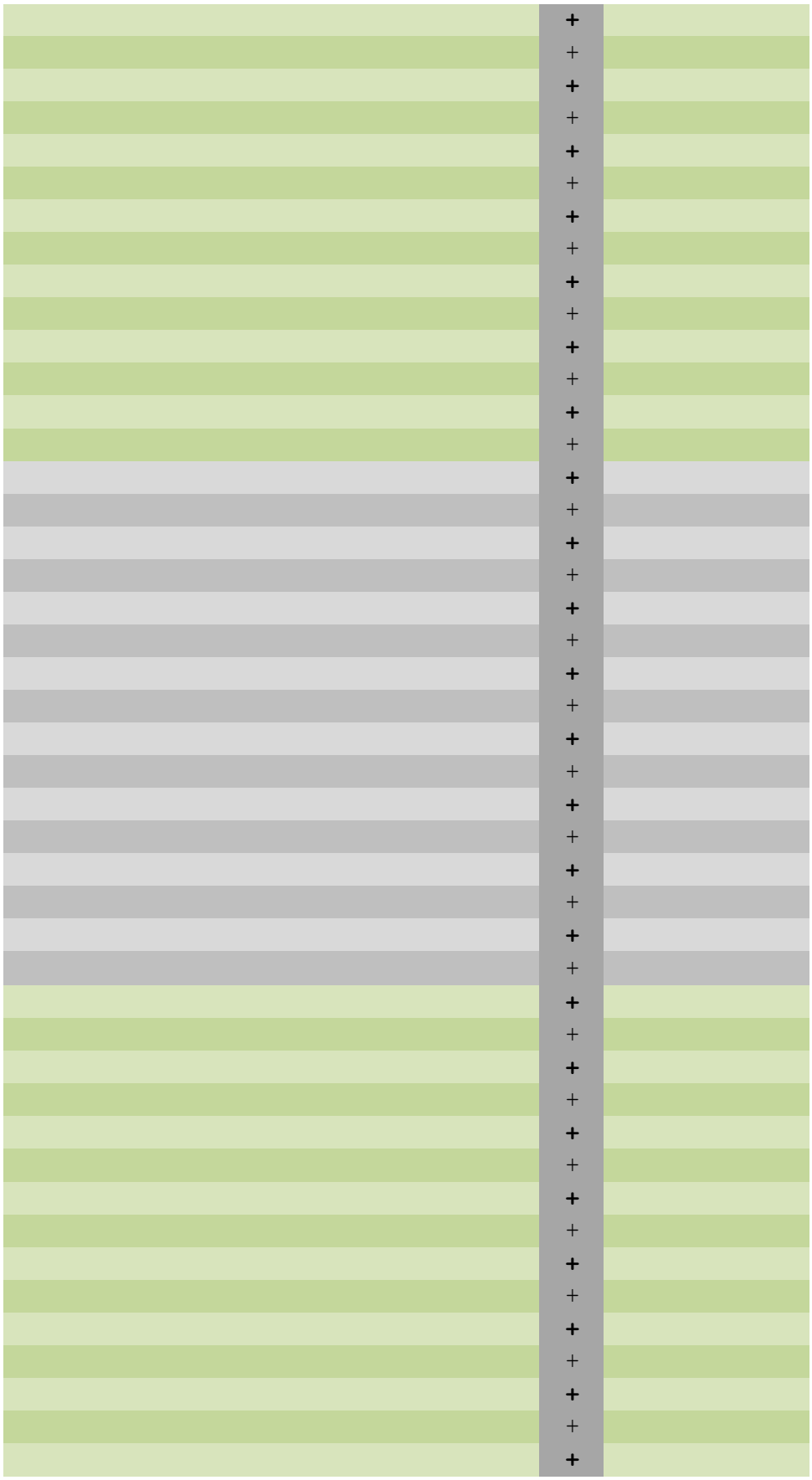




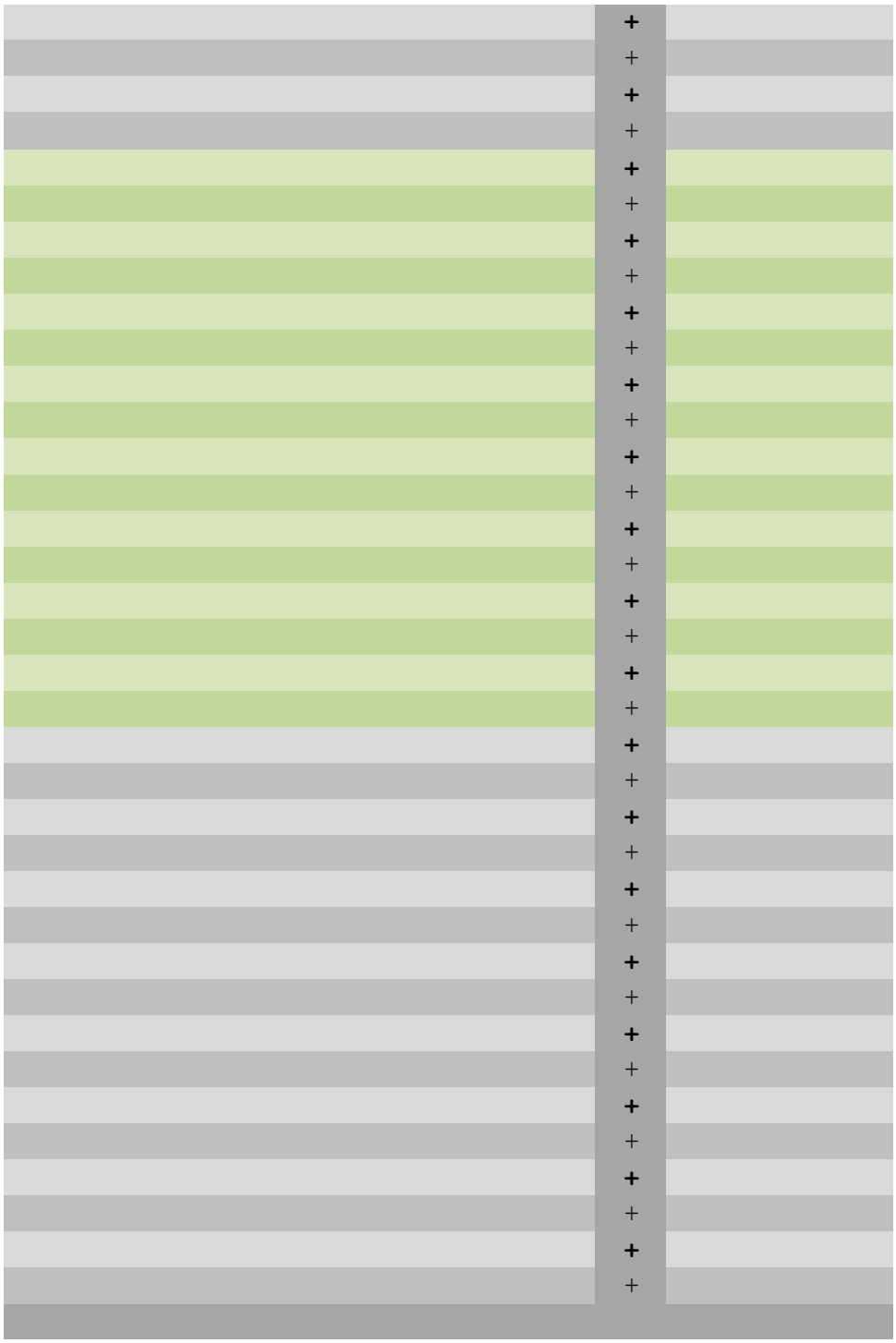
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	







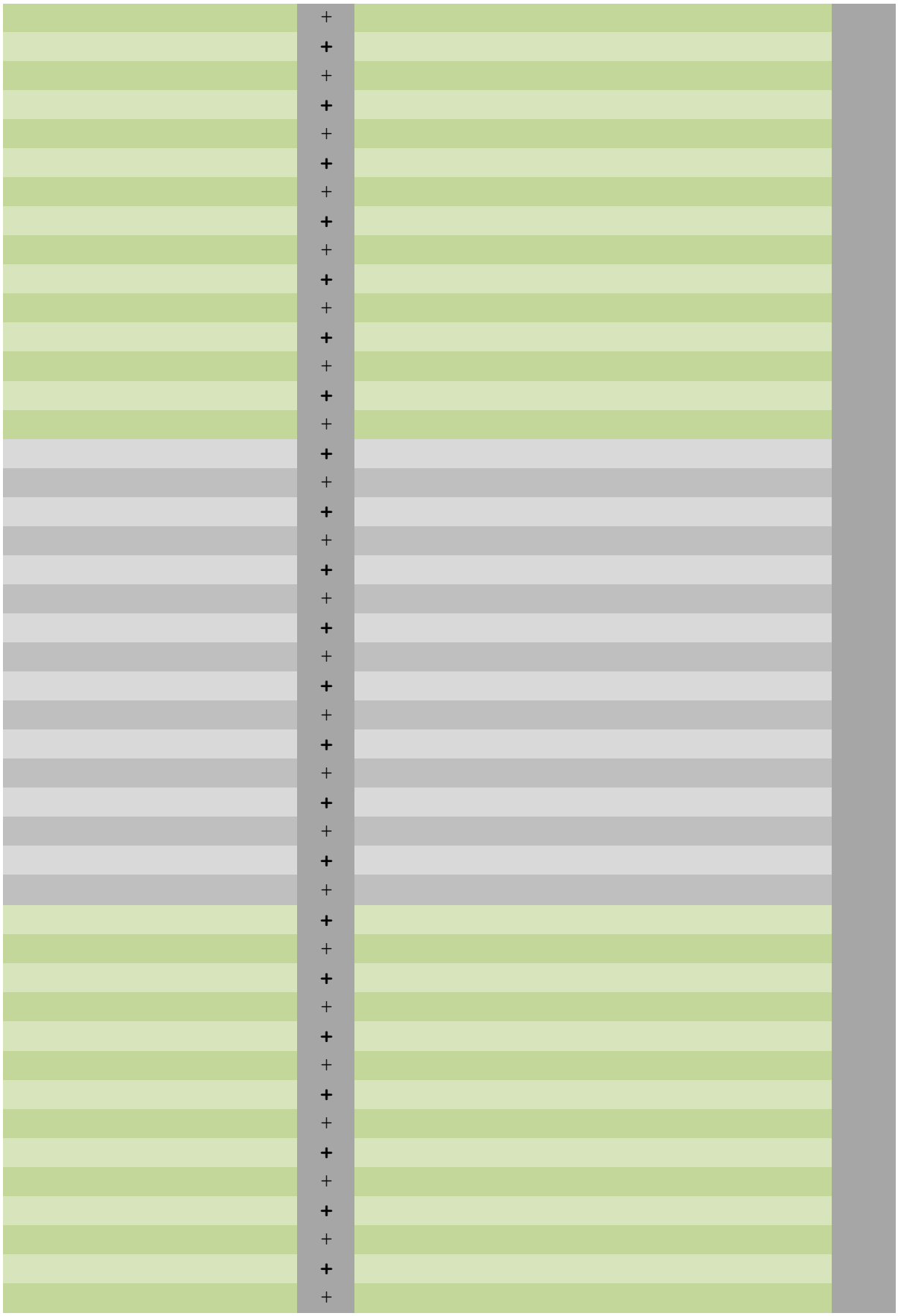
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	
	+	

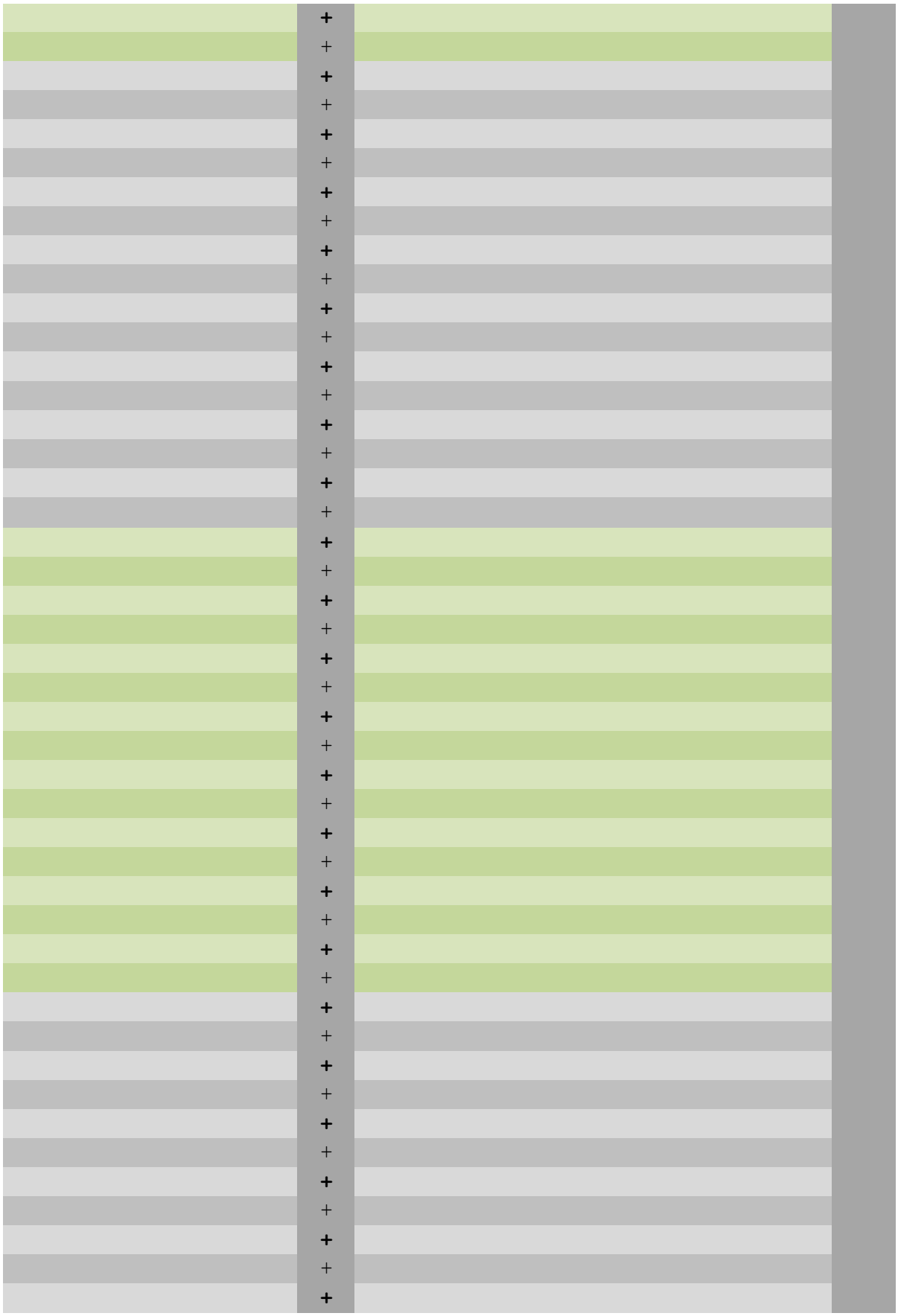


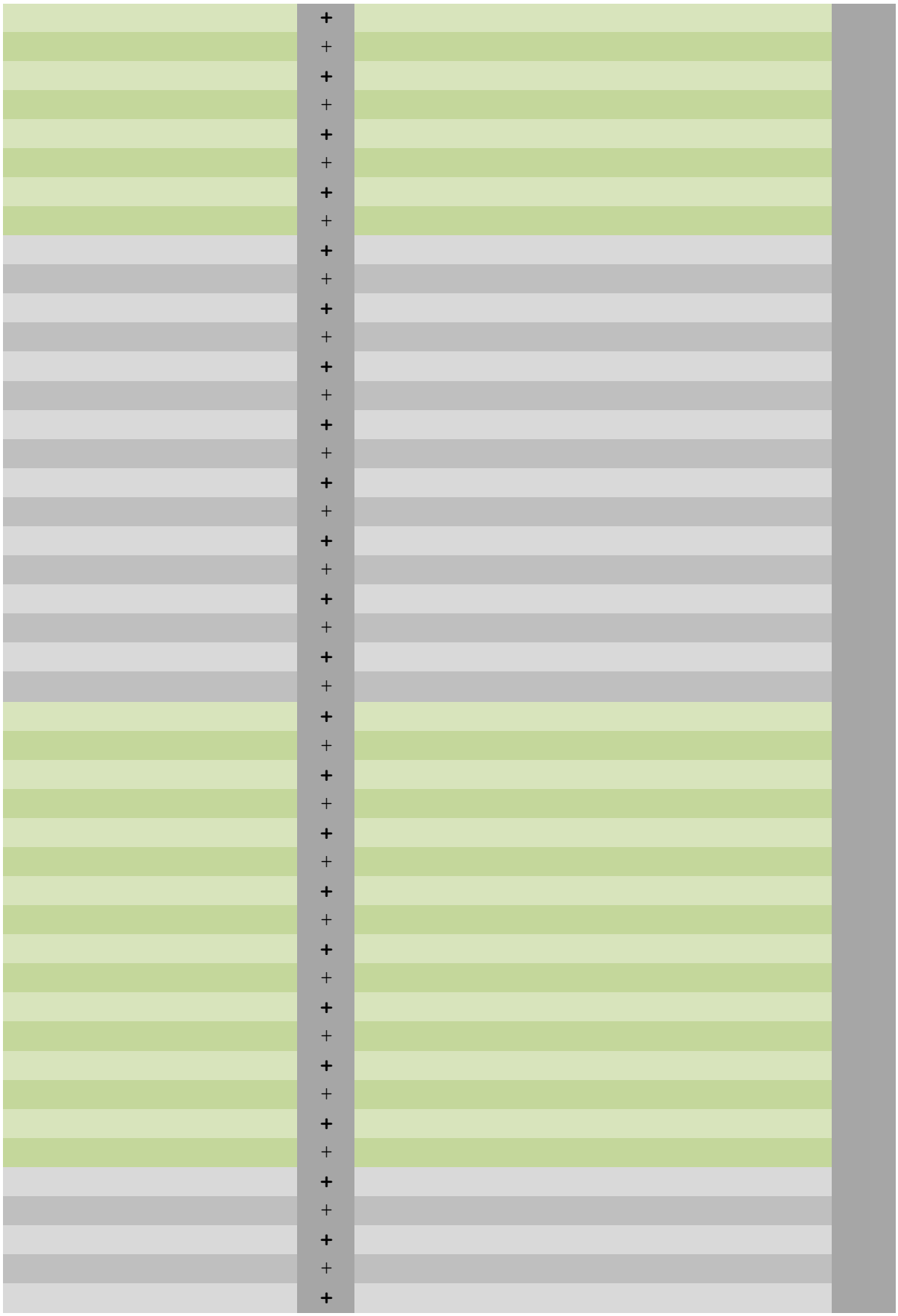
MPLATE

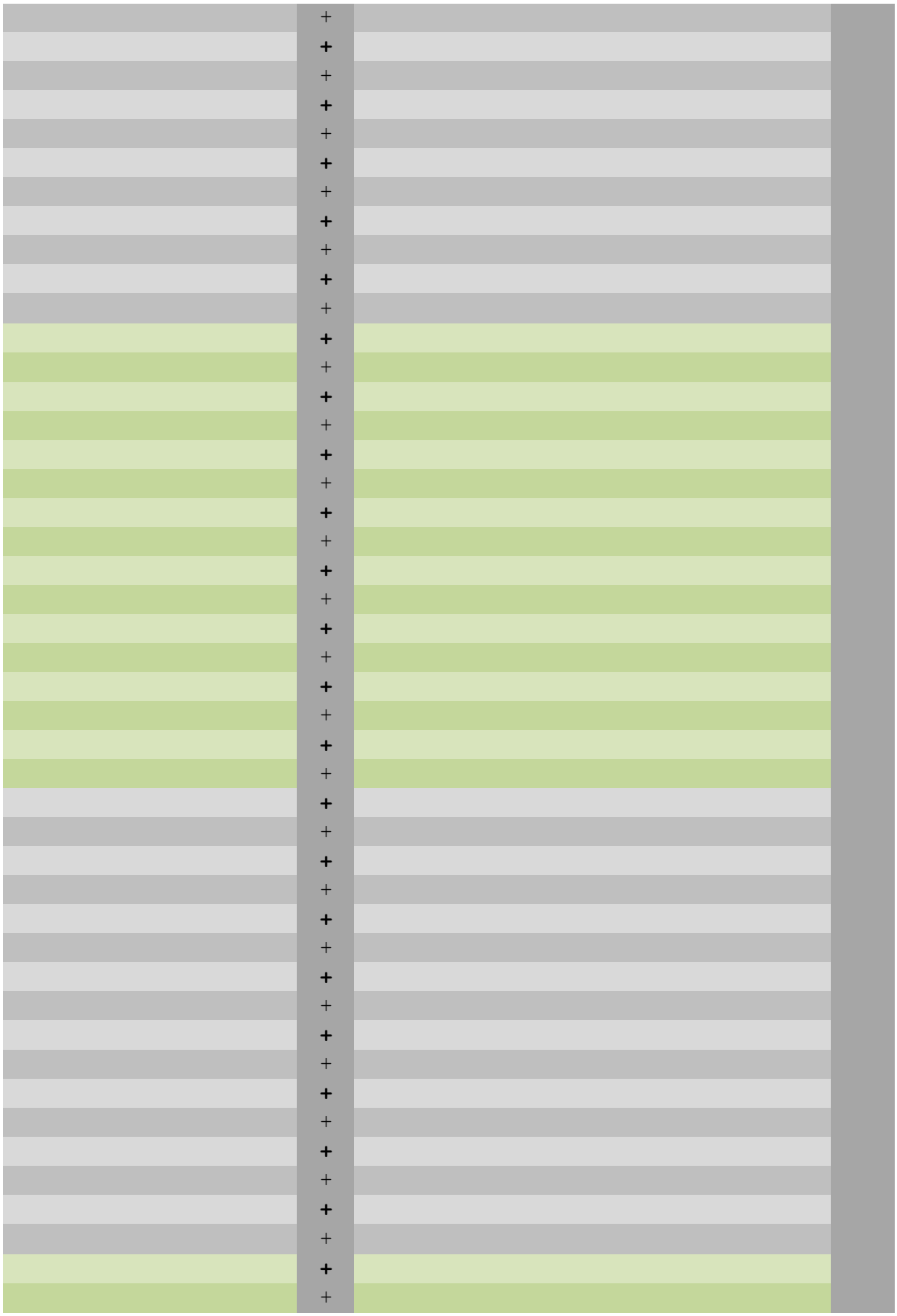
Generate PickLists

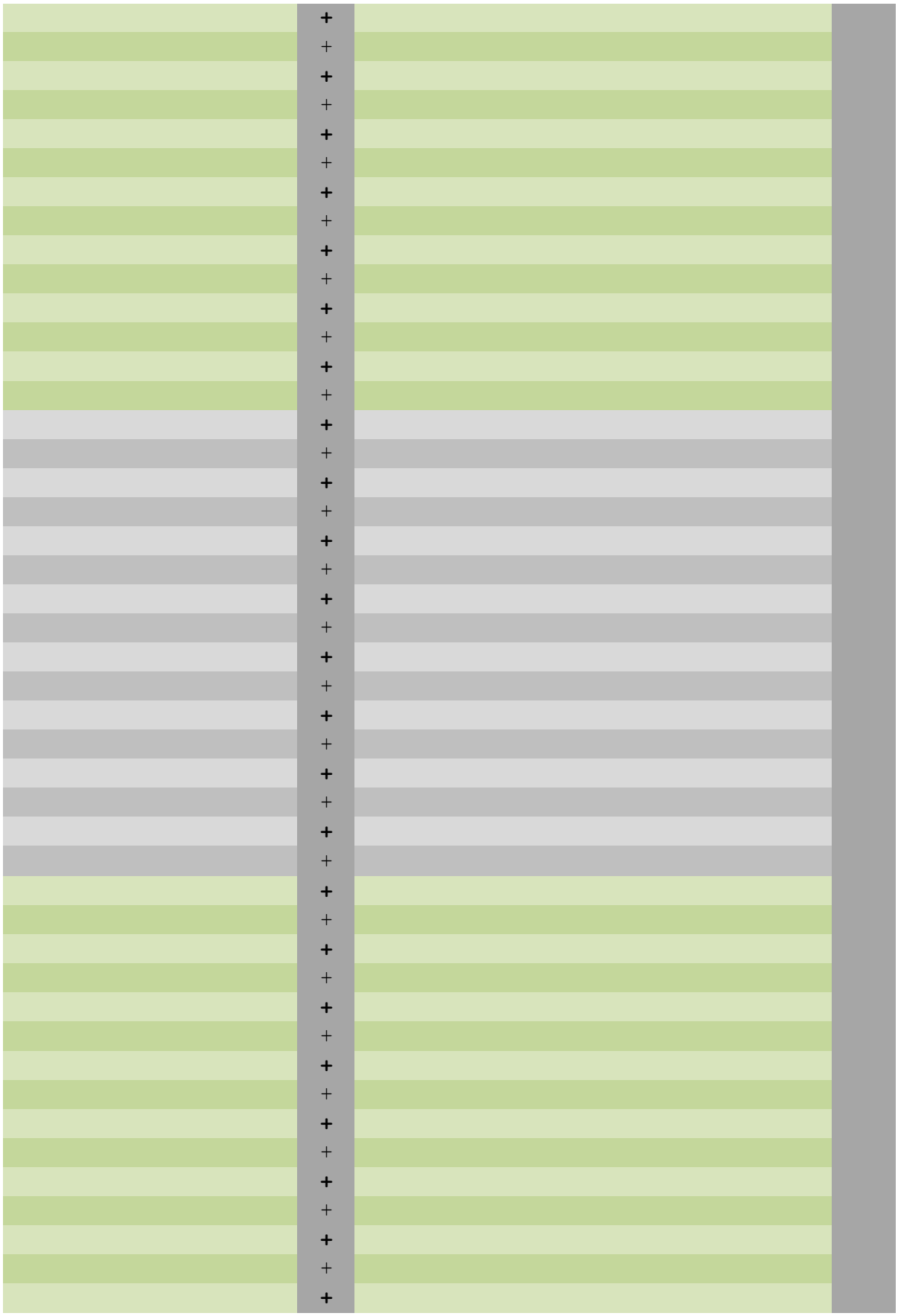
PLASMID 3		[source well C°] (ng/µL)	PLASMID 4
Plasmid Name		Quantity (ng)	Plasmid Name
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		
	+		

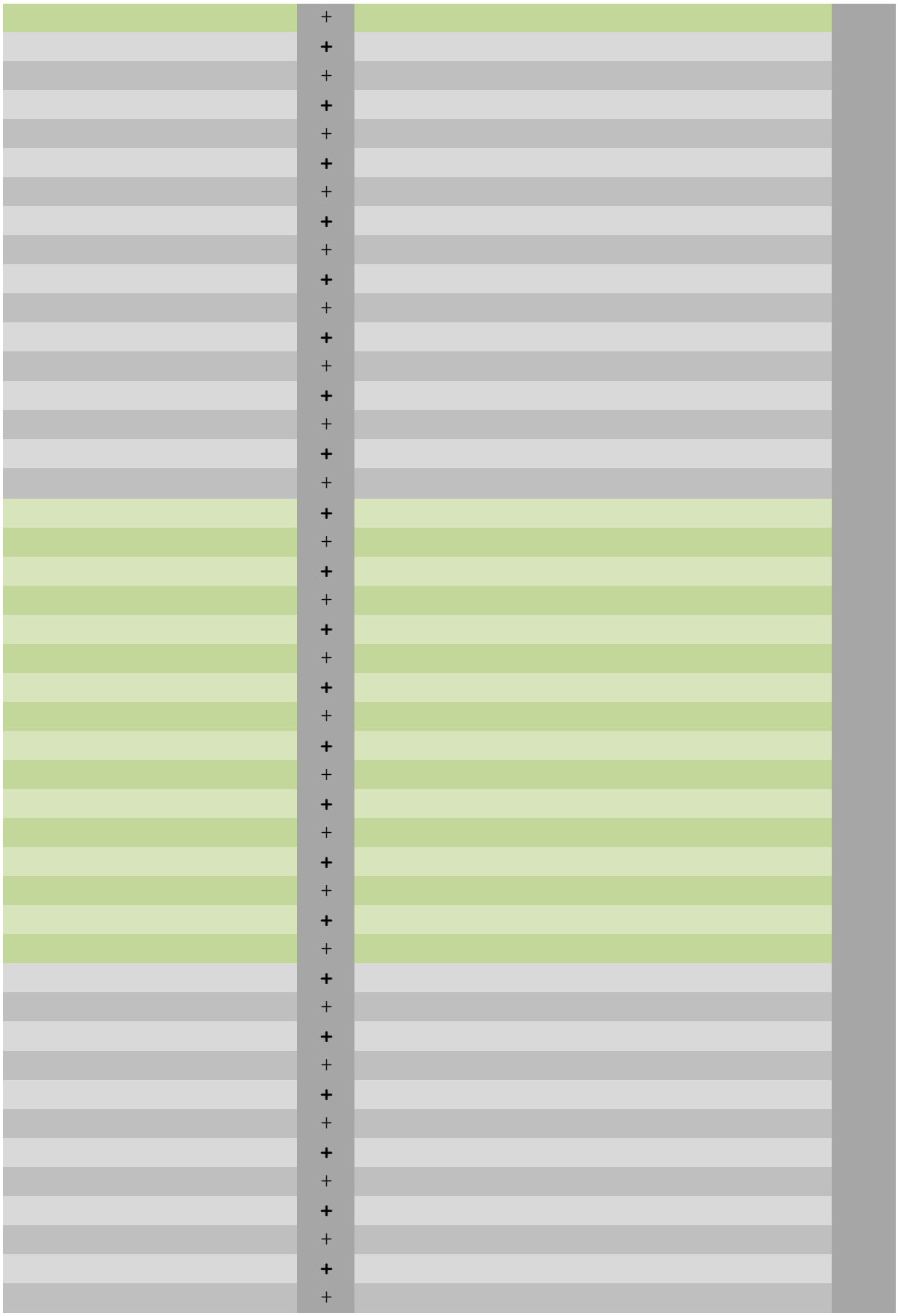


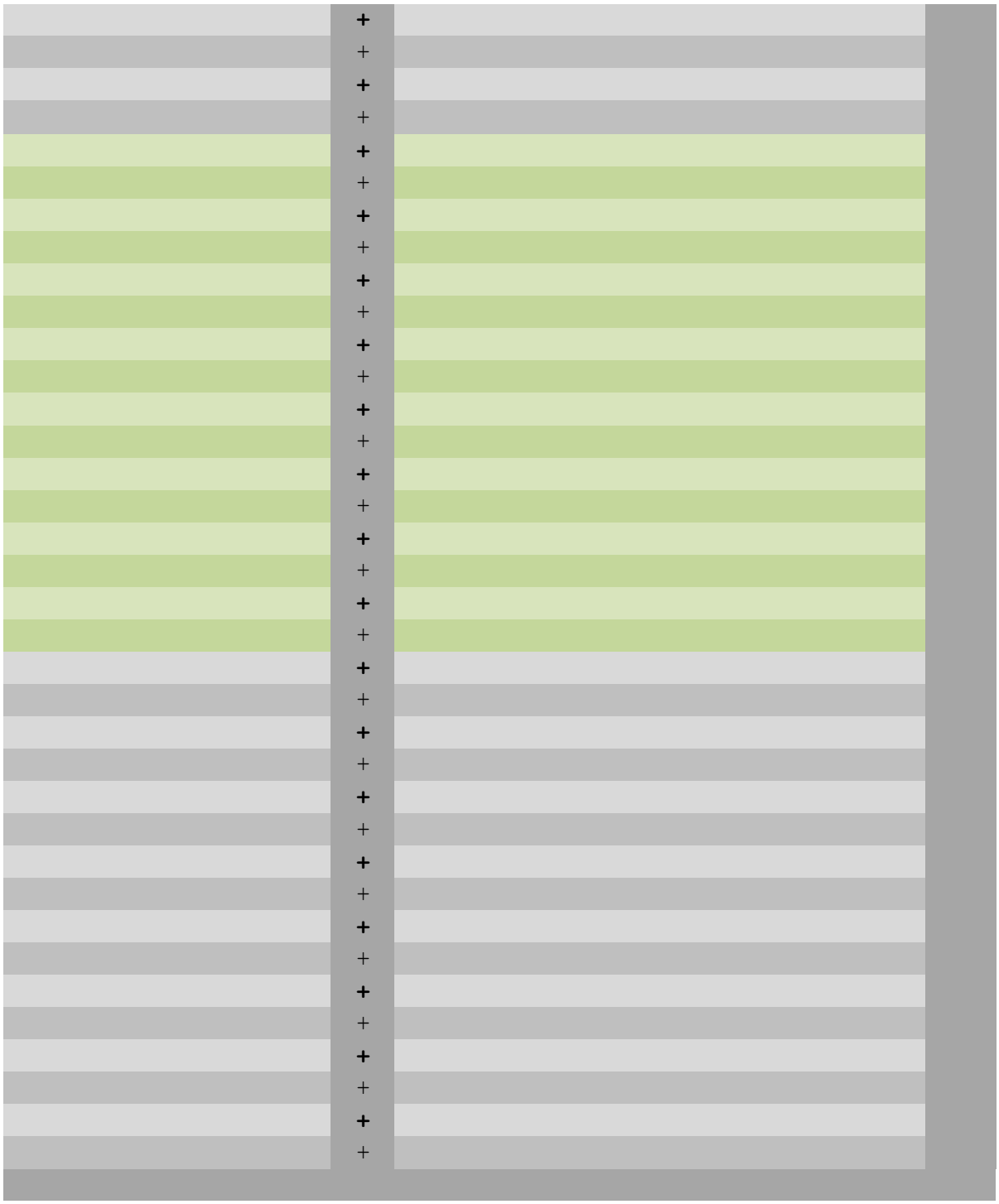












Source Plate n°1

(minimal volume to fill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

Source Plate n°2

(minimal volume to fill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								

P																							

Source Plate n°3

(minimal volume to fill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

Source Plate n°4

(minimal volume to fill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								

N																							
O																							
P																							

Source Plate n°5

(minimal volume to fill)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
E																								
F																								
G																								
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O																								
P																								

Well	Plate	Name
A01	Source Plate 1	mVenus
B01	Source Plate 1	tdTomato
C01	Source Plate 1	Transfection Reagent
D01	Source Plate 1	Transfection Reagent

Concentration	Volume	Well,Plate,Name,Concentra
100	7.6	A01,Source Plate 1,mVenus
100	7.6	B01,Source Plate 1,tdTomat
	11.5	C01,Source Plate 1,TR,100,1
	8.5	D01,Source Plate 1,TR,100,8

ıtion,Volume
,100,7.60
to,100,7.60
11.50
3.50

SAGE PUBLICATIONS LICENSE
TERMS AND CONDITIONS
Jan 25, 2019

This Agreement between INSERM DR NORD OUEST -- Cyril Couturier ("You") and SAGE Publications ("SAGE Publications") consists of your license details and the terms and conditions provided by SAGE Publications and Copyright Clearance Center.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	4515380890316
License date	Jan 24, 2019
Licensed Content Publisher	SAGE Publications
Licensed Content Publication	SLAS Discovery
Licensed Content Title	High-Throughput DNA Plasmid Transfection Using Acoustic Droplet Ejection Technology
Licensed Content Author	Béatrice Colin, Benoit Deprez, Cyril Couturier
Licensed Content Date	Oct 5, 2018
Licensed Content Volume	0
Licensed Content Issue	0
Licensed Content Pages	9
Type of Use	Journal
Requestor type	Publisher
The intended publisher of the new work is...	Not listed below
Format	Electronic
Portion	Figure/table
Number of figures/tables	3
Will you be translating?	No, only English
circulation	1000
Title of new article	High-throughput DNA Plasmid Multiplexing and Transfection using Acoustic Nanodispensing Technology
Lead author	Couturier
Title of targeted journal	Journal of Visual Experiment (JoVE)
Publisher	Not listed below
Expected publication date	Feb 2019
Order reference number	2019000793
Portions	Main results from figures 1, 2 and 3 will be presented in one new "representative results" figure.
Requestor Location	INSERM DR NORD OUEST 6 rue du Professeur Laguesse Lille, 59045 France Attn: N. Dekeyne
Customer VAT ID	FR31180036048
Billing Type	Invoice
Billing Address	INSERM DR NORD OUEST 6 rue du Professeur Laguesse Lille, France 59045 Attn: N. Dekeyne
Customer reference info	U1177 INSERM
Total	197.57 EUR

Terms and Conditions

SAGE Terms and Conditions for Permissions Administered Through Rightslink

You, the Requestor, are requesting to use the material specified in the permission request (the “Work”). Your agreement to the terms and conditions herein and completion of a permission request constitutes a Permission Request. You are in no way required to use the Work; however, should you decide not to use the Work after you complete this request, you agree to cancel your order through this website. Under the above conditions, and the following terms and conditions, permission to use Work (“Permission”) is granted solely to you:

1. SAGE reserves the right to revoke any Permission, at SAGE’s sole discretion, within two (2) business days of the request.
2. The number of copies (“Copies”) for print use is defined as the total number of copies made for distribution or for repurposing, and the number of copies (“Copies”) for electronic use is defined as the total number of viewers of the Work, recipients of

copies of the Work, and individuals or entities who may have access to the Work. The Copies must not exceed the Copies as stated in the Permission Request.

3. Requests to post a full article on a website, internet, intranet, or any publicly accessible site must be submitted to the Publisher at permissions@sagepub.com.
4. Permission is granted only for the type of use specified in the Permission Request. If any information pertaining to your Permission Request changes, you must cancel this request and resubmit a new request with the correct and current permission request information. SAGE may exercise its right to revoke Permission, if SAGE finds, in SAGE's sole opinion, that the context in which you have used or repurposed the Work is considered libelous, in violation of any right of privacy, or otherwise unlawful; infringement or in violation of any copyright or other proprietary right of others; or can be construed to possibly cause harm or injury. You agree that use of Work will be professional, in the context of fact-based and generally acceptable professional practices.
5. **Permission does not include the use within Custom Publishing Programs, and all use within such programs is explicitly prohibited.**
6. **Permission does not include use of the material within Massive Open Online Courses (MOOC's). For permission to include material in a MOOC, please contact SAGE directly at permissions@sagepub.com.**
7. If your Permission Request includes the right to translate the Work, you agree that the translation of the material shall be made faithfully and accurately, and that abbreviations and/or alterations in the text and/or title of the Work shall be made only with SAGE's prior the written consent. Requestor shall not own or acquire any copyright or other proprietary rights in the Work or any other material furnished by SAGE, including without limitation translations or transcriptions thereof, all of which rights shall be owned by and/or are hereby assigned to SAGE. Requestor shall indemnify SAGE against any and all claims, including without limitation attorneys' fees and legal costs, that concern or relate to (a) inaccurate translation or transcription of the Work, (b) infringement claims arising out of the inclusion of material not furnished by SAGE or (c) infringement or other claims asserted by anyone retained by Requestor to translate the Work. Requestor agrees that the name of the Author (s), Copyright Holder, and Publisher shall appear in due prominence on the title page of every copy of the translation and in all advertisements for the translation, and that the translation shall include: (1) the Work's original copyright notice and original American title, both in English, and (2) notice in granted translated language in identifying the SAGE Publications as the original publisher and stating the translation is published by arrangement with SAGE. The rights licensed to Requestor are not transferrable. **The translated article reprint must include the following disclaimer in English and in the language of the reprint: "While every effort has been made to ensure that the contents of this publication are factually correct, neither the authors nor the publisher accepts, and they hereby expressly exclude to the fullest extent permissible under applicable law, any and all liability arising from the contents published in this Article, including, without limitation, from any errors, omissions, inaccuracies in original or following translation, or for any consequences arising therefrom. Nothing in this notice shall exclude liability which may not be excluded by law. Approved product information should be reviewed before prescribing any subject medications.**"
8. Permission is granted for prospective use only, and does not apply to any use that has occurred prior to the date of the Permission Request.
9. Permission does not apply to any material (reprints, illustrations, figures, tables, etc.) not controlled by SAGE. Requests for permission to re-use third-party material should be submitted to the original copyright holder, as indicated in the credit line for the materials.
10. Full acknowledgment of your source must appear in every copy of your work as follows:
    - Author(s), Journal Title (Journal Volume Number and Issue Number)
    - pp. xx-xx, copyright © YEAR by (Copyright Holder)
    - Reprinted by Permission of SAGE Publications, Inc.
11. Unless specified in the request or by prior arrangement with SAGE, payment is due from you within sixty (60) days after the completion of Permission.
12. It is assumed that the Requester is using the selection in question, and is subject to billing and collections procedures, unless otherwise noted.
13. Permission Requests for reuse of text excerpts shall not exceed 50% of the article's content.
14. No more than 20% of any one SAGE journal issue may be reused at one time.

Other Terms and Conditions:

v4.2

You will be invoiced within 48 hours of this transaction date. You may pay your invoice by credit card upon receipt of the invoice for this transaction. Please follow instructions provided at that time.
To pay for this transaction now; please remit a copy of this document along with your payment. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK502984565.

Make payments to "COPYRIGHT CLEARANCE CENTER" and send to:

Copyright Clearance Center
29118 Network Place
Chicago, IL 60673-1291
Please disregard electronic and mailed copies if you remit payment in advance
Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

SAGE PUBLICATIONS LICENSE
TERMS AND CONDITIONS

Jan 24, 2019

This Agreement between INSERM DR NORD OUEST -- Cyril Couturier ("You") and SAGE Publications ("SAGE Publications") consists of your license details and the terms and conditions provided by SAGE Publications and Copyright Clearance Center.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	4515380890316
License date	Jan 24, 2019
Licensed Content Publisher	SAGE Publications
Licensed Content Publication	SLAS Discovery
Licensed Content Title	High-Throughput DNA Plasmid Transfection Using Acoustic Droplet Ejection Technology
Licensed Content Author	Béatrice Colin, Benoit Deprez, Cyril Couturier
Licensed Content Date	Oct 5, 2018
Licensed Content Volume	0
Licensed Content Issue	0
Licensed Content Pages	9
Type of Use	Journal
Requestor type	Publisher
The intended publisher of the new work is...	Not listed below
Format	Electronic
Portion	Figure/table
Number of figures/tables	3
Will you be translating?	No, only English
circulation	1000
Title of new article	High-throughput DNA Plasmid Multiplexing and Transfection using Acoustic Nanodispensing Technology
Lead author	Couturier

Title of targeted journal	Journal of Visual Experiment (JoVE)
Publisher	Not listed below
Expected publication date	Feb 2019
Order reference number	2019000793
Portions	Main results from figures 1, 2 and 3 will be presented in one new "representative results" figure.
Requestor Location	INSERM DR NORD OUEST 6 rue du Professeur Laguesse Lille, 59045 France Attn: N. Dekeyne
Customer VAT ID	FR31180036048
Billing Type	Invoice
Billing Address	INSERM DR NORD OUEST 6 rue du Professeur Laguesse Lille, France 59045 Attn: N. Dekeyne
Customer reference info	U1177 INSERM
Total	197.57 EUR

Terms and Conditions

SAGE Terms and Conditions for Permissions Administered Through Rightslink

You, the Requestor, are requesting to use the material specified in the permission request (the "Work"). Your agreement to the terms and conditions herein and completion of a permission request constitutes a Permission Request. You are in no way required to use the Work; however, should you decide not to use the Work after you complete this request, you agree to cancel your order through this website. Under the above conditions, and the following terms and conditions, permission to use Work ("Permission") is granted solely to you:

1. SAGE reserves the right to revoke any Permission, at SAGE's sole discretion, within two (2) business days of the request.
2. The number of copies ("Copies") for print use is defined as the total number of copies made for distribution or for repurposing, and the number of copies ("Copies") for electronic use is defined as the total number of viewers of the Work, recipients of copies of the Work, and individuals or entities who may have access to the Work. The Copies must not exceed the Copies as stated in the Permission Request.
3. Requests to post a full article on a website, internet, intranet, or any publicly accessible site must be submitted to the Publisher at permissions@sagepub.com.
4. Permission is granted only for the type of use specified in the Permission Request. If any information pertaining to your Permission Request changes, you must cancel this request and resubmit a new request with the correct and current permission request information. SAGE may exercise its right to revoke Permission, if SAGE finds, in SAGE's sole opinion, that the context in which you have used or repurposed the Work is considered libelous, in violation of any right of privacy, or otherwise unlawful; infringement or in violation of any copyright or other proprietary right of others; or can be construed to possibly cause harm or injury. You agree that use of Work will be professional, in the context of fact-based and generally acceptable professional practices.
5. **Permission does not include the use within Custom Publishing Programs, and all use within such programs is explicitly prohibited.**

6. **Permission does not include use of the material within Massive Open Online Courses (MOOC's). For permission to include material in a MOOC, please contact SAGE directly at permissions@sagepub.com.**
7. If your Permission Request includes the right to translate the Work, you agree that the translation of the material shall be made faithfully and accurately, and that abbreviations and/or alterations in the text and/or title of the Work shall be made only with SAGE's prior the written consent. Requestor shall not own or acquire any copyright or other proprietary rights in the Work or any other material furnished by SAGE, including without limitation translations or transcriptions thereof, all of which rights shall be owned by and/or are hereby assigned to SAGE. Requestor shall indemnify SAGE against any and all claims, including without limitation attorneys' fees and legal costs, that concern or relate to (a) inaccurate translation or transcription of the Work, (b) infringement claims arising out of the inclusion of material not furnished by SAGE or (c) infringement or other claims asserted by anyone retained by Requestor to translate the Work. Requestor agrees that the name of the Author (s), Copyright Holder, and Publisher shall appear in due prominence on the title page of every copy of the translation and in all advertisements for the translation, and that the translation shall include: (1) the Work's original copyright notice and original American title, both in English, and (2) notice in granted translated language in identifying the SAGE Publications as the original publisher and stating the translation is published by arrangement with SAGE. The rights licensed to Requestor are not transferrable. **The translated article reprint must include the following disclaimer in English and in the language of the reprint: "While every effort has been made to ensure that the contents of this publication are factually correct, neither the authors nor the publisher accepts, and they hereby expressly exclude to the fullest extent permissible under applicable law, any and all liability arising from the contents published in this Article, including, without limitation, from any errors, omissions, inaccuracies in original or following translation, or for any consequences arising therefrom. Nothing in this notice shall exclude liability which may not be excluded by law. Approved product information should be reviewed before prescribing any subject medications.**"
8. Permission is granted for prospective use only, and does not apply to any use that has occurred prior to the date of the Permission Request.
9. Permission does not apply to any material (reprints, illustrations, figures, tables, etc.) not controlled by SAGE. Requests for permission to re-use third-party material should be submitted to the original copyright holder, as indicated in the credit line for the materials.
10. Full acknowledgment of your source must appear in every copy of your work as follows:
    - Author(s), Journal Title (Journal Volume Number and Issue Number)
    - pp. xx-xx, copyright © YEAR by (Copyright Holder)
    - Reprinted by Permission of SAGE Publications, Inc.
11. Unless specified in the request or by prior arrangement with SAGE, payment is due from you within sixty (60) days after the completion of Permission.
12. It is assumed that the Requester is using the selection in question, and is subject to billing and collections procedures, unless otherwise noted.
13. Permission Requests for reuse of text excerpts shall not exceed 50% of the article's content.
14. No more than 20% of any one SAGE journal issue may be reused at one time.

Other Terms and Conditions:

v4.2

You will be invoiced within 48 hours of this transaction date. You may pay your invoice by credit card upon receipt of the invoice for this transaction. Please follow instructions provided at that time.
To pay for this transaction now; please remit a copy of this document along with your payment. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK502984565.

Make payments to "COPYRIGHT CLEARANCE CENTER" and send to:

Copyright Clearance Center
29118 Network Place
Chicago, IL 60673-1291
Please disregard electronic and mailed copies if you remit payment in advance

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

plate adapter

plate adapter