

Reverse Transfection Guide

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This guide describes a microarray-based system for the functional analysis in mammalian cells of many genes in parallel. Mammalian cells are cultured on a glass slide printed in defined locations with solutions containing different DNAs. Cells growing on the printed areas take up the DNA, creating spots of localized transfection within a lawn of non-transfected cells. We call this approach 'reverse transfection' and we have developed two methods to reverse transfect cells.

The image below (Figure 1) is an example of the microarrays that can be created with this approach. It is a laser scan image of a 14 X 10 microarray of cell clusters expressing Green Fluorescent Protein (GFP). Each cluster is about 120 μm in diameter and contains 50-80 transfected cells.

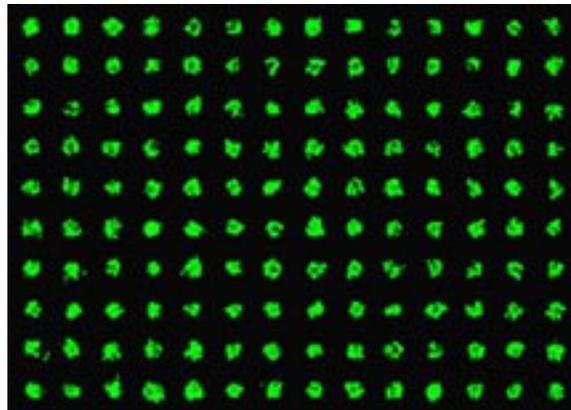


Figure 1: GFP array.

By printing sets of complementary DNAs (cDNAs) cloned in expression vectors, we can make microarrays whose features are groups of live cells that express a defined cDNA at each location. These 'transfected cell microarrays' should be of broad utility for the high-throughput expression cloning of genes, particularly in areas such as signal transduction and drug discovery.

The schematic (Figure 2) depicts the steps in making transfected cell microarrays. Plasmid DNA dissolved in an aqueous gelatin solution is printed on a glass slide using a robotic arrayer. The slide is dried and the printed array covered with a lipid-based transfection reagent. After removal of the transfection reagent, the slide is placed in a culture dish and covered with cells in media. The transfected cell microarray forms in 1-2 days and is then ready for downstream assays. The method illustrated is the 'gelatin-DNA' method of the reverse transfection approach. We have also developed a variant called the 'lipid-DNA' method in which the lipid-based transfection reagent is added to the DNA prior to printing. This guide describes how to implement both methods.

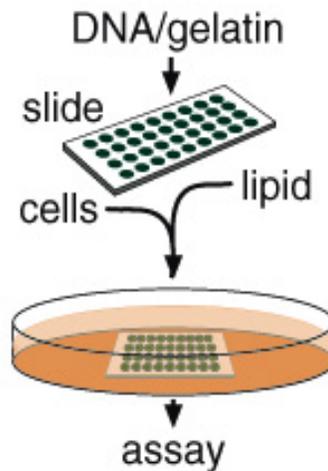


Figure 2: Schematic for making transfected cell microarrays.

Gelatin Preparation, DNA Purification, Sample Preparation and Microarray Printing

Materials

- Gelatin, Type B: 225 Bloom (Sigma, catalog #G-9391)
- sterile MilliQ water
- Purified DNA plasmids (see below)
- Gamma-Amino Propyl Silane (GAPS) slides (Corning catalog #2549)
- Costar 384-well plates (VWR catalog #7402)
- Stealth Micro Spotting Pins (Telechem International, Inc. catalog #SMP4)
- PixSys 5500 Robotic Arrayer (Cartesian Technologies, Model AD20A5)
- Vacuum Desiccator with Stopcock 250 mm, NALGENE (VWR catalog #24987-004),
- DRIERITE Anhydrous Calcium Sulfate (VWR catalog #22890-229)
- Light Microscope (4x-20x) to visualize printed arrays.

Preparation of 0.2% (w/v) Gelatin Solution

- 1) Dissolve gelatin powder in sterile MilliQ water by gently swirling mixture for 15 minutes in a 60°C water bath.
- 2) Cool the 0.2% gelatin solution at room temperature, and, while still warm (~37-40°C), filter it through a 0.45 µm cellular acetate membrane (CA). We usually prepare 100 ml at a time and store 50 ml aliquots of the filtered gelatin solution at 4°C.

Notes on DNA Purification

DNA can be purified via any method that gives supercoiled plasmid DNA with a 260/280 absorbance ratio greater than 1.7. To purify many plasmids in parallel, we use the Qiagen Turbo Miniprep Kit. We seed bacterial clones in 1.3 ml of terrific broth (TB) in a 96-Deep-Well Plate and shake them at 250 rpm for 18-24 hours at 37°C. Of course, DNA of equivalent or better quality can be purified via other methods and is also suitable for making transfected cell microarrays.

Preparation of Samples to Print

To prepare samples for printing, in a 96-well plate dilute purified plasmid DNAs with 0.2% gelatin to a final DNA concentration of 0.030-0.040 µg/µl. Make sure the final gelatin concentration remains greater than 0.17%. This requires DNA solutions of relatively high concentration. After dilution we mix the DNA-gelatin samples and transfer them to a 384-well plate for printing with a robotic arrayer.

Notes on Microarray Printing

We use a PixSys5500 Robotic Arrayer with Telechem's ArrayIt Stealth Pins (SMP4), but have also successfully tested other arrayers. We program the arrayer to transfer the gelatin-DNA solution to the slide by touching the pins to the slide surface for 25-50 ms. Using SMP4 pins, the printed spots have diameters of 100-120 µm and we normally print them 300-400 µm apart. We always maintain a 55% relative humidity environment during the arraying and perform a thorough wash step between each dip into a DNA-containing sample. The dried spots on the slide are very difficult to see because they contain low amounts of salt. Figure 3 is a photograph

of a dried 14 X 10 array and was taken with a light microscope. We have transfected slides as soon as one hour after printing, but we normally keep them for at least 16 hours before using them.

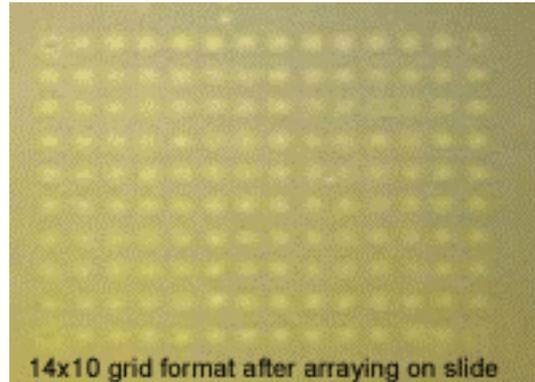


Figure 3: Dried printed microarray.

Note on Storage of Printed Slides

We store printed slides at 4°C or at room temperature (~20-25°C) in a vacuum desiccator containing anhydrous calcium sulfate pellets. We have not observed a detectable deterioration in performance after storage for greater than 3 months. See Tips and Information for other information on storage conditions.

Cell Preparation

We optimized conditions for reverse transfection with the HEK 293T cell line. We culture these adherent cells in DMEM containing 10% IFS, 50 units/ml penicillin and 50 µg/ml streptomycin. In this media the cells have a doubling time of about 20 hours and we split them every 3-4 days to avoid confluency.

To prepare HEK 293T cells for reverse transfection:

- 1) 24 hours before needed, plate 10×10^6 cells in 10 ml media in a 10 cm dish. Alternatively, 5×10^6 cells can be cultured for 2 days before transfection. In both cases, cells are still actively growing when harvested.
- 2) Immediately before reverse-transfection, in a tissue culture hood harvest cells from a 10 cm dish as follows:
 - a) Remove media via vacuum aspiration.
 - b) Rinse cells with 2 ml of 4°C trypsin-EDTA (Life Technologies, 25300-054). Allow the solution to spread over the plate and then remove it immediately via vacuum aspiration.
 - c) Add 1 ml trypsin-EDTA to the cells and evenly spread over plate.
 - d) Bang the plate with the palm of your hand to dislodge cells from the surface of the plate.
 - e) Allow cells to sit in the tissue culture hood for 3-5 minutes.
 - f) Add 10 ml of 37°C full medium to the plate of cells.

- g) Pipet up and down 12-15 times with a 10 ml pipet until a single cell suspension is obtained. Try to avoid creating too much froth.
- h) Count cells in a hemocytometer.
- i) Aliquot 10×10^6 cells into a sterile 50 ml conical tube.
- j) Add warmed full medium to bring volume up to 25 ml.
- k) Close tube and mix by inverting 3-4 times.
- l) Cells are now ready to add to microarrays. If cells are ready before transfection time is over, we keep the 50 ml tube containing the cells in the tissue culture incubator. We have kept cells in the 50 ml tube for up to 30-40 minutes before needed and have seen no adverse effects. Thus, cells can be harvested and prepared before beginning transfection of arrays. Always invert cell mix 2-3 times immediately before adding to arrays.

We also tested other adherent cell lines (cos7, NIH 3T3, HeLa, A549) and found them to give transfection efficiencies of 30-50% of HEK293T cells. For these cell lines we also used 10×10^6 cells.

Reverse Transfection of slides printed with gelatin-DNA microarrays

Materials

- Gelatin-DNA- arrayed GAPS slides (above)
- Effectene Transfection Reagent (Qiagen catalog #301425)
- INTEGRID 100 x 15 mm Tissue Culture Square Petri Dishes (Becton Dickinson: Falcon catalog #35-1012)
- CoverWell Incubation Chambers (Grace Bio-Labs catalog #PC200) or HybriWell Sealing Systems (Grace Bio-Labs catalog #HB2240)
- Forceps to manipulate slides
- Human Embryonic Kidney (HEK) 293T cells

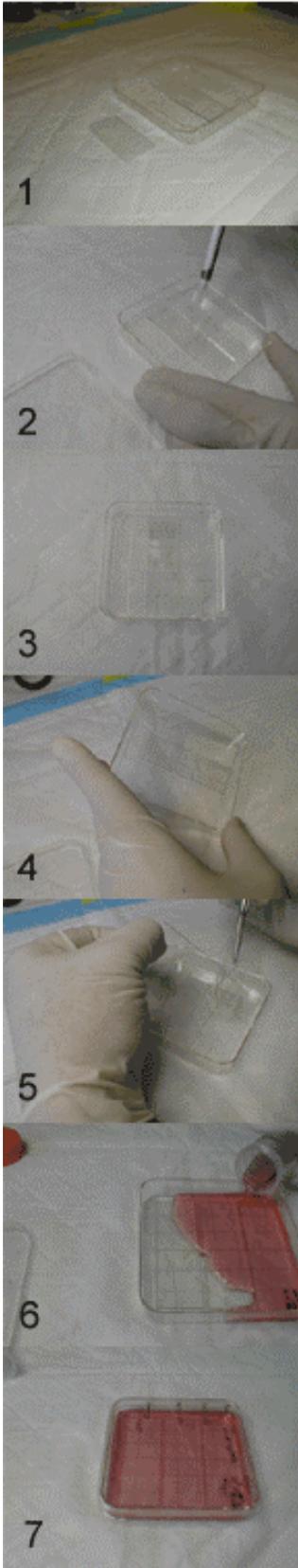
Note: Enhancer, EC buffer and Effectene are components of the Effectene Transfection Reagent kit.

For each slide to be transfected:

- 1) In a 1.5 ml micro-centrifuge tube add 16 μ l Enhancer to 150 μ l EC Buffer.
- 2) Mix and incubate for 5 minutes at room temperature.
- 3) Add 25 μ l Effectene Transfection Reagent and mix by lightly vortexing for 3-4 seconds (setting 4 on Vortex Genie 2, VWR).

Follow steps 4-8 if using HybriWells and 9-12 if using CoverWells. **We now routinely use only HybriWells.**

HybriWell Procedure

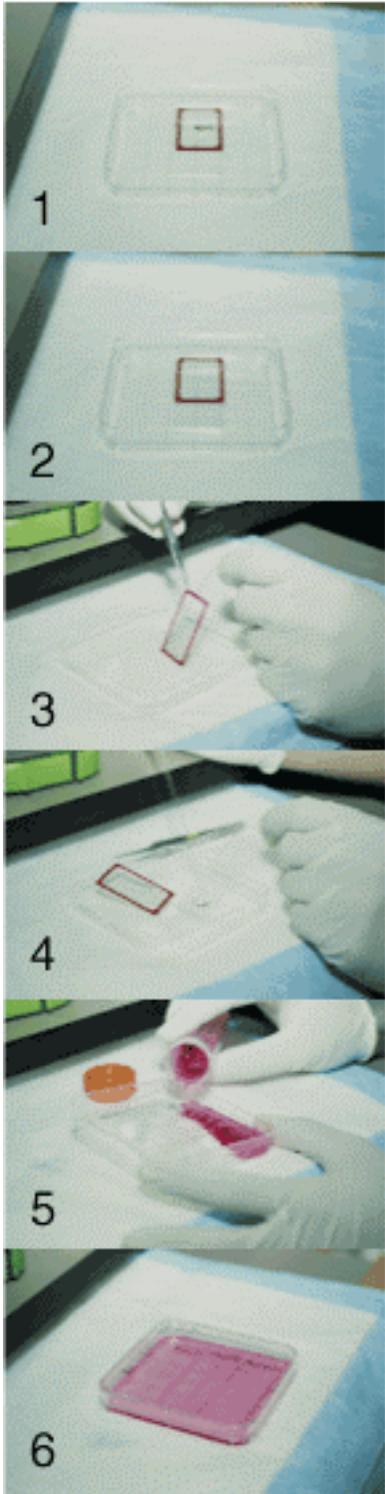


- 4) To use HybriWells, peel off the adhesive from the HybriWell and attach the HybriWell over the area of the slide containing the array, making sure an air-tight seal is formed.
- 5) Pipet the transfection mix into one of the ports of the HybriWell --the mix will evenly distribute over the array. Depending on the size of HybriWell used volumes may be adjusted appropriately. We use 200 μ l of transfection mix for the HB2240 HybriWell.
- 6) Expose the array to the transfection reagent for 6-20 minutes. For HEK293T cells we find that 15-20 minutes is optimal.
- 7) After the incubation with the transfection mix is complete, in a tissue culture hood suction off the mix by inserting into one of the ports the tip of a pasteur pipet attached to a vacuum source. Suction off most of the transfection reagent but do not worry about removing the last drop that will remain under the center of the HybriWell.
- 8) Pull off the HybriWell using a thin tipped forceps. The adhesive attaching it to the slide is strong and a firm and uniform pull is needed to detach the HybriWell.

Go to Step 13 from here.

OR

CoverWell Procedure



- 9) To use CoverWells, pipet the entire mix from each tube onto a CoverWell (40 x 20 mm).
- 10) Gently place a printed slide upside down onto the CoverWell such that the solution covers the entire arrayed area while creating an airtight seal between the slide and the CoverWell.
- 11) Expose the array to the transfection reagent for 6-20 minutes. For HEK293T cells we find that 15-20 minutes is optimal.
- 12) After the incubation with transfection mix is complete, in a tissue culture hood use a thin-tipped forceps to gently pry off the CoverWell from the slide. Carefully remove the majority of the remaining transfection mix using a pasteur pipet attached to a vacuum source.

- 13) During the incubation of the printed slide(s) with the transfection reagent, harvest HEK293T cells and suspend 10×10^6 cells in 25 ml medium (DMEM with 10% FBS, 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin). See below for how to harvest cells. 10×10^6 cells are enough cells for transfecting three slides in one dish.
- 14) Place the slide with the printed-side facing up in a sterile 100 x 100 x 10 mm square dish (3 slides fit side-by-side in one plate). In case we are transfecting less than three slides, we use non-printed dummy slides to prevent the printed slides from moving around the dish.
- 15) Gently pour the cells onto the slides while avoiding direct pouring on the printed areas.
- 16) Place the dish in a 37°C, 5% CO₂ humidified incubator. The microarrays of transfected cells form in ~40 hours and are then ready for use.

If required for downstream assays fix the slides as follows:

- 17) Rinse slides by dipping them for 10 seconds into a Coplin jar containing room temperature PBS.
- 18) Transfer the slides into another Coplin jar containing room temperature fixative (PBS with 3.7% paraformaldehyde and 4.0% sucrose)
- 19) Fix for 20 minutes.
- 20) Before processing by immunofluorescence or other detection methods, rinse the fixed slides for 1-2 minutes in PBS.
- 21) Slides can be stored at 4°C in PBS for at least 5 days before processing or examination.

Detection Methods

If the expressed proteins or phenotypes of interest can be detected with fluorescent reagents, transfected cell microarrays can be viewed and photographed with a fluorescent microscope or with a laser scanner. If detection is based on radioactivity, the slides can be fixed and the signal detected with film or emulsion autoradiography. A "Western blot" protocol can be used to detect phenotypes or expressed proteins after transfected cell microarrays are carefully transferred to a nitrocellulose membrane.

Immunofluorescence

All steps are performed at room temperature.

- 1) Fix cells as above.
- 2) Permeabilized by placing slide in 0.1% Triton X-100 in PBS for 15 minutes
- 3) After permeabilization, rinse each slide twice with PBS.
- 4) Block slide for 1 hour with 500 μl of blocking solution (1.0% BSA/1:100 normal donkey serum in PBS).

- 5) Remove blocking solution by vacuum aspiration and add 500 μ l of primary antibody diluted in antibody dilution solution (0.5% BSA in PBS) for 1 hour. Do not rinse after removal of blocking solution. If using two primary antibodies, add them together.
- 6) Wash slide 2X 5 minutes in PBS. Perform incubation in a humid chamber.
- 7) Re-blocked slide for 20 minutes with 500 μ l of blocking solution.
- 8) Remove blocking solution and add 500 μ l of secondary antibody in antibody dilution solution for 40 minutes.
- 9) Wash slide 3X 5 minutes in PBS.
- 10) Mount coverslip using vectashield (Vector Laboratories) and stored in the dark at 4°C.

Notes on imaging: Microarrays can be imaged with a conventional fluorescent microscope or a laser fluorescence scanner. We have used a ScanArray 5000 (GSI Lumonics) that can image with a resolution of up to 5 μ m. GFP and Cy3 emissions are measured separately after sequential excitation of the FITC and Cy3 fluorophores, respectively. Slides CANNOT be mounted when using this particular scanner as they will not fit in the entry port.

Figure 4 is a laser scan image (10 μ m resolution) of a large array expressing GFP (green), HA-GST (red) and V5-PDGF receptor (red).

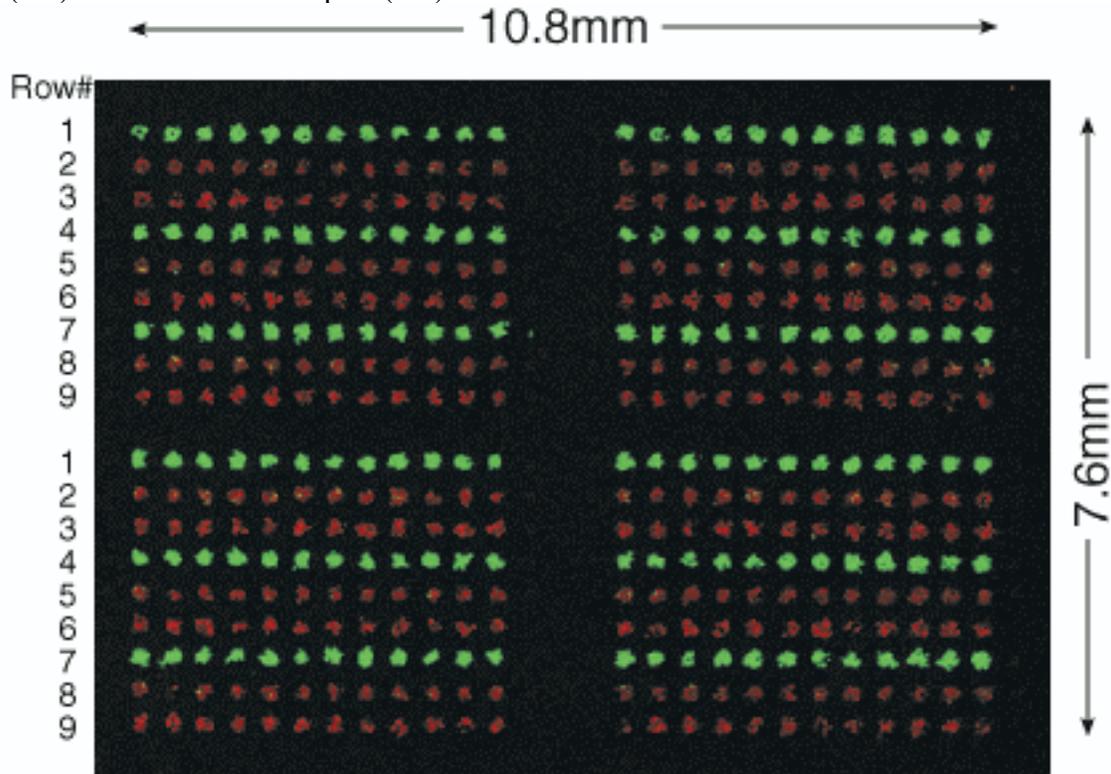


Figure 4: Example of microarray detected by immunofluorescence.

Autoradiography

Radiolabelled compounds are added directly to the cell media containing the microarrays. We added the compounds we tested for 1 hour before rinsing the slides once with room temperature PBS and fixation. Before autoradiography, slides can be processed for immunofluorescence and imaged with a laser fluorescence scanner or a fluorescence microscope. We exposed slides to tritium sensitive film (Hyperfilm cat# RPN 535, Amersham) which was developed as recommended by the manufacturer. When used, autoradiographic emulsion (EM-1 cat# RPN 41, Amersham) was also processed as described by the manufacturer.

Transfer to nitrocellulose and "Western Blotting" of transfected cell microarrays

We have developed an alternative to immunofluorescence for detecting protein expression in the transfected cell microarray. We transfer the arrays onto nitrocellulose membranes (0.45 μ m pure nitrocellulose membrane; cat. 162-0116, BioRad) and detect the proteins with standard western blotting protocols. Figure 5 is an example of an array of myc-tagged proteins detected via enhanced chemiluminescence using a standard anti-myc western blotting protocol. The middle two rows (horizontally) are printed with half the amount of the expression construct as the top and bottom rows. The signal was detected with Kodak Biomax MR film and each spot is \sim 150 μ m in diameter. Other methods compatible with proteins affixed to membranes (e.g. far-western and southwestern blotting) may also work with our system, but we have not tested this possibility.

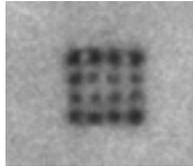


Figure 5: 'western blot'

Transfer procedure:

- 1) When the cells are ready to be processed, use forceps to lift the slide from the culture dish and quickly rinse it with PBS in a Coplin Jar.
- 2) After the rinse, remove excess PBS from the slide by briefly blotting its edge with an absorbent paper towel.
- 3) Place the slide with the cells facing up on a flat surface, immobilize it with tape and allow it to dry for 2-3 minutes (this time can vary depending on how much PBS remains on the slide).
- 4) Very carefully place a nitrocellulose membrane (about two to three times the area of the slide) on the slide, in a similar manner as is done for traditional plaque lifts (i.e. center first). It is very important to not permit any horizontal movement of the membrane or slide at this step.
- 5) Keep the membrane on the slide for 1-3 minutes or until the PBS has wetted the entire area of the membrane that covers the slide. Do not press down on or roll a pin over the membrane as this will invariably cause the membrane to slip and destroy the array. Also, do not allow all the moisture on the slide to be transferred to the

membrane as this will cause the membrane to stick to the slide and it will tear when it is lifted off.

- 6) After transfer, carefully lift off the membrane from the slide surface with forceps and allow it to air dry for 2 hours.
- 7) After drying dip the membrane into a pH 11 CAPS-methanol transfer buffer for 1-2 minutes and transfer it into a standard western blot blocking solution. CAPS-methanol transfer buffer: 2.2g/l CAPS, 10% methanol, pH 11 (usually requires 3-4 NaOH pellets/liter for this pH).
- 8) Process the membrane with primary and secondary antibodies as in any standard western blotting protocol.

Tips and Information

Below are some tips and notes for several steps of our system: gelatin preparation, DNA purification, slides, microarray printing, reverse-transfection and storage conditions. Suggestions for further information are welcome.

Gelatin Preparation

- Do not use a microwave to dissolve gelatin in water because we have found that the resulting solutions behave extremely variably in our system. Gelatin dissolved by heating the gelatin-water mix at 60°C for 15 minutes in a water bath gives the most consistent results.
- Gelatin should filter quite easily through a 0.45 µm cellular acetate (CA) membrane when cooled down from 60°C to approximately 37°-40°C. If it doesn't filter easily, the gelatin may have cooled too much.
- Do not store gelatin at room temperature because it not only degrades faster, but also becomes easily contaminated. We have found that gelatin solutions do not deteriorate for at least 1 month when stored at 4°C.
- When diluting DNA in 0.2% gelatin, the DNA concentration must be high enough such that the final gelatin concentration is greater than 0.17%.
- We tested a variety of types of gelatin and found that 'type A' gelatins do not work well in our system. Gelatin from ICN (cat.#901771) and from Sigma (cat # G-9391) work the best.

DNA Purification

- High purity DNA is important for successful transfection and for limiting cytotoxicity to cells on the arrays. We always use DNA with a 260/280 absorbance ratio greater than 1.7. Transfection does work with DNA of lower ratios, but we observe more cytotoxicity. As in conventional transfections, supercoiled plasmid DNA works best.

Slides

- Although we have made most of our arrays using Gamma Amino-Propyl Silane (GAPS) coated slides (Corning, Inc.), we have also tested other slide types, including poly L-lysine coated and polystyrene slides. Using poly L-lysine coated slides from several manufacturers we found comparable and sometimes even better results (i.e. higher transfection efficiencies) than those obtained with GAPS slides. However, we found substantial batch-to-batch variability of the poly L-lysine slides, which introduced unacceptable variability into the

experiments. Polystyrene slides also work well with our system, but they tend to float in the media which makes working with them difficult.

- We have recently tested QMT Epoxy-coated slides (Quantifoil, Jena, Germany). With our current methods (optimized for GAPS-coated slides) we reproducibly see about 50% of the transfection efficiencies we obtain with GAPS -coated slides.

Microarray Printing

- To prevent clogging of the arraying pins it is important to maintain a 55% relative humidity environment and to implement a thorough wash step between each dip into a new well of gelatin-DNA samples.
- We routinely examine our pins with a microscope for clogging and clean them in a sonicating 60°C hot water bath containing a detergent cleaning solution (ArrayIt Micro Cleaning Solution, cat.# MCS-1 from Telechem International).
- If pin clogging is a persistent problem, try warming (to ~37°C) the water that cycles through the wash station of the robotic arrayer.

Reverse-Transfection

- Although Effectene (Qiagen, Inc.) has been the most effective transfection reagent to date with HEK293T cells, CytoFectene (Bio-Rad Laboratories, Inc.) has shown comparable results. The efficiency of transfection is somewhat lower, but probably can be improved by optimizing protocols for this reagent.
- If using CoverWells (Grace Bio-Labs), slides should be placed gently onto the CoverWells containing the transfection mix so that the array is not disturbed.
- If using HybriWells (Grace Bio-Labs), adjust volume of the transfection mix (keeping the ratio of the components unchanged) to cover the entire surface (we use 200µl for HB2240, Grace Bio-Labs). Pipetting should be gradual so as not to disturb the array on the slide. We have found that Hybriwells perform better than the Coverwells for arrays containing greater than 500 spots.

Storage Conditions of Printed Slides and of Samples

- Although printed slides have been successfully stored in a vacuum dessicator at room temperature, we have recently found that the quality of 'transfected cell microarrays' produced can be improved by storing the slides at 4°C. Storage of slides at lower temperatures (-20°C, -80°C) has not shown any additional improvement.
- For re-arraying purposes, the DNA-gelatin samples may be sealed in their 384-well plates with ThermoWell Sealers (Corning, Inc.) and stored at 4°C for at least 4 weeks. Storage times beyond 4 weeks have not been tested.

Lipid-DNA Method

This page describes an alternative to the 'gelatin-DNA' method in which the lipid-based transfection reagent ('lipid') is pre-mixed with the DNA before printing. In the 'gelatin-DNA' method the lipid-based transfection reagent is added to all the samples once they have been printed on the slide. The 'lipid-DNA' method allows greater flexibility in optimizing conditions for different cell lines. We have found that Effectene, Lipofectamine 2000 and Fugene 6 work with this protocol.

I. Gelatin Preparation and DNA Purification

Materials

- Gelatin, Type B: 225 Bloom (Sigma, catalog #G-9391)

Preparation of 0.2% (w/v) Gelatin Solution

- 1) Dissolve gelatin powder in sterile MilliQ water by gently swirling mixture for 15 minutes in a 60°C water bath.
- 2) Cool the 0.2% gelatin solution at room temperature, and, while still warm (~37-40°C), filter it through a 0.45 µm cellular acetate membrane (CA). We usually prepare 100 ml at a time and store 50 ml aliquots of the filtered gelatin solution at 4°C.
- 3) Dilute 0.2% to 0.05% using MilliQ water and store at 4°C.

DNA Purification

DNA can be purified via any method that gives supercoiled plasmid DNA with a 260/280 absorbance ratio greater than 1.7. To purify many plasmids in parallel, we use the Qiagen Turbo Miniprep Kit. We seed bacterial clones in 1.3 ml of terrific broth (TB) in a 96-Deep-Well Plate and shake them at 250 rpm for 18-24 hours at 37°C. Of course, DNA of equivalent or better quality can be purified via other methods and is also suitable for making transfected cell microarrays.

II. Sample Preparation, Microarray Printing, Slide Storage, and Cell Addition

Materials

- Effectene Transfection Reagent (Qiagen catalog #301425)
- EC buffer (from Effectene Transfection Kit) containing 0.2-0.4M sucrose
- Purified cDNA
- Sucrose (Life Technologies)
- 0.05% gelatin prepared by diluting 0.2% gelatin with MilliQ water
- INTEGRID 100 x 15mm Tissue Culture Square Petri Dishes (Becton Dickinson: Falcon catalog #35-1012)
- Costar 384-well plates (VWR catalog #7402)
- Stealth Micro Spotting Pins, (Telechem International, Inc. catalog #SMP4)
- PixSys 5500 Robotic Arrayer (Cartesian Technologies, Model AD20A5)
- Gamma-Amino Propyl Silane (GAPS) slides (Corning catalog #2549)
- Vacuum Desiccator with Stopcock 250 mm, NALGENE (VWR catalog #24987-004)
- DRIERITE Anhydrous Calcium Sulfate (VWR catalog #22890-229)

- Thin-tip forceps to manipulate slides
- Human Embryonic Kidney (HEK) 293T cells
- Tissue Culture hood

Sample Preparation

- 1) In a 1.5 ml tube, add 0.80-1.60 μg DNA to 15 μl of DNA-condensation buffer (Buffer EC from Effectene Kit) in which sucrose has been dissolved to a concentration of 0.2- 0.4M. The sucrose in the 'lipid-DNA' mix is necessary to stabilize the Effectene (or other lipid-based transfection reagent) after printing.
- 2) Add 1.5 μl of Enhancer solution (from Effectene Kit), mix the contents by pipetting up and down five times, and incubate the mixture at room temperature for 5 minutes.
- 3) Next add 5 μl Effectene transfection reagent (from Effectene Kit), mix the solution with gentle vortexing.
- 4) Incubate at room temperature for 10 minutes.
- 5) Add a 1X volume of 0.05% gelatin, remix the solution and pipette 20 μl into a well of a 384-well plate.

Notes on Microarray Printing and Slide Storage

After transferring to a 384-well plate, we print samples onto GAPS slides with a PixSys5500 Robotic Arrayer equipped with Telechem's ArrayIt Stealth Pins (SMP4). The pins transfer the 'lipid-DNA' solution to the slide while touching the surface of the slide for 50-500 ms (this method requires longer times than the 'gelatin-DNA' method). We print spots 300-400 μm apart from other spots. To prevent clogging of the pins we maintain a 55% relative humidity environment. After printing, slides are stored at room temperature in a vacuum desiccator with anhydrous calcium sulfate pellets. Cells can be added to printed slides as soon as one hour after printing.

Cell Addition

Cells are prepared and added exactly as in the 'gelatin-DNA' method.

Reference:

Junaid Ziauddin and David M. Sabatini. Microarrays of cells expressing defined cDNAs. *Nature*, in press.

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We welcome all suggestions for the improvement of this guide.