Technical Overview

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Automated Quantification of Transfection Efficiency for Assay Optimization

Large-format cell culture imaging with Lionheart FX

Introduction

Chemical transfection is a well-established approach to introduce plasmid DNA for gene expression into mammalian host cells. The transfection efficiency (TE) of a given plasmid is quantified by deriving a ratio between the number of transfection cells and the total number of cells counted. This is typically accomplished using fluorescent reporters and a microscope, with one or more image fields acquired within the culture vessel where the transfection took place. When scaling from a microplate format to a large culture vessel, such as a 75 cm^2 flask, it is crucial to optimize both the amount of plasmid required, as well as the transfection period, before downstream applications are undertaken. In this technical overview, an efficient and robust single-instrument workflow for optimizing transfection reactions is demonstrated using the Agilent BioTek Lionheart FX automated microscope wide field of view (WFOV) optics, open-stage design, and integrated multiple-region-of-interest (multiROI) features and automated image analysis tools of the Agilent BioTek Gen5 microplate reader and imager software (Figure 1).

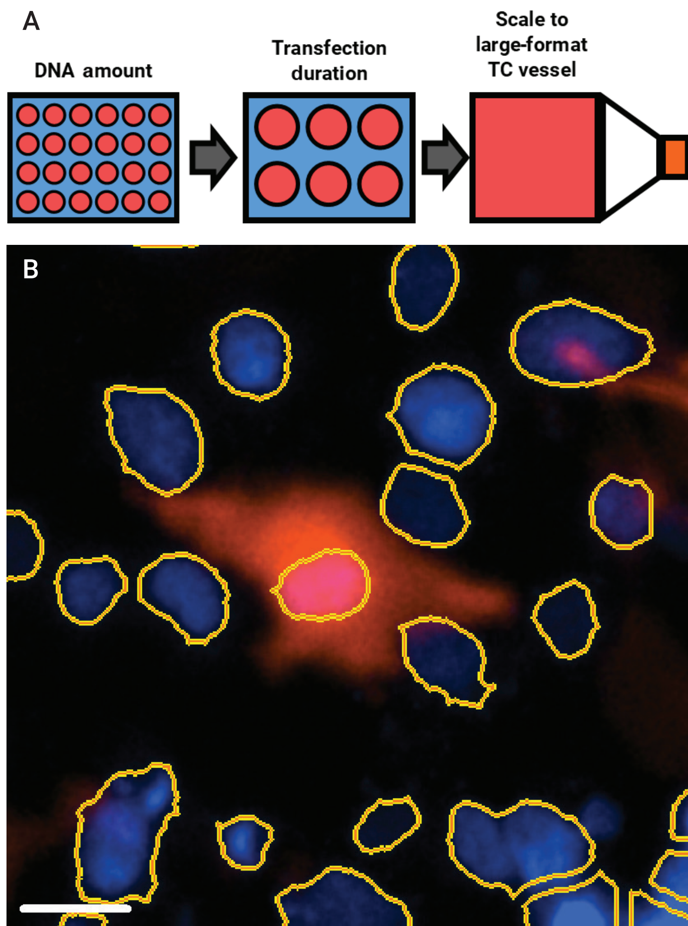


Figure 1. Large -format scale-up optimization. (A) Workflow of transfection optimization in a 6-well format before scaling up to large-format tissue culture (TC) vessel (75 cm^2 flask). (B) Image of a singly transfected COS7 cell among nontransfected neighboring cells. Scale bar = 20 µm.

Experimental

Cell lines

COS7 cell lines (part number CRL-1651) were purchased from ATCC (Manassas, VA).

Extracellular matrix coating and cell seeding

Costar 24-well (part number 3524), 6-well (part number 3516), and 75 cm^2 flasks (part number 430641U) from Corning (Corning, NY) were coated with 10 µg/mL fibronectin, which was diluted in DPBS from a 1 mg/mL stock, part number F1141 (MilliporeSigma; St. Louis, MO) for 30 minutes at room temperature (RT), immediately prior to seeding. Cells were seeded in 24-well plates at a density of 5 × 10^4/well and allowed to settle and attach to the fibronectin-coated well bottom for 15 minutes at RT.

Plates were then transferred to a TC incubator and allowed to adhere overnight at 37 °C. Seeding densities for 6-well microplates and 75 cm^2 flasks were scaled proportionally based on cell growth area.

Plasmid DNA and transfection

A mammalian expression construct containing cDNA encoding the membrane-targeting CAAX motif from HRas fused to tagRFP-T, driven by the CMV promoter, was employed to assess TE. The tagRFP-T excitation and emission spectra best align with the Agilent TRITC filter cube (part number 1125125). Plasmid DNA was transfected into COS7 cells using X-tremeGENE HP transfection reagent, part number 6366236001 (MilliporeSigma; St. Louis, MO) according to the manufacturer's protocol. Prior to transfection, each well was treated with 250 nM Hoechst 34580 (Thermo Fisher Scientific; Wilmington, DE) for 1 hour, followed by three washes of DPBS; then, 0.5 mL fresh culture (no Hoechst) was added. For the 24-well plate, cells were transfected with decreasing amounts of DNA: 1,000, 500, 250, 100, 50, or 0 ng for 18 hours, followed by three washes with DPBS and fresh media added (Figure 2A).

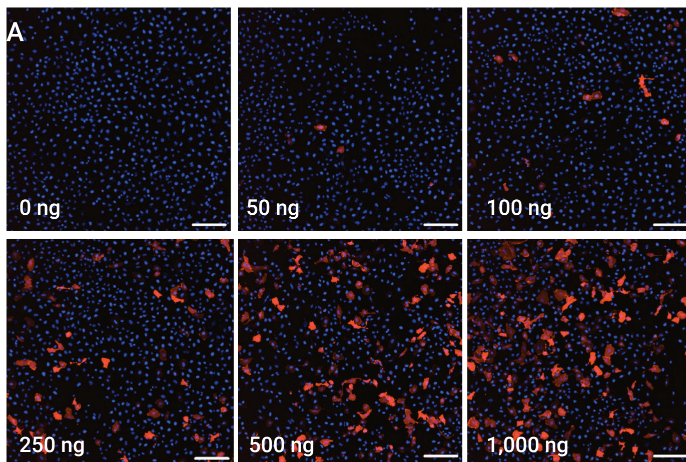
For the 6-well plate (Figure 3) and 75 cm^2 flask (Figure 4), the DNA amount was scaled up according to the maximal TE found for the 24-well dilution series in Figure 1.

Imaging

For the 24- and 6-well plates, live cells were placed in a humidity chamber, and a three-channel, 3 x 3 (24-well) or 4 x 4 (6-well) ROI matrix was captured within each well: phase contrast, DAPI, and TRITC. Laser autofocus Z-height was set using the phase contrast channel, with optimal Z-heights for the DAPI and TRITC channel set appropriately as offsets. For the 75 cm^2 flask, a 6 x 9 ROI matrix was captured. To capture the transfection time lapse in the 6-well plate (Figure 3), a kinetic run was set up in experiment mode initiated within 30 minutes of adding the transfection cocktail, with 15-minute kinetic intervals over a 24-hour period.

Image processing and cellular analysis

A preprocessing background reduction step was applied to the DAPI and TRITC channels of the captured images with a rolling ball set to 25 and 100 µm, respectively. For the kinetic run analyzed for Figure 3 an initial kinetic alignment using the phase contrast channel was performed prior to any further preprocessing or cellular analysis steps. Because of the nature of the tagRFP-T-HRasCAAX marker, fluorescent signal is diffusely observed throughout the plasma membrane, including membrane above and below the nucleus. Therefore, tagRFP-T signal was measured within a secondary mask generated within the DAPI primary mask (Figure 1B).



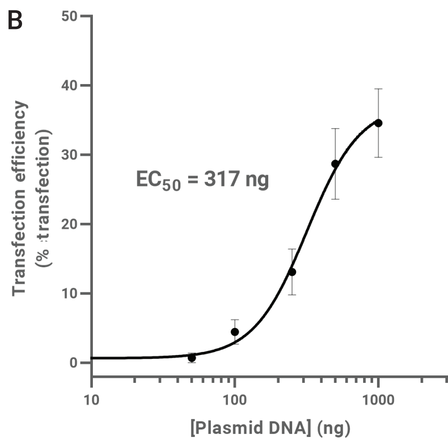
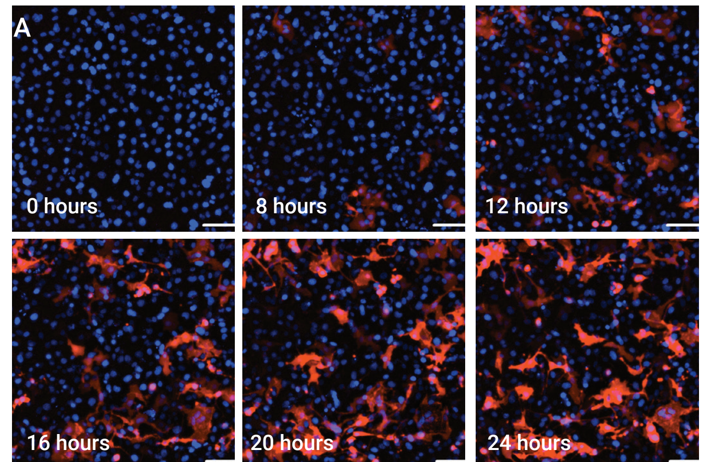


Figure 2. DNA transfection titration. (A) Images of COS7 cells transfected with the indicated amount of tagRFP-T-HRasCAAX plasmid DNA. Blue = Hoechst 34580; Red = tagRFP-T-HRasCAAX. Scale bar = 20 µm. (B) DNA was transfected in increasing amounts (ng) to identify the amount that produced maximal TE.



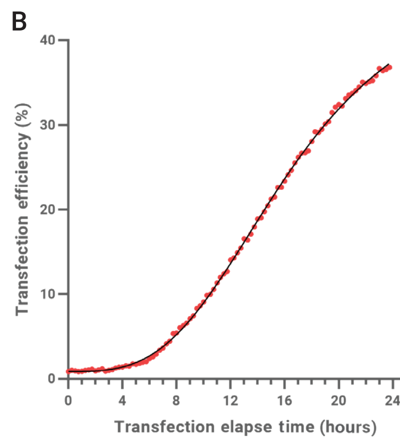


Figure 3. Transfection duration optimization. The duration of transfection that produced maximal expression was determined using 1 µg / 5.0 x 10^4 cells. Representative FOVs at various time points are represented in (A) whereas the integrated cellular signal is plotted over time in (B).

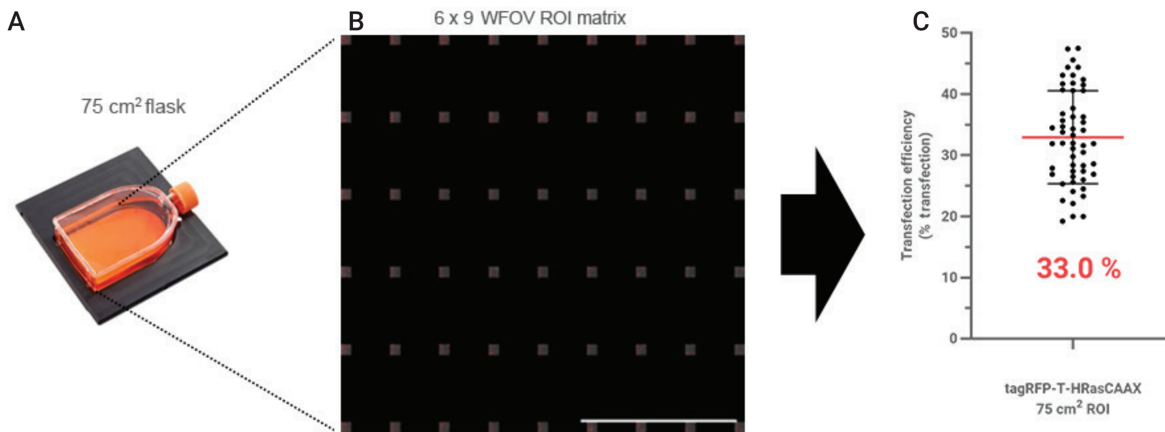


Figure 4. Transfection scale up to a 75 cm^2 flask. Once the transfection conditions are optimized as shown in Figures 1 and 2, the transfection can be scaled to a 75 cm^2 flask (A). The Agilent BioTek Lionheart FX automated microscope with WFOV, combined with the Agilent BioTek Gen5 software ROI array, provides a robust sampling (B) that enables extensive statistical evaluation of large-format culture vessels (C).

Transfected cells were further identified by establishing a subpopulation where mean tagRFP-T signal of the primary mask reached or exceeded a set fluorescence threshold based on the background fluorescence signal from a control well containing nontransfected cells. TE was calculated by deriving the ratio of transfected cells to total cells in an image field, reported as a percentage.

Results and discussion

When scaling a transfection to a large format culture vessel, such as a 75 cm^2 flask, it is important to first optimize two parameters: 1) the amount of plasmid DNA needed to maximize TE, and 2) the optimal transfection duration that gives sufficient expression levels (Figure 1A). To these ends, pilot transfections were conducted in 24- and 6-well microplates. A six-point DNA dilution series was carried out in a 24-well TC plate, ranging from 10 to 1,000 ng total transfected DNA (Figure 2A). From these images, the TE was determined using the ratio of transfected cells to total cells and plotted as a function of transfected DNA amount (Figure 2B). From this, maximum TE and EC_{50} can be determined.

Secondly, the transfection incubation duration that gives maximal TE can be determined by performing a kinetic run encompassing the point when transfection is initiated through a predetermined end point. Based on previous experiences with the tagRFP-T-HRasCAAX construct, the kinetic run spanned 24 hours with 15-minute kinetic intervals (Figure 3A). TE was calculated at each interval and was plotted as a function of the transfection duration (Figure 3B).

The information gained from the DNA titration and kinetic data aid to maximize the amount and transfection duration for a large-vessel format (75 cm^2 flask).

Maximal TE was achieved with 1 µg DNA in the 6-well culture plate, which has a growth area of 9.5 cm^2—an eight-fold difference from a 75 cm^2 flask. Therefore, a transfection was performed in a 75 cm^2 flask with 8 µg DNA with an end point set to 36 hours. To ensure sufficient sampling from the 75 cm^2 flask, a 6 x 9 ROI matrix was generated (Figure 4B). Each three-channel image is 0.1389 cm^2, which means that 10.4 cm^2 (or 13%) of total growth area was sampled, which took 7.5 minutes to acquire. Because image statistics can be derived from each ROI independently, the TE from each ROI can be treated as a sample *n* value for a single transfection ($n = 54$), which is represented in the grouped scatter plot in Figure 4A.

Conclusion

Compatibility with large-format cell culture vessels makes the Agilent BioTek Lionheart FX automated microscope an ideal imaging platform for assay optimization studies, including transfection reactions. The workflow and robust quantification techniques described here are also applicable to transduction studies and other genome modifying techniques.

The faster stage movement of the Lionheart FX ensures that capture time is expeditious, while the multiROI feature of Agilent BioTek Gen5 software expands the statistical power of capturing multiple ROIs within a single culture vessel.

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