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Abstract

To fully exploit the potentials of reprogramming the epigenome through CRISPR/dCas9 systems for epigenetic editing, there is a growing need for improved transfection methods. With the utilization of constructs often with large sizes and the wide array of cell types used to read out the effect of epigenetic editing in different biological applications, it is evident that ongoing optimalization of transfection protocols tailored to each specific experimental setup is essential. Whether the goal is the production of viral particles using human embryonic kidney (HEK) cells or the direct examination of epigenomic modifications in the target cell type, continuous refinement of transfection methods is crucial. In the hereafter outlined protocol, we focus on optimization of transfection protocols by comparing different reagents and methods, creating a streamlined setup for transfection efficiency optimization in cultured mammalian cells. Our protocol provides a comprehensive overview of flow cytometry analysis following transfection not just to improve transfection efficiency, but also to assess the expression level of the utilized construct. We showcase our transfection protocol optimization using HEK293T Lenti-X™ and breast cancer MCF-7 cell lines, using a single-guide RNA-containing plasmid. Specifically, we incorporate heat shock treatment for increased transfection efficiency of the MCF-7 cell line. Our detailed optimization protocol for efficient plasmid delivery and measurement of single-cell plasmid expression provides a comprehensive instruction for assessing both transient and sustained effects of epigenetic reprogramming.

Running head: Plasmid delivery and single cell analysis for epigenetic editing

Key words: transfection efficiency – single cell analysis – CRISPR/dCas9 – epigenetic editing – MCF-7 cells

1. Introduction

In recent years, epigenetic editing has gained popularity as a potential way of treating various diseases. The CRISPR/dCas9 platform, which uses a deactivated Cas9 protein for genome targeting, lends itself especially well for epigenetic editing. By fusing the catalytic domain of epigenetic effectors to dCas9, the effectors can easily be guided to the genomic region of interest using single guide RNAs (sgRNAs) [1]. During research procedures, however, transfection efficiency can often be a limiting factor when using CRISPR-based (epi)genetic editing systems [1-3].

Transfection of the dCas9-effector constructs or co-transfection of these constructs with the necessary sgRNA plasmids can pose issues when transfection efficiency is low, especially when stable integration of either or both components of the epigenetic editing system is undesirable. For example, when studying whether transient expression of the dCas9-effector-based system is able to induce sustained epigenetic changes, stable integration of dCas9 effectors is undesirable. In some cases, combining the established cell lines that stably express dCas9-effector fusions with the transient expression of sgRNAs via transfection, can be a suitable solution [4]. This does not negate the need for high transfection efficiency but reduces the need for co-transfection. Efficient transfection would greatly facilitate to quantitatively measure cell-to-cell transcription variability following epigenetic reprogramming at different time points. For instance, single molecule RNA fluorescence in situ hybridisation (smRNA FISH) is a powerful technique to localize and quantify nascent and mature transcripts for studying epigenetic regulation and state switching [5]. However, to ascertain the sustained effects of transiently induced epigenetic reprogramming, efficient transient transfection of both the dCas9-effector constructs and the sgRNAs would be preferable. This approach would minimize potential side effects associated with the constitutive expression of the dCas9-effector fusion protein.

Optimising transfection is therefore essential, especially when working with difficult-to-transfect cell lines like human primary fibroblasts or breast cancer cell line MCF-7. While working with more easy-to-transfect cell lines like human embryonic kidney (HEK) cells may allow for initial testing and fine tuning of assays, the resulting protocol needs to be applicable to the research-specific cell type. For example, commonly used transfection reagents like Lipofectamine™ 2000 may have a transfection efficiency as high as 93% in HEK cells, but only ~33% in MCF-7 cells [6-7]. Such low transfection efficiencies leave only a small portion of cells available for analysis, and although cell sorting can be applied to isolate the transfected cells, sorting itself can cause sorting induced cell stress (SICS) [8]. Combining the stress of transfection and the exposure to sorting could result in an even smaller pool of cells for continued experiments and analysis. Therefore, having a larger initial pool of transfected cells available is advantageous.

Fluorescence-activated cell sorting (FACS) is a useful tool to separate cells expressing the protein of interest from non-expressing cells (*see* Fig. 1). With FACS, the measured fluorescence intensity of the fluorescent protein expressing cells serves as an indicator of the protein expression level for each individual cell [9]. This is especially advantageous as the optimal expression level of the dCas9-effector fusion proteins may differ per effector [10]. Attachment of a fluorescent protein to the dCas9 fusion protein enables sorting of cells with different expression levels and their subsequent culturing. This way, the effect of an epigenetic effector domain expressed with for instance low, mid, or high expression levels can be determined. Selecting a transfection method tailored to each specific cell line and experimental context is crucial to achieve applicable expression levels of the analysed epigenetic effectors.

The following protocol details a streamlined setup for the optimisation of transfection experiments using chemical transfection reagents and using flow cytometry/FACS for expression analysis of the transfected plasmids, aiming to find the most suitable composition of transfection mixes in terms of reagent per plasmid DNA amount. We showcase the visualisation of flow cytometry gating and data analysis using MCF-7 and HEK293T Lenti-X™ optimisation experiments, using several different transfection reagents and kits (*see* Fig. 1 and Fig. 2). To visualise protein expression using flow cytometry and optimise transfections for their intended use, transfections were performed using a blue fluorescent protein (BFP) containing sgRNA expression plasmid (*see* Fig. 3). For MCF-7 cells, previous and our current detailed experiments have shown that including a 42 °C heat shock step ~4 hours after transfection can considerably increase the transfection efficiency, especially for lipid-based transfection reagents [11-12]. By following the described methodology using available transfection reagents and/or equipment, the optimal transfection reagent as well as reagent-to-DNA ratio for the utilised cell line can be determined. For difficult to transfect cell lines and/or constructs, the MCF-7 described heat shock treatment can be considered as well. Our comprehensive optimisation protocol for efficient plasmid delivery and induction of single-cell expression, provides detailed instructions to assess both transient and sustained effects of epigenetic reprogramming.

2. Materials

Store all reagents, cell culture media and buffers at 4 °C, unless indicated otherwise. Make use of aseptic laboratory techniques.

2.1 Cell culture and transfection

1. Cell type specific complete culture medium
2. Mammalian cells for transfection, e.g. HEK293T Lenti-X™ (Catalogue number 632180, Takara bio/Clonetech) or MCF-7 cells (ATCC-HTB-22)
3. 1x Trypsin-EDTA solution
4. Sterile phosphate-buffered saline (PBS)
5. Transfection Reagent
6. Opti-MEM™ I Reduced Serum Medium (Gibco)
7. Plasmid DNA (pDNA) of interest (*see* **Note 1**)
8. 12-well flat-bottom cell culture plates
9. CO_2 incubator, 37 °C, 5% CO_2
10. Incubator, 42 °C (*see* **Note 2**)

2.2 Analysis of transfected cells using flow cytometry

1. Sterile PBS
2. 1x Trypsin-EDTA solution
3. Cell type specific complete culture medium
4. 15 ml conical tubes
5. Flow cytometry buffer: PBS supplemented with 5% FBS (*see* **Note 3**)
6. Round-bottom 12 x 75 mm tubes

3. Methods

3.1 Transfection optimization

Standard culturing conditions, such as incubating at 37 °C and 5% CO_2, should be maintained unless otherwise specified. Cells should be carefully and routinely cultured prior to transfection to maintain proper growth patterns and viability. Medium and PBS should be warmed to 37 °C prior to use. The Trypsin solution should be pre-warmed to room temperature prior to use.

1. Seed the cells approximately 16 hours prior to transfection. MCF-7 cells should be at 50-60% confluency at time of transfection, and HEK293T Lenti-X™ cells at 70-80% confluency (*see* **Note 4**). Take care to seed cells evenly across the surface of the well, as overly clustered cells may transfect poorly. Always seed enough wells to include a non-transfected control sample, and if possible, a positive control as well. All quantities given in this protocol are specified for cells seeded in 12-well plates but can be adjusted according to your requirements (*see* **Note 5**).
2. Incubate cells overnight.
3. Warm the reagent to room temperature and vortex gently (*see* **Note 6**).
4. Prepare four sterile 1.5 ml tubes per reagent. In each tube, combine 200 µl Opti-MEM™ I Reduced Serum Medium or Reagent Buffer with 2 µg of plasmid DNA (*see* **Note 7**). Vortex for 1 second and subsequently spin down for 1-5 seconds to collect all liquid at the bottom of the tubes.
5. Label the tubes with the reagent and Reagent:DNA ratio to be used (µl reagent per µg pDNA). Standard ratios to test are 2:1, 3:1, 4:1 and 5:1 (*see* **Note 8**).
6. Add the appropriate amount of reagent per tube and mix by gently pipetting up and down. Do not vortex after addition of reagent (*see* **Note 9**).
7. Incubate the Reagent:DNA complexes at room temperature for the duration specified in the manufacturer's protocol.
8. Divide each Reagent:DNA complex equally between two wells, allocating half to each well, to create technical duplicates using 1 µg of pDNA per well. For each well, add the complex in different areas of the well in a drop-wise manner. In case of more fragile cells, add the mixture very gently.
9. Gently rock the plate back-and-forth and side-to-side to evenly distribute the Reagent:DNA complex (*see* **Note 10**). Incubate the cells at 37 °C, 5% CO_2.
10. **MCF-7 only:** Four hours after transfection, transfer the plates to a 42 °C stove and incubate for 10 minutes. Subsequently transfer the plates back to a 37 °C stove and continue incubation at 37 °C, 5% CO_2.
11. Refresh the medium 24 hours after transfection, or at the time specified in the reagent's instruction manual.
12. Prepare to harvest 48 hours post-transfection.

3.2 Harvesting of cells and protein induction analysis using flow cytometry

1. Start with the non-transfected control sample. Aspirate medium and wash once with 0.5 ml pre-warmed PBS per well. Aspirate PBS (*see* **Note 11**).
2. Add approximately 0.5 ml of 1x Trypsin-EDTA solution to each well, incubate at 37 °C for 5-10 minutes.

3. Add 1 ml of complete culture medium to each well to neutralize the Trypsin treatment. For each sample, pipette up and down 20 times to break up cell clumps and transfer the cell suspension to a conical 15 ml tube (*see* **Note 12**).
4. Centrifuge the cells at 200 g for 5 minutes. Carefully aspirate the medium without disturbing the cell pellet (*see* **Note 13**).
5. Wash the cell pellet with PBS, centrifuge the cells at 200 g for 5 minutes, and carefully aspirate.
6. Resuspend the cell pellet in 250-500 µl flow cytometry buffer and transfer the sample to a 12x75 mm round bottom tube (*see* **Note 14**). Aim for a concentration of ~1000 cells/µl.
7. Run samples through the flow cytometer/FACS machine as per manufacturer instructions, using the appropriate laser and sensor for the tested fluorescent protein. Aim for 50.000-100.000 cells per sample. Use appropriate forward and side scatter gating for the respective cell type and select the data points for protein induction analysis (*see* Fig. 1).
8. After data collection, use flow cytometry analysis software to compensate and gate your data based on the non-transfected control, to appropriately identify the cell population expressing the respective fluorescent protein and the non-expressing negative control cells (*see* **Note 15**) (*see* Fig. 3).
9. Export the overall statistics, and the compensated single-cell event data of your cell population expressing the respective fluorescent protein (*see* **Note 16**).
10. Log transform your single-cell event data using $Y=\log_{10}(Y)$. Create a violin plot of the transformed data, using a linear Y-axis and antilog ticks (*see* **Note 17)** [14]**.**
11. Create bar graphs for your mean fluorescence intensity (MFI) and transfection efficiency. Use the percentage of fluorescence positive cells as the measurement for transfection efficiency (*see* Fig. 2).

4. Notes

1. Make sure the plasmid DNA is of acceptable purity (260/280 ratio ~1.8 or higher), and that plasmids are isolated using DNA precipitation purification or using endotoxin-free plasmid isolation kits, as some cell lines can be sensitive to impurities.
   We here made use of Plasmid pU6-sgRNA EF1Alpha-puro-T2A-BFP: A lentiviral U6-based single-guide RNA (sgRNA) expression plasmid derived from pSICO, expressing Blue fluorescent protein and a puromycin-resistance cassette, separated by a T2A sequence, from an EF1α promoter. pU6-sgRNA EF1Alpha-puro-T2A-BFP was a gift from Jonathan Weissman (Addgene plasmid #60955; RRID:Addgene_60955) [13]. The plasmid is stored in TE buffer at -20 °C.
2. Since the 42 °C incubation step is only for 10, this incubator does not strictly need to have CO_2 regulation.
3. Standard FACS/flow cytometry buffer consists of PBS, supplemented with either 0.5-1% BSA or 5-10% FBS, with optional addition of 0.1% NaN_3 sodium azide. Sodium azide inhibits metabolic activity; do not add sodium azide to buffers if cells are also collected for functional assays or if you have concerns about cell function recovery. When not using antibodies for analysis, PBS can be used without supplementation as well.

4. MCF-7 breast cancer cells tend to grow in clumps, causing the confluency to appear lower while the number of cells is in principle similar to other cultured cells at time of transfection.
5. Using plates with 6 wells or up to 24 wells is optimal for transfection optimization. This is due to the increased number of cells that can be harvested for analysis, and the relatively low surface area to volume ratio. Reagent:DNA complexes formed using highly positively charged reagents (e.g. cationic polymers, nanoparticles, liposomal systems) can react with the plastic, thereby interfering with the transfection efficiency. For this reason, it is advisable to initially optimize any protocol in a 6-24 well format before scaling down.
6. The manufacturer's instructions will detail whether vortexing of the reagent is appropriate or not. In general, vortexing for 1-2 seconds at medium speed is acceptable.
7. Most transfection reagents will need to be mixed with Opti-MEM™ I Reduced Serum Medium, but a few will provide their own buffer that will be used to make the Reagent:DNA complex instead (e.g. PolyPlus reagents like JetPRIME and JetOPTIMUS).
8. For most reagents a ratio of 2-5 µl of reagent per µg plasmid DNA is appropriate. Always check the manufacturer instructions to see if your reagent of interest has a different range of optimal ratios, for instance 0.5-3 µl reagent per µg DNA. Adjust the ratios used accordingly.
9. When comparing several different reagents for the same experiment, ensure that the addition of reagents is appropriately spaced out in terms of timing. Adjust this to allow yourself enough time to prepare and incubate the Reagent:DNA complexes, but more importantly to harvest each sample after 48 hours exactly without having to rush or under/over-incubate some samples relative to others.
10. Always rock, never swirl. Swirling can cause the Reagent:DNA complex to pool in the middle of the well and not distribute evenly across the entire surface of the well, impairing transfection efficiency.
11. In order to minimize contaminations, always start each step of the harvesting and sample preparation process at the non-transfected control sample before moving to the plasmid transfected samples.
12. When using 24 or 12 well plates, if you are concerned about the number of surviving cells being too low for effective analysis, combining the technical replicates in the same tube can provide a solution. Aim for a minimum of 100,000 cells per sample for analysis. When using 6 well plates the seeding density is high enough to circumvent this issue even when using reagents that cause low viability.
13. In case the cell pellet is disturbed during aspiration or if you cannot aspirate all medium without disturbance, remove as much medium as possible and proceed to the washing step. If deemed necessary, include an additional PBS washing step to ensure no interference from particles or medium colouration during flow cytometry occurs.
14. If analysis will take place immediately after harvesting, keeping samples and buffer at room temperature is acceptable. If there is a waiting period or you have many samples; make sure the flow cytometry buffer is at 4 °C and keep your samples in the dark and on ice, or at 4 °C, until the scheduled time of analysis.
15. Most FACS analysis programmes will have their own workflow or pipeline to calculate the necessary compensation and gating, when the negative and positive control have been measured as part of the experiment. In the case of using only a negative control,

compensate for autofluorescence and manually establish the gate for positive versus negative cell populations by referring to the histogram of the negative (non-transfected) control. This is known as subjective gating.

16. Minimal statistics to export for each sample are: The percentage of positive and negative cells, mean or median fluorescence intensity, standard deviation of the fluorescence intensity. Depending on the distribution of your data the median fluorescence intensity may give a more accurate representation than the mean fluorescence intensity.
17. Due to the wide distribution of (single cell) fluorescence data, using a logarithmic scale makes the data easier to interpret. But, as outlined by GraphPad in their Knowledgebase article #2183, using a logarithmic scale on a violin plot may result in potentially misleading representation of the data [14]. Therefore, it is advised to first log transform the data and plot it afterwards, rather than using a logarithmic Y-axis on the non-transformed data. Using antilog ticks on the Y-axis provides visually easy-to-interpret data plots. Use the distribution of the relative fluorescence unit (RFU) of the fluorescence positive cells as the measurement for the induced protein induction level.

References

1. Nakamura M, Gao Y, Dominiguez AA, Qi LS (2021) CRISPR technologies for precise epigenome editing. Nat Cell Biol 23: 11–22.
2. Glass Z, Lee M, Li Y, Xu Q (2018) Engineering the delivery system for CRISPR-based genome editing. Trends Biotechnol 36(2): 173-185.
3. Lino CA, Harper JC, Carney JP, Timlin JA (2018) Delivering CRISPR: a review of the challenges and approaches. Drug Delivery 25(1): 1234-1257.
4. Goubert D, Koncz M, Kiss A, Rots MG (2018) Establishment of cell lines stably expressing dCas9-fusions to address kinetics of epigenetic editing. In: Jeltsch, A., Rots, M. (eds) Epigenome Editing. Methods in Molecular Biology, vol 1767.
5. Beckman W, Vuist IM, Kempe H, Verschure PJ (2018) Cell-to-cell transcription variability as measured by single molecule RNA FISH to detect epigenetic state switching. In: Jeltsch, A., Rots, M. (eds) Epigenome Editing. Methods in Molecular Biology, vol 1767.
6. Maurisse R, De Semir D, Emamekhoo H, Bedayat B, Abdolomohammadi A, Paris H et al (2010) Comparative transfection of DNA into primary and transformed mammalian cells from different lineages. BMC Biotechnol 10, 9.
7. Wang T, Larcher LM, Ma L, Veedu RN (2018) Systematic Screening of Commonly Used Commercial Transfection Reagents towards Efficient Transfection of Single-Stranded Oligonucleotides. Molecules 23(10):2564.
8. Ryan K, Rose RE, Jones DR, Lopez PA (2021) Sheath fluid impacts the depletion of cellular metabolites in cells afflicted by sorting induced cellular stress (SICS) Cytometry A 99(9): 921-929.
9. Butts CL, Sternberg EM (2009) Flow Cytometry as a tool for measurement of steroid hormone receptor protein expression in leukocytes. Methods Mol Biol. 505: 35-50.
10. Omachi K, Miner JH (2022) Comparative analysis of dCas9-VP64 variants and multiplexed guide RNAs mediating CRISPR activation. PLoS One 17(6):e0270008.

11. Pipes BL, Vasanwala FH, Tsang TC, Zhang T, Luo P, Harris DT (2005) Brief heat shock increases stable integration of lipid-mediated DNA transfections. BioTechniques 38(1): 48-52.
12. Milani A, Bolhassani A, Rouhollah F, Naseroleslami M (2021) Which one of the thermal approaches (heating DNA or cells) enhances the gene expression in mammalian cells? Biotechnol Lett. 2021 43(10): 1955-1966.
13. Gilbert LA, Horlbeck MA, Adamson B, Villalta JE, Chen Y, Whitehead EH et al (2014) Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 159(3): 647-61.
14. GraphPad (2023) Violin Plots and Logarithmic Axes. GraphPad Knowledgebase #2183, accessed 12-08-2023, URL: https://www.graphpad.com/support/faq/violin-plots-and-logarithmic-axes/ .

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Figures and figure legends

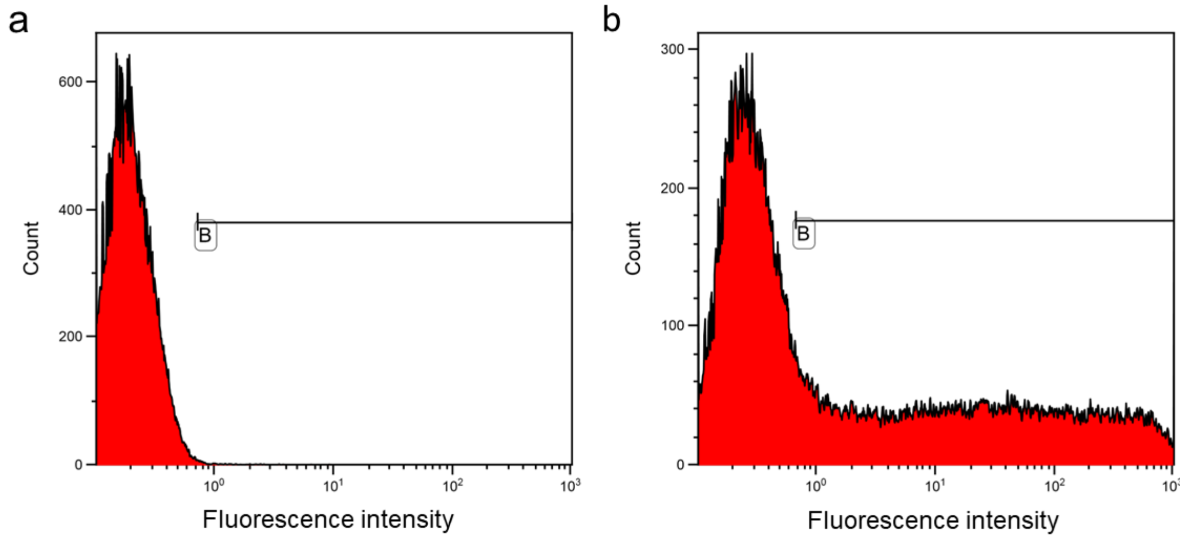

Fig. 1 Subjective gating of flow cytometry data using a non-transfected control. Histograms only include events that have been gated based on forward and side scatter to identify healthy single MCF-7 cells. Following the gating of the initial event data, the histograms depicting fluorescence intensity per event can be used for further gating to distinguish between BFP expressing and non-expressing cells (gate B). The Y-axis of the histograms represents the number of events (event count), and the X-axis the fluorescence intensity using log-scale. (**a**) The histogram illustrates the fluorescence intensity per counted event of the non-transfected negative control sample. Since the detection of a few false-BFP positive events cannot be avoided, gate B has been set by considering the histogram slope and the percentage of false-BFP positive events. 0.24% of the non-transfected cell population was counted as the relevant BFP positive cells using this positioning of gate B. (**b**) The histogram illustrates the fluorescence intensity per counted event of the cells subjected to heat shock treatment following transfection, with a ViaFect reagent to DNA ratio of 4:1. Gate B is applied to the events of this sample, resulting in 45.45% being positive for BFP expression.

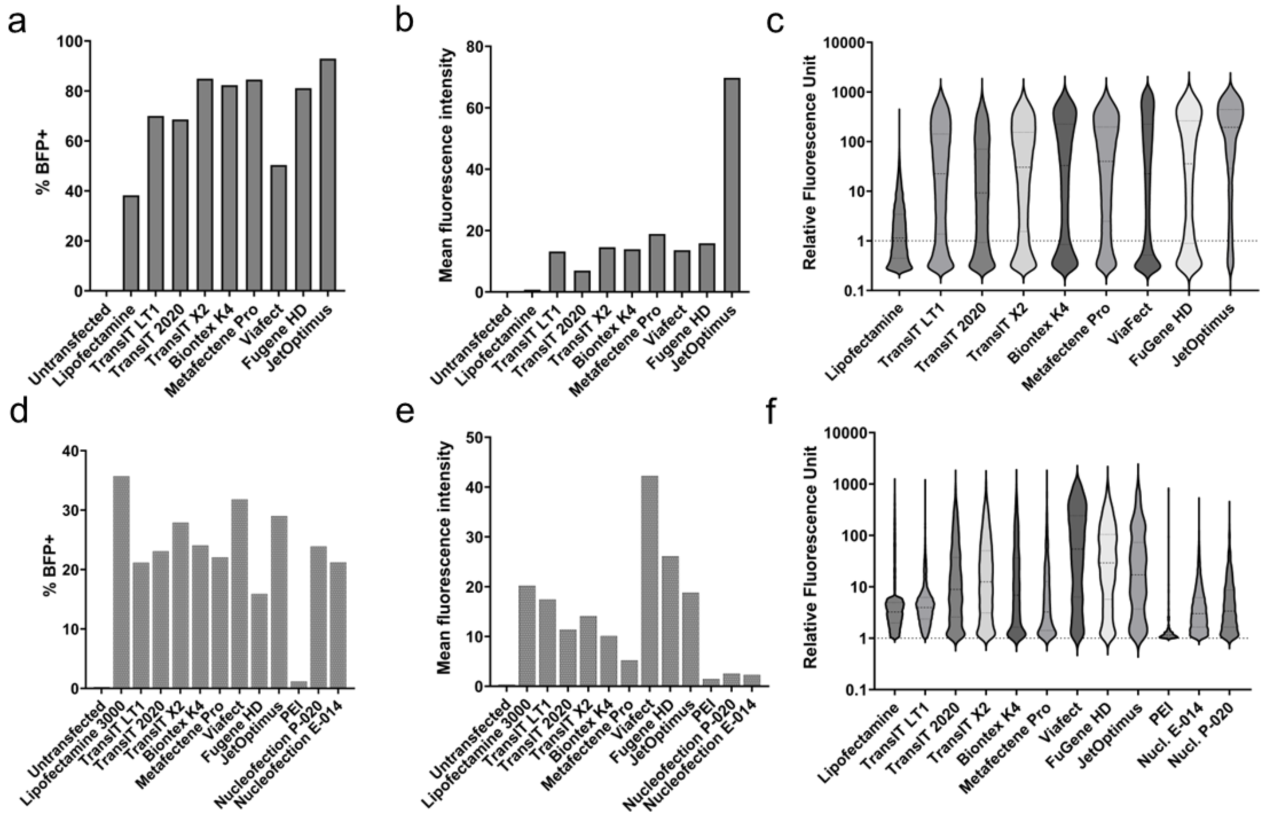

Fig. 2 Transfection efficiency, mean fluorescence intensity and single cell fluorescence data of HEK293T Lenti-X and MCF-7 breast cancer cells 24 hours post-transfection with several reagents and kits, using standard protocols. All reagents represent transfection chemicals, apart from nucleofection with the Nucleofector® electroporation system. The two different Nucleofector® programs used are indicated by program codes, P-020 and E-014, respectively. Panels A, B and C represent data from HEK293T Lenti-X samples, D, E and F from MCF-7 samples. Transfection efficiency is based on the percentage of cells positive for the reporter gene, blue fluorescent protein (BFP). Mean fluorescence intensity (MFI) is based on the geometric mean. The single cell fluorescence plot visualises the log-transformed relative fluorescence unit (RFU) data for each positive event. (**a**) Transfection efficiencies of different transfection reagents in HEK293T Lenti-X cells are shown. The sample treated with JetOptimus exhibits the highest transfection efficiency ~93%, followed by Trans*IT* X2 Dynamic Delivery System ~84%. (**b**) The mean fluorescence intensity of different transfection reagents in HEK293T Lenti-X cells is shown. The sample treated with JetOptimus has a higher MFI than the other samples. (**c**) The violin plots illustrate the single cell RFU data for each sample, effectively showing the distribution between high, mid and low fluorescence. The JetOptimus sample exhibits a high expression/BFP fluorescence distribution, whereas for instance Lipofectamine 3000 shows a bulk BFP concentration at the low end. (**d**) The transfection efficiencies of different transfection reagents and kits in MCF-7 cells are shown. Lipofectamine 3000 and ViaFect give the most promising results, showing a ~35% and ~32% transfection efficiency, respectively. (**e**) The mean fluorescence intensity is shown for different transfection reagents in MCF-7 cells. Based on the results obtained with the tested transfection reagents, ViaFect induces the highest mean BFP expression. (**f**) The violin plots illustrate the single cell RFU data for each sample. PEI, Lipofectamine 3000 or Trans*IT* LT-1 resulted in a higher proportion of low expressing cells, whereas ViaFect exhibited a more balanced distribution of BFP expression among the transfected cells.

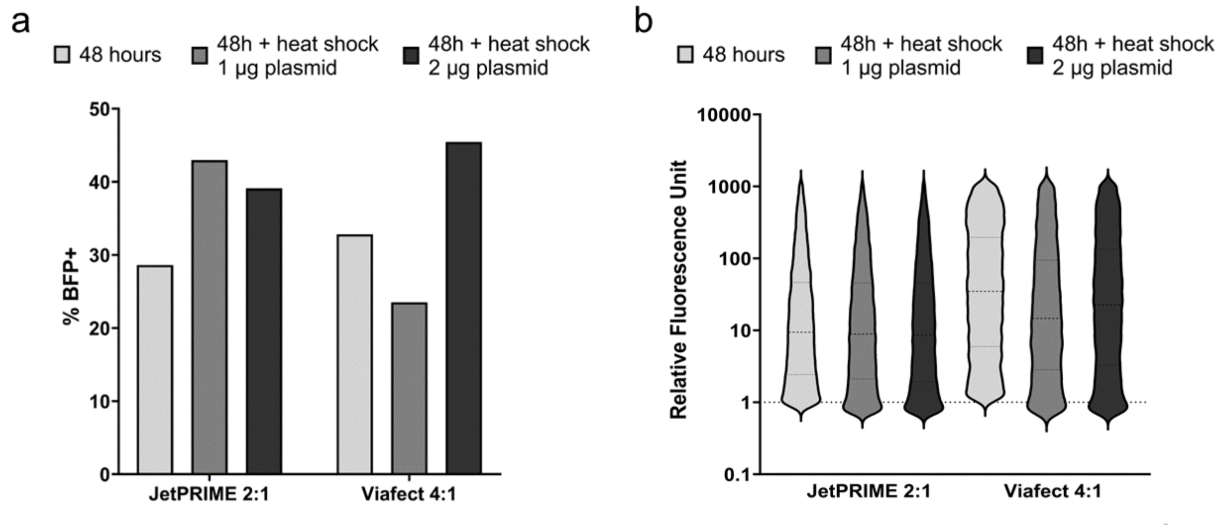

Fig. 3 Transfection efficiency and single-cell fluorescence profiles with JetPRIME and ViaFect as transfection reagents in MCF-7 breast cancer cells. MCF-7 cells were transfected with a reagent to DNA ratio of 2:1 for JetPRIME and 4:1 for ViaFect. Cells were either exposed to heat shock treatment or left untreated and measurements were taken 48 hours post-transfection. For samples without heat shock, 1 μg of plasmid was used for the transfection. (**a**) The transfection efficiency of MCF-7 cells as indicated by the percentage of blue fluorescent protein (BFP) expressing cells is shown. Samples transfected using the standard method, with heat shock, and with heat shock and double the amount of plasmid DNA, i.e. 2 μg plasmid instead of 1 μg. Incorporating heat shock into the protocol increased the total efficiency by ~13% for both reagents. The optimal cell response to ViaFect was observed when employing heat shock and using 2 μg pDNA per well, resulting in a transfection efficiency of 45.45%. (**b**) Violin plots of single-cell fluorescence in relative fluorescence units (RFU) are shown. Among the samples treated with ViaFect, the BFP expression is most uniformly distributed, making it the preferred choice for transfection. Subsequent sorting could be performed to isolate a relatively balanced proportion of high, mid and low-expressing cells.