Thank you for using our product. Please note that suboptimal concentrations of DNA and/or reagent may result in unexpectedly high toxicity to your cells, as is the case with many products of this nature. **In order to provide the highest possible transfection efficiency, it is imperative to optimize our product to work best with your specific cells.** Once you have determined the optimal concentrations of reagent, DNA, and cell number, you may proceed to the next step of the protocol. Please note, these concentrations are guidelines and routinely work well for many cell types. It is possible that certain cells may require one or more additional groups (Continuum™ & DNA) in order to ensure proper optimization. However, this is only recommended if our optimization suggestions do not produce the expected results.

Important Guidelines for Transfection

1. Prepare high-quality plasmid DNA at 0.5–5 µg/µl in deionized water or TE buffer. Make sure the plasmids are endotoxin-free and have A260/280 absorbance ratio of 1.8–2.0. A GFP (green fluorescent protein) plasmid can be used to determine transfection efficiency.
2. Use regular high glucose DMEM **without serum** to make Continuum™ and nucleic acid mix. Do not use $NaCl_2$ solution or PBS.
3. Maintain the same seeding conditions between experiments. Use low passage cells; make sure that cells are healthy and greater than 90% viable before transfection.
4. It is important to have the cells in proliferation state and 70-90% confluence at the time of DNA transfection.
5. Increasing the number of cells plated per well or decreasing the Continuum™ amount will minimize the effect of transfection on cell growth and viability. With careful optimization, this can be achieved while keeping the highest transfection efficiency.
6. Do not use antibiotics in the culture medium during the first 24 hours of transfection.

Protocol

1. DNA Transfection

1.1 Cell Seeding: For optimal DNA transfection conditions, we recommend using cells which are 70% to 90% confluent at the time of transfection. Typically, for experiments in 6-well plates, 150,000-250,000 adherent cells are seeded per well in 2ml of cell growth medium without antibiotics 24 hours prior to transfection. For the different culture formats, refer to Table 1.

Table 1. Recommended number of cells to seed the day before transfection in culture medium without antibiotics:

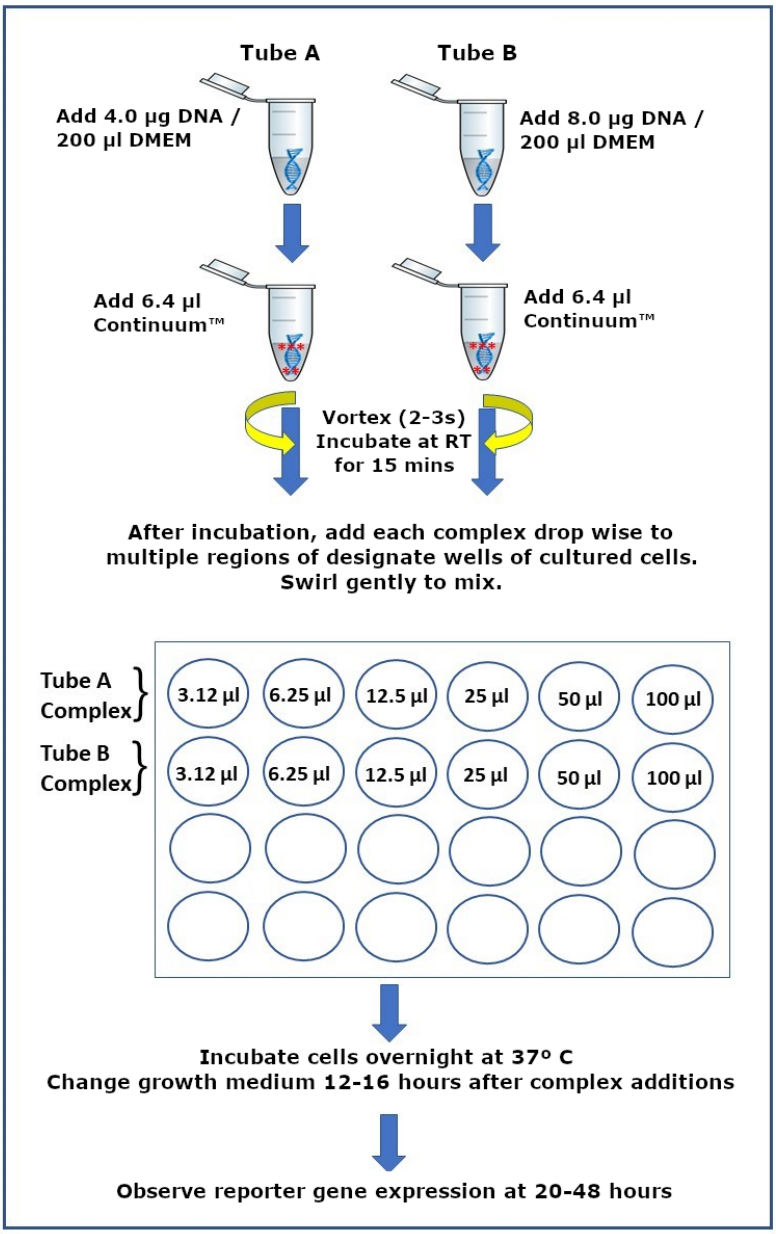
Culture Vessel	Number of Adherent cells to seed (Suspension Cells)	Surface Area per well (cm^2)	Volume of medium per well to seed the cells (ml)
96-well	7,500-10,000 ($4x10^4$)	0.3	0.1
24-well	50,000-80,000 ($2x10^5$)	1.9	0.5
12-well	80,000-150,000 ($4x10^5$)	3.8	1
6 well / 35mm	150,000-250,000 ($8x10^5$)	9.4	2
60mm / flask 25 cm^2	250,000-800,000 ($2x10^6$)	25-28	5
100mm / flask 75 cm^2	$1x10^6$-$2x10^6$ ($6x10^6$)	75-78.5	10
150 mm / flask 175 cm^2	$2x10^6$-$5x10^6$ ($1.3x10^7$)	153-175	25

1.2 DNA Transfection: If this is the first time that you are using Continuum™ on a specific type of cells, first, transfect the cells according to Table 2a for optimization **(The optimization procedures are crucial for successful transfection. Different cell types can have different levels of sensitivity to Continuum™; therefore, the amount of Continuum™ needed for maximum transfection can drastically vary on different cell types.)**.

The following procedures and Figure 1 are provided as an example of the optimization process on a 24-well plate.

1. Bring Continuum™ and DMEM to room temperature before starting.
2. Add 200µl DMEM into two 1.5ml tubes (Tubes A and B).
3. Add 4.0 µg of DNA to Tube A and 8.0 µg of DNA to Tube B.
4. Mix Continuum™ prior to preparing complexes. Add 6.4 µl of Continuum™ to both Tube A and Tube B containing 200µl of different concentration of DNA solution (20.0 µg/ml and 40.0 µg/ml respectively). Vortex for 2-3 seconds. Incubate each tube for 15 minutes at room temperature.
5. After incubation, add 3.12, 6.25, 12.5, 25, 50, and 100 µl of Continuum™ complexes dropwise directly to the corresponding wells of the 24-well cell culture plate (See Figure 1). Swirl plate gently.
6. Incubate the cells at 37°C in a CO_2 incubator
7. Change growth medium 12-16 hours later.
8. Expression of reporter gene activity should generally be assessed at 20-48 hours post-transfection. GFP expression is maximal at 40-48 hours post-transfection.

Figure 1



After you have completed the optimization steps, choose the amount of DNA and Continuum™ that gave you the optimal balance of potency and low cytotoxicity (which will usually be the lowest dose that gave you the same high transfection efficiency as other higher doses did) for all of your future experiments on this specific cell type.

1.3 Scale Up or Down Transfection: Use Table 2 to scale the volumes for your transfection experiment.

Table 2. Scaling Up or Down Transfection

Culture Vessel	Multiplication Factor[1]	Vol. Complex DMEM per well (µl)	DNA (µg)	Continuum™ (µl)
96-well	0.17	10	0.05	0.04-0.2
48-well	0.50	25	0.125	0.1-0.5
24-well	**1.00**	**50**	**0.25**	**0.2-1.0**
12-well	2.00	100	0.5	0.4-2.0
6-well	5.00	200	1.25	1.0-5.0
60-mm	11.05	500	2.75	2.3-11.5
10-cm	28.95	1000	7	5.8-29
T75	39.47	1500	10	7.9-39

[1]After determining the optimal reagent amount, use the multiplication factor to determine the reagent amount needed for your new plate format.

2. siRNA Transfection

2.1 Cell Seeding: For optimal siRNA transfection conditions, we recommend using cells that are 50% confluent at the time of transfection. Typically, for experiments in 6-well plates, 100,000 to 150,000 cells are seeded per well in 2ml of growth medium without antibiotics 24 hours prior to transfection. For other culture formats, refer to Table 3.

Table 3. Recommended number of cells to seed the day before transfection in culture medium without antibiotics

Culture Vessel	Number of Adherent cells to seed	Surface area per well (cm^2)	Medium per well to seed the cells (ml)
24-well	25,000-40,000	1.9	0.5
12-well	50,000-80,000	3.8	1
6-well/35mm	100,000-150,000	9.4	2
60 mm/flask 25 cm^2	200,000-500,000	25-28	5
100 mm/flask 75 cm^2	$0.5x10^6$-$1x10^6$	75-78.5	10

2.2 siRNA Transfection: For optimal siRNA-mediated silencing, we recommend using 10 to 50 nM siRNA (final concentration). The following conditions are given per well of a 6-well plate. For other culture formats, please refer to Table 4.

1. Dilute 22 to 110 pmoles siRNA (final concentration: 10 to 50 nM) into 200µl of regular high glucose DMEM without serum. Mix by vortexing.
2. Briefly vortex Continuum™, and add 1.0 to 5.0 µl into the diluted siRNA. Immediately vortex for 10s.
3. Incubate for 15 min at RT.

4. Add the transfection mixture drop-wise into each well.
5. Gently rock the plates back and forth and from side to side, and return the plate to the 37˚C CO2 incubator.
6. Analyze after incubating for 24 h or longer.

Table 4. siRNA transfection guidelines according to the cell culture vessel per well.

Culture Vessel	siRNA (pmole) 10 nM	siRNA (pmole) 50 nM	Continuum™ (µl)	DMEM (µl)	Growth Medium (ml)	Final Volume in the well (ml)
24-well	5.5	27.5	*0.2-1.0	50	0.5	0.55
12-well	11	55	*0.4-2.0	100	1	1.1
6-well / 35 mm	22	110	1.0-5.0	200	2	2.2
60 mm/ flask 25 cm^2	44	220	2.3-11.5	400	4	4.4
100 mm/ flask 75 cm^2	121	605	5.8-29.0	1100	11	12.1

**Dilute Continuum™ 1:5 with H_2O prior to application (4 µl reagent + 16 µl H_2O), and then use 5 times of the volume in the table for accurate pipetting.*