**si**FEX™ **Transfection of siRNA into 3T3-L1 cells**

3T3-L1 (ATCC® CL-173) cells are a mouse embryonic fibroblast cell line. ATCC has achieved 40-60% knockdown of target genes using the protocol described below. Your results will vary based on your gene of interest, quality of RNA, assay method and timing. Optimal conditions for your application will need to be experimentally determined.

**General considerations for using the siFEX RNAi Transfection Reagent:**

* **Cell conditions.** Cells should be passaged at least once post-thaw and the use of low-passage cells is recommended. Passage the cells 18-24 hours before transfection to ensure the cells are actively dividing and that they will be at the appropriate cell density at the time of transfection. Make sure that the cells are healthy, contaminant-free and are ≥ 90% viable, prior to transfection.
* **Seeding density.** Cell density should be 30-50% confluent on the day of transfection. See recommended cell type specific seeding densities in the cell line specific protocols. If extended culture is required post transfection, lower seeding densities (10-20%) may be required.
* **Presence of antibiotics and inhibitors.** Antibiotics can increase toxicity and should be omitted during transfection. Culture medium containing polyanions such as heparin, heparin sulfate or dextran sulfate can inhibit transfection. Medium containing these compounds should not be used during transfection. However, the medium can be replaced with complete growth medium (containing polyanions and/or antibiotics) 8-24 hours after transfection.
* **RNA.** Use high quality, sterile, validated RNA sequences for transfection. If needed, dilute stock solutions of RNA according to manufactures recommended instructions. We do not recommend using water. The optimal final RNA concentration will need to be experimentally determined; we recommend a starting point of 10 nM (approximate final concentration) and testing a range of 10-50 nM.
* **Volume of siFEX reagent.** The optimal reagent volume will need to be experimentally determined. See **Table 1** for suggested volumes for a variety of well sizes.
* **Complex formation conditions.** Prepare RNA complexes in serum-free medium. We recommend Opti-MEM I Reduced-serum Medium (Life Technologies).
* **Post-transfection incubation time.** The optimal incubation time is generally 24–72 hours post transfection, but will vary depending on cell type, RNA, and assay method.

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| Material Required | Catalog No.  |
| 3T3-L1  | ATCC® CL-173 |
| Complete growth media  | See cell line product sheet |
| siFEX™ RNAi Transfection Reagent | ATCC® ACS-4006™ |
| Opti-MEM® I Reduced-Serum Media | Life Technologies™ 31985-062 |
| siRNA or miRNA precursors (10 μM stock) |

**Suggested starting protocol:**

The following describes a protocol for the transfection of siRNA into 3T3-L1 cells using the siFEX Reagent in a **single well of a** **12-well plate.** The reaction may be scaled up or down as needed to account for alternate vessel sizes, multiple wells and/or account for pipetting error. Please refer to **Table 1** for recommended reaction conditions for other dish or plate sizes. A similar protocol is recommended when using miRNA precursors or other short RNA sequences.

1. **Prepare the cells for transfection**

The day before transfection:

* + 1. Count and measure cells for density and viability.
		2. Plate cells in complete growth medium. Cell density should be such that cells are **40-50%** confluent on the day of transfection. Plate cells at a density of 2-3 × 105 cells/well.
	1. Incubate cells overnight.

The day of transfection:

1. Remove old media.
2. Add fresh growth media (omit polyanions and antibiotics) to a total volume of 1.0 mL.
3. **Prepare the siRNA : siFEX transfection complexes**
	1. Bring the **si**FEX reagent and Opti-MEM I Reduced-serum Medium to room temperature. Vortex the **si**FEX reagent briefly to mix.
	2. Pipette 100 µL Opti-MEM I Reduced-serum Medium into a sterile microcentrifuge tube.
	3. Add 1.2 µL siRNA (of a 10 µM stock solution). Gently mix.
	4. Add 4 µL **si**FEXReagent to the diluted RNA mixture.
	5. Mix **si**FEX : siRNA complexes thoroughly.
	6. Incubate **si**FEX : siRNA complexes at room temperature for 5-10 minutes.
4. **Add transfection complexes to cells**
	1. Distribute the **si**FEX : siRNA complexes to the cells by adding the complexes drop-wise to different areas of the wells.
5. **After transfection**
	1. OPTIONAL: Change media 8-24h after transfection if excessive cytotoxicity occurs.
	2. Incubate until ready to assay for target knockdown. Typically knockdown at the mRNA level is apparent 24-48 hours post-transfection while protein knockdown may take 72 hours or more.

 **Table 1: Recommended conditions (per well) for different size culture vessels.**

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| Culture Vessel | 96-well plate | 24-well plate | 12-well plate | 6-well dish |
| Surface area | 0.2 cm2 | 1.9 cm2 | 3.8 cm2 | 9.6 cm2 |
| 3T3-L1 Cells  | 3-6x103 | 3-7x104 | 2-3x105 | 4-9x105 |
| Growth Medium | 0.1 mL | 0.5 mL | 1.0 mL | 2 mL |
| Opti-MEM I Reduced Serum Medium | 10 µL | 50 µL | 100 µL | 200 µL |
| siRNA or miRNA precursors | 0.6-1.2 pmol | 3-6 pmol | 6-12 pmol | 24-48 pmol |
| siFEX RNAi Transfection Reagent | 0.1-0.4 µL | 0.5-2 µL | 1-4 µL | 2-10 µL |

**Additional Notes**

* **Optimization.** The provided transfection protocols are guidelines and for will need to be empirically validated and optimized. Transfection efficiency, gene silencing and toxicity will vary based on numerous factors including culture conditions, RNA sequence, duration, and assay method.
* **Controls.** We recommend including validated positive and negative control RNAs with every experiment. A positive control is a RNA sequence previously validated to target a specific mRNA transcript. A negative control is a non-targeting or “scrambled” RNA sequence previously validated to not target any known mRNA transcripts.
* **Reverse transfection protocol.** The provided protocol is for a *forward transfection* where the transfection complexes are added to cells that have been seeded into plates 24 hours prior. Alternatively, the transfection reagent and RNA can be initially combined within the culture vessel to form complexes and cells in medium are added subsequently. This *reverse transfection* procedure is faster as it does not require pre-plating cells but may increase cytotoxicity in some cell lines. We do not provide cell line specific reverse transfection protocols.
* **Serum.** We generally recommend performing transfections in cells growing in the recommended complete growth media (see product sheet) which often contain serum. Unless specified on the cell line specific protocol we have not tested performing the transfection in serum-free media. Serum **must** be omitted from the media used to form transfection complexes.
* **Small volumes.** The volume of **si**FEX reagent or RNA may be too small to pipette accurately. If necessary, we recommend diluting **si**FEX 10-fold in sterile water and transferring a 10X higher volume. Unused diluted **si**FEX reagent should not be stored for later use. Refer to the RNA manufacturer recommendations for the appropriate dilution buffer and storage conditions.
* **Fluorescently labeled RNA.** We have not extensively tested the effectiveness of fluorescently labeled RNAs with **si**FEX and do not recommend their use as controls.