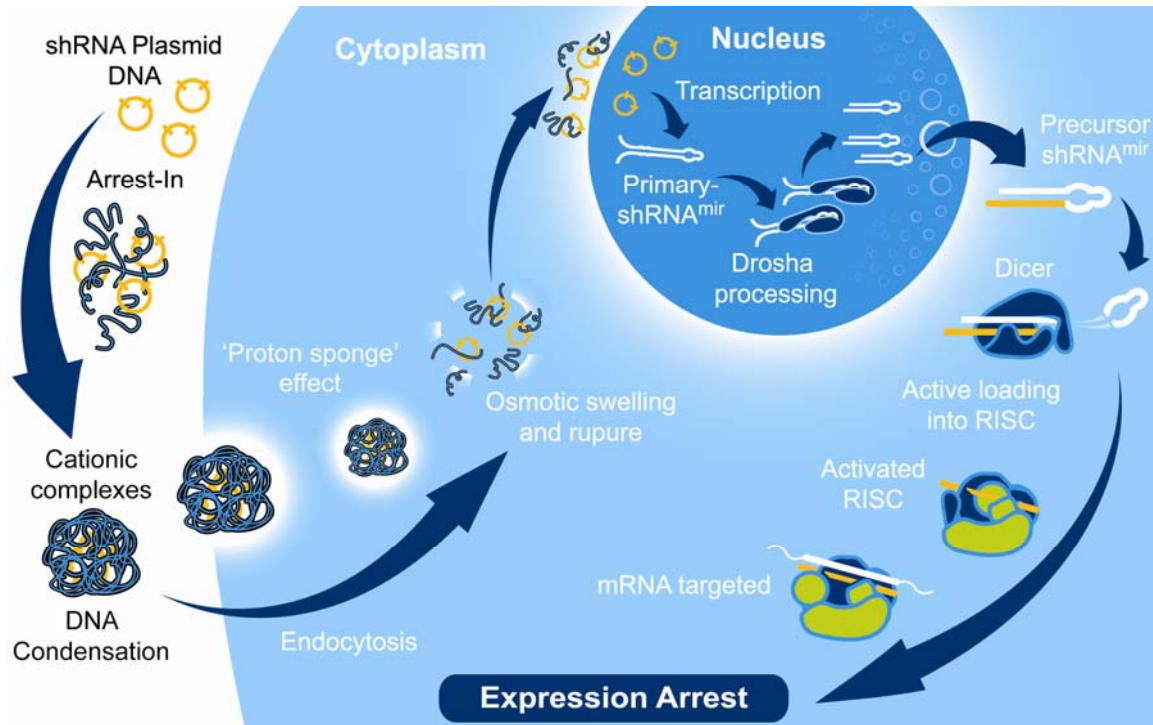


Fluorescent Arrest-In™ Transfection Reagent for shRNA delivery
 Catalog#s ATR4164, ATR4165, ATR4166, ATR4167



Product: Fluorescent Arrest-In™ Transfection Reagent for shRNA delivery

Catalog #s ATR4164, ATR4165, ATR4166, ATR4167

Product Description

Fluorescent (FI) Arrest-In™ transfection reagent is a polyethylenimine (PEI)-based lipopolymeric formulation, developed and optimized for highly efficient delivery of shRNA plasmid DNA into the nucleus of cultured eukaryotic cells. FI Arrest-In's proprietary formulation also provides an enhanced uptake efficiency of the shRNA plasmid DNA into cells. Once in the cells, FI Arrest-In promotes the entry of the shRNA-containing plasmid into the nucleus where it is transcribed into a hairpin which enters the cytoplasm and is processed by the endogenous RNAi machinery into functional siRNAs. See schematic on cover page.

PEI is well suited for gene delivery because of the high number of secondary amines that are protonable at low pH, leading to the influx of chloride ions and, consequently, to osmotic swelling and rupture of the lysosome. The early escape of the PEI-DNA complex from the lysosome, arising from the "proton sponge" effect, and the avoidance of lysosomal degradation makes FI Arrest-In transfection reagent very efficient at getting DNA into the nucleus of cells¹⁻⁴.

FI Arrest-In™ is rhodamine-conjugated Arrest-In. Excitation is at 560nm and emission at 580nm. This dual function transfection reagent allows the real-time visualization of transfected complexes (Fig.1), permitting biodistribution and double labeling studies (Fig.2) as well as promoting gene delivery. FI Arrest-In produces transfection efficiencies comparable to that described for Arrest-In (Fig.3).

Visualize transfection complexes

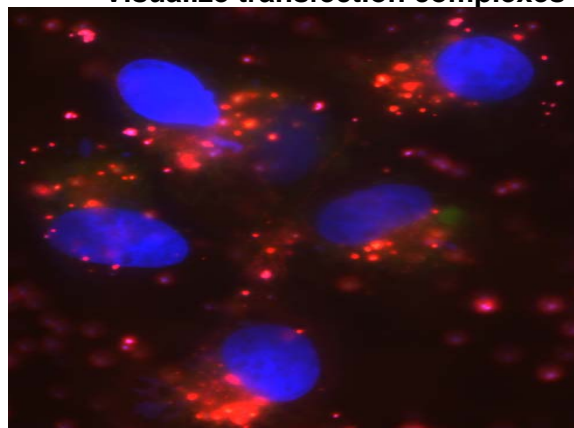


Fig. 1: Cos-1 cells transfected with FI Arrest-In/DNA and visualized after 5 hours. Cells were labeled with DAPI to visualize nuclei (blue).

FI Arrest-In™ is easy to use, robust, and exhibits very low toxicity. One milliliter is sufficient for approximately 100 transfections on 35mm tissue culture dishes using 2µg of DNA.

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Visualize gene delivery

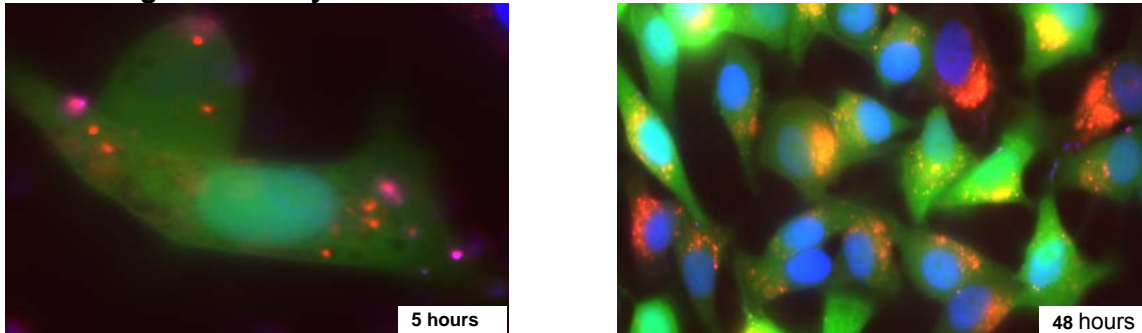


Fig.2: Transfected FI Arrest-In/DNA complexes are visible in HeLa cells overexpressing GFP (SeaGlow HeLa cells are available through Open Biosystems) at 5 hours and at 48 hours after transfection.

- Red** Transfection Complexes (FI Arrest-In/DNA (5 h) or FI Arrest-In/DNA (48 h))
- Green** GFP Expression
- Yellow** Co-localized transfection Complexes & GFP Expression
- Blue** Nucleus

Transfection efficiency of FI Arrest-In™ is comparable to Arrest-In™

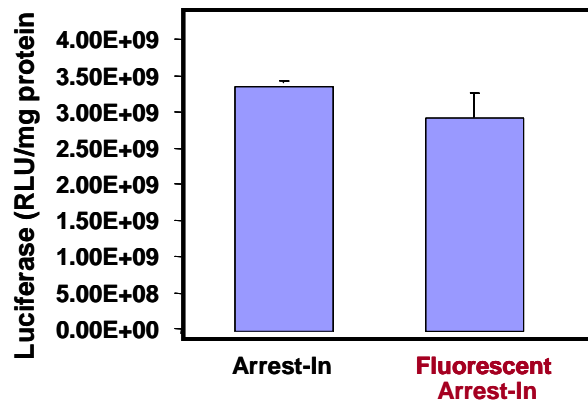


Figure 3. Luciferase activity measured after transfection of Cos-1 cells with Arrest-In or FI Arrest-In. The data are an average of two separate transfection experiments. Luciferase expression is not significantly different using either reagent.

FI Arrest-In is non-toxic to transfected cells

Arrest-In and FI Arrest-In display very low cytotoxicity. The graph below shows cell viability (determined by CellTiter 96 Assay) following *in vitro* transfection of Cos-1 cells with Arrest-In or FI Arrest-In. Cell viability assays were conducted 48 hours after transfection.

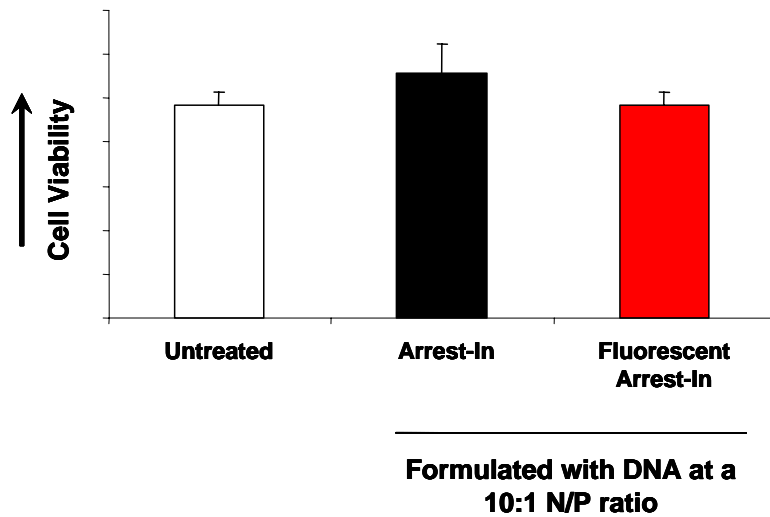


Figure 4: Cos-1 cells were transfected with DNA using Arrest-In or FI Arrest-In. 48 hours later cell viability was measured using the Celltiter 96 assay. Both reagents were non-toxic when compared against untreated cells.

Contents and Storage Conditions:

FI Arrest-In™ is supplied in amber tubes at a concentration of 1 mg/ml. 0.5ml, 1ml, 5x1ml and 10x1ml sizes are available. Store at 4°C in the dark for up to 12 months.

Quality Control:

Each lot of Fluorescent Arrest-In™ is functionally tested by transfection of Cos-1 or Hek293T cells with 1µg reporter plasmid. Following transfection, the cells are assayed at 48hrs and typically >70% of the cells will be positive for reporter activity. Each lot is tested by NMR and elemental analysis.

Protocols:

1) Transient Transfection of Adherent Cells with Plasmid DNA containing Reporters or shRNA constructs

1. The day before transfection, plate the cells so that they are approximately 60-80% confluent on the day of transfection.

***Warm FI Arrest-In™ reagent to ambient temperature (approximately 10-15 minutes at room temperature) prior to use. Always mix well by vortex or inversion prior to use. Do not add antibiotics to cell media during transfection.*

2. See Table 1 to determine the appropriate starting amounts of plasmid DNA, FI Arrest-In™ reagent, and culture medium based on your culture vessel size.

Form DNA/FI Arrest-In™ transfection complexes as follows:

- a. For each well in transfection, dilute plasmid DNA in 50µl of serum-free medium.
 - b. Dilute the appropriate amount of FI Arrest-In™ in 50µl serum-free medium.
 - c. Add the diluted DNA (step a) to the diluted FI Arrest-In™ reagent (step b), mix rapidly then incubate for 10 minutes at room temperature.
 - d. Add the remaining volume of serum-free medium required for your culture vessel size (Table1).
3. Add the DNA/FI Arrest-In™ complex mixture to the cells and incubate for 3-6 hrs in a CO₂ incubator at 37°C.

Note –DNA/FI Arrest-In™ complexes should be made in the absence of serum. Following complex formation the DNA/FI Arrest-In™ mixture can be added directly to the cells in the presence of serum if this is preferred. Only a slight reduction in transfection efficiency has been observed in the presence of serum.

4. Following the 3-6 hr incubation add an equal volume of growth medium containing twice the amount of normal serum to the cells (i.e. to bring the overall concentration of serum to what is typical for your cell line). Alternatively the transfection medium can be aspirated and replaced with the standard culture medium (see Note). Return the cells to the CO₂ incubator at 37°C.

*Note – FI Arrest-In™ has displayed low toxicity in the cell lines tested. Removal of transfection reagent is not required for many cell lines. In our hands, higher transfection efficiencies have been achieved if the transfection medium is **not** removed. However, if toxicity is a problem, aspirate the transfection mixture after 3-6 hrs and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.*

5. After 48-96 hrs of incubation, assay cells for reduction in gene activity, compared to untreated or non-specific/irrelevant shRNA controls, by qRT-PCR and Western blot or functional assay. Optimal length of incubation from the start of transfection is dependent on cell type and stability of the protein being analyzed.

6. If selecting for stably transfected cells, transfer the cells to medium containing puromycin for selection. It is important to wait at least 48 hours before beginning selection.

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Note – The working concentration of puromycin varies between cell lines. We recommend you determine the optimal concentration of antibiotic required to kill your host cell line prior to selection for stable shRNA transfectants. Typically the working concentration ranges from 1-10µg/ml.

Table 1 - Suggested amounts of DNA, medium and FI Arrest-In™ reagent for transfection of shRNA constructs into adherent cells

Tissue Culture Dish	Surface area per well (cm ²)	Total serum free media volume per well (ml)	shRNA plasmid DNA (µg)*	FI Arrest-In™ (µg)**
60 mm	20	2	4	21
35 mm	8	1	2	10
6-well	9.4	1	2	10
12-well	3.8	0.5	1	5
24-well	1.9	0.25	0.5	2.5
96-well	0.3	0.1	0.1–0.2	0.5–1

*Recommended starting amount of DNA. May need to be optimized for the highest efficiency.

**Recommended starting amounts of FI Arrest-In™ reagent. See Transfection Optimization.

II) Transfection of Suspension Cells in Presence of Serum Containing Medium

****Warm FI Arrest-In™ reagent to ambient temperature (approximately 10-15 minutes at room temperature) prior to use. Always mix well by vortex or inversion prior to use. Do not add antibiotics to cell media during transfection.**

1. Add 4-6 X 10⁵ cells in 500µl of growth medium with serum but without antibiotics.
2. The protocol below is for Jurkat cells in a 12-well plate, if a different culture dish is used adjust all amounts in proportion to the change in surface area. Form DNA/FI Arrest-In™ transfection complexes as follows:
 - a. For each well in transfection, dilute 2µg DNA into 50µl serum free medium.
 - b. For each well in transfection, dilute 10µg FI Arrest-In™ into 50µl serum free medium.
 - c. Add the diluted DNA (step a) to the diluted FI Arrest-In™ (step b), mix rapidly and incubate for 10 minutes to form the transfection complexes.
3. Add the DNA/FI Arrest-In™ complex mixture to the cells in culture and incubate for 48-96 hrs in a CO₂ incubator at 37°C.

Note - FI Arrest-In™ has shown low toxicity in the cell lines tested. Therefore removal of transfection reagent is not required for many cell lines. If toxicity is a problem, remove the transfection mixture after 4-6 hrs and/or dilute with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

4. After 48-96 hrs of incubation, assay cells for reduction in gene activity, compared to untreated or non-specific/irrelevant shRNA controls, by qRT-PCR and Western blot or

functional assay. Optimal length of incubation from the start of transfection is dependent on cell type and stability of the protein being analyzed.

5. If selecting for stably transfected cells, transfer the cells to medium containing puromycin for selection. It is important to wait at least 48 hrs before beginning selection.

Note – The working concentration of puromycin varies between cell lines. We recommend you determine the optimal concentration of antibiotic required to kill your host cell line prior to selection for stable shRNA transfectants. Typically the working concentration ranges from 1-10 µg/ml.

III) Co-Transfection of Adherent Cells with Reporters and shRNA constructs

***Warm FI Arrest-In™ reagent to ambient temperature (approximately 10-15 minutes at room temperature) prior to use. Always mix well by vortex or inversion prior to use. Do not add antibiotics to cell media during transfection*

1. The day before transfection, plate the cells so that they are approximately 70-80% confluent on the day of transfection.
2. The protocol below is optimized for co-transfection of a reporter and a shRNA construct into Hek293T cells in a 24-well plate (2.8-3 x 10⁴) cells per well seeded the day before transfection). If a different culture dish is used adjust all amounts in proportion to the change in surface area. Form shRNA DNA/FI Arrest-In™ transfection complexes as follows:
 - a. For each well in transfection, in a sterile microcentrifuge tube dilute into 40µl serum-free medium the following:
 - i. 50ng of the reporter DNA to be targeted by the shRNA (eGFP or Luciferase)
 - ii. 20ng of a non-targeted reporter, to be used as a transfection efficiency control (e.g.β-gal)
 - iii. 500ng of the appropriate shRNA plasmid DNA
 - iv. Add serum free medium as required to bring the final volume to 50µl
 - b. For each well in transfection, in a sterile microcentrifuge tube dilute 2.85µl (2.85µg) FI Arrest-In™ into 48µl serum free medium.
 - c. Add the 50µl of medium containing the diluted DNA (step a) to the diluted FI Arrest-In™ (step b), mix and incubate for 15 minutes to form the transfection complexes.
3. Add the transfection complex mixture to the cells and incubate in a CO₂ incubator at 37°C.

Method 1– Add the transfection complex mixture directly to the cells in culture.

Tap the plate and shake carefully to mix. Return the cells to a CO₂ incubator at 37°C. The following day replace medium with fresh growth medium as required.

Method 2 – Serum free transfection for difficult to transfect lines, to increase efficiency, or sensitive cell lines.

First aspirate the growth medium from the cells. Add an additional 300µl of serum free medium to the tube containing the transfection complexes, mix well, then overlay onto the cells. Return the cells to the CO₂ incubator at 37°C for 3-6 hrs. Following the incubation add an equal volume of growth medium containing twice the amount of normal serum to the cells (i.e. to bring the overall concentration of serum to what is typical for your cell line). Alternatively the transfection medium can be aspirated and replaced with the standard culture medium (see Note). Return the cells to the CO₂ incubator at 37°C.

Note – FI Arrest-In™ has displayed low toxicity in the cell lines tested therefore removal of transfection reagent is not required for many cell lines. In our hands higher transfection efficiencies have been achieved if the transfection medium is not removed. However, if toxicity is a problem, aspirate the transfection mixture after 3-6 hrs and replace with fresh growth medium. Additionally, fresh growth medium should be replenished as required for continued cell growth.

4. After 48 hrs of incubation, assay cells for reduction in gene activity, compared to reporter alone, non-silencing shRNA, or other negative controls.
5. Calculate decrease in reporter activity compared to % of control as follows:
 - a. Calculate the mean of your replica samples (R1 + R2...) of shRNA/reporter transfections and the non-silencing/reporter or other negative controls.
 - b. Subtract the mean of a 'no plasmid DNA' control (background) from each sample.
 - c. Divide each of the background subtracted replica means by the mean of the Non-silencing/Reporter transfections (or other negative control transfection). This results in your sample:control ratio.
 - d. Assay for the non-targeted reporter activity (e.g. β-gal, Renilla Luciferase) to determine if transfection efficiency varied across wells. If significant variation exists either repeat transfection under tightly controlled conditions or normalize transfection efficiency prior to comparing across wells.

Note: Factors affecting transfection efficiency are not limited to but include purity of plasmid DNA, health of transfected cells, inconsistencies in number of cells plated, insufficient mixing of transfection complexes.

Transfection Optimization using FI Arrest-In™

It is essential to optimize transfection conditions to achieve the highest transfection efficiencies and lowest toxicity with your cells. The most important parameters for optimization are transfection reagent to DNA ratio, DNA concentrations and cell confluency. We recommend that you initially begin with 70-80% confluent cells, and with the FI Arrest-In™ and DNA amount indicated in Table 1.

Additional Factors Influencing Successful Transfection

Concentration and purity of nucleic acids – Determine the concentration of your DNA using 260 nm absorbance. Avoid cytotoxic effects by using pure preparations of nucleic acids.

Transfection in serum containing or serum-free media – Our studies indicate that FI Arrest-In™ /DNA complexes should always be formed in the absence of serum. In the cell lines tested we found that the highest transfection efficiencies can be obtained if the cells are exposed to the transfection complexes in serum free conditions followed by the addition of medium containing twice the amount of normal serum to the complex medium 3-5 hrs post transfection (leaving the complexes on the cells). However, the transfection medium can be replaced with normal growth medium if high toxicity is observed.

Presence of antibiotics in transfection medium – The presence of antibiotics can adversely affect the transfection efficiency and lead to increased toxicity levels in some cell types. It is recommended that these additives be initially excluded until optimized conditions are achieved, then these components can be added, and the cells can be monitored for any changes in the transfection results.

High protein expression levels – Some proteins when expressed at high levels can be cytotoxic; this effect can also be cell line specific.

Cell history, density, and passage number – It is very important to use healthy cells that are regularly passaged and in growth phase. The highest transfection efficiencies are achieved if cells are plated the day before, however, adequate time should be allowed to allow the cells to recover from the passaging (generally >12 hours). Plate cells at a consistent density to minimize experimental variation.

References

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2. Boussif, O., Lezoualc'h, F., Zanta, A., Mergny, M. D., Scherman, D., Demeneix, B. & Behr, J.-P. (1995) *Proc. Natl. Acad. Sci. USA* 92, 7297–7301.
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