siPOOL transfection protocol

Introduction:

Concentration, efficiency and specificity:

What is critical for the efficiency and specificity of an RNAi experiment is not the siRNA concentration in the medium but inside the cells. Optimal transfection conditions will deliver an amount of siRNAs into the cytoplasm sufficient for a saturating knock-down of the target gene but avoiding an overload that may lead to unspecific side effects (e.g. by displacement of endogenous miRNAs). The efficiency of siRNA delivery is largely dependent on the cell type and transfection reagent used.

Cells:

This protocol is intended for the transfection of standard adherent mammalian cells lines as HeLa, A549, MCF7 or Hek293 for which the method will yield efficient transfection at low nano-molar siPOOL concentrations.

Many types of primary cells but also certain cell lines such as MDCK, BJ or GH3 are significantly harder to transfect. Optimal transfection conditions for these cells may require the testing of different transfection reagents and frequently higher siPOOL concentrations. (For advice and help please contact info@sitools.de.)

Transfection Reagents:

As with every other RNAi reagent, siPOOLs require the use of transfection reagents for efficient transfer into the cytoplasm. There is a great choice of commercially available transfection reagents, which vary in their efficiency and toxicity in different cell lines. For many adherent cell types, we have observed that Lipofectamine RNAi Max from Thermo gives close to optimal results and recommend it for starting transfection optimization.

Guidelines on critical parameters:

For any transfection reagent and cell line, the 3 most important parameters defining transfection efficiency, toxicity and assay compatibility are a) siRNA concentration, b) transfection reagent concentration and c) cell seeding density. For adaptations of the basic protocol below, follow these general guide lines:

- Keep siPOOL concentration (the total concentration of all siRNAs composing the siPOOL) as low as possible. For optimal results, quantify silencing efficiency for a test gene by qPCR (e.g. GAPDH) or phenotypic readout (e.g. PLK1) to establish the siPOOL concentration where silencing starts to saturate. For most cell lines and genes, this concentration will be between 1 and 3 nM.
- Transfection reagent concentration must be adapted to cell seeding density. High reagent concentration and low cell seeding density will lead to target gene independent toxicity that may mask or alter target gene specific phenotypes.
- Adapt your cell seeding density to your assay protocol. The protocol below is intended for analysis 24h post-transfection as recommended for qPCR analysis of silencing efficiency. Reduce cell seeding density according to the doubling rate of your cell line and the duration of your assay. You may have to reduce transfection reagent concentration to avoid toxicity. For further information, please contact us: info@sitools.de

Dishes:

- **Plates:** Most reader or microscopy based assays are designed for multi-titer plates, 96-well being a common standard. As pipetting volumes are low and experiments are frequently done in replicates, the protocol for 6, 24, 96 and 384-well plates indicates both: the precise volumes for one single well (Table 1) AND practical volumes with excess for one experimental triplicate (Table 2).
- **Dishes:** Biochemical analysis often requires larger amounts of cell material and is performed in petri dishes. Our protocols for 6 cm, 10 cm and 15 cm dishes are calculated for one single petri dish without excess (Table 1).

In all cases we recommend to prepare the transfection mix in separate containers (tubes or plates) for optimal mixing and complex formation.

Forward and reverse transfection:

The complex of siPOOL and transfection reagent (transfection mix) may be added onto settled, previously seeded cells (forward transfection) or can be mixed with cells during the seeding process (reverse transfection).

- For **forward** transfection, cells are classically seeded 24h prior to transfection. This approach may be better suited for certain screening procedures and may have lower toxicity in some cell lines. Note that cells will have to be seeded at lower density to reach the same confluency at the time point of analysis.
- **Reverse** transfection is the protocol of choice for most applications, as it frequently yields better transfection efficiency and facilitates the experimental protocol by combining cell seeding and transfection into a single step. Also it allows for longer assay duration. The protocol indicated below is a reverse transfection protocol.

Required solutions:

transfection reagent:	Lipofectamine RNAiMax (Thermo)
dilution solution:	Opti-MEM (Thermo)
siPOOL pre-dilution stock :	concentration required for **1 nM** and **3 nM** final siRNA concentration. use: siPOOL solution **50 nM** or **150 nM** for 384/ 96/ 24 or 6-well plates use: siPOOL solution **0,5 µM** or **1,5 µM** for 6/ 10 or 15 cm dishes

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siPool reverse transfection protocol for adherent cells with 1 or 3 nM final siRNA concentration (for amounts and volumes see Tables 1 and 2)

Table 1: precise volumes for single well/dish

plate type	surface area (cm^2)	final volume (µl)	Optimem for siPOOL dilution (µl)	siPOOL pre-dilution stock **0,05** or **0,15** µM (µl)	Optimem for RNAiMax dilution (µl)	RNAiMax (µl)	transfection mix/ well (µl)	cell seeding number	cell seeding density (cells/ml)	cell susp. volume (µl)
6	9	2000	210	40	246	4	500	300 000	200 000	1500
24	1,8	500	40	10	49	1	100	60 000	150 000	400
96	0,3	100	8	2	9,8	0,2	20	10 000	125 000	80
384	0,1	25	2	0,5	2,4	0,1	5	3 300	165 000	20

dish diameter (cm)	surface area (cm^2)	final volume (ml)	Optimem siPOOL dilution (µl)	siPOOL pre-dilution stock **0,5** or **1,5** µM (µl)	Optimem for RNAiMax dilution (µl)	RNAiMax (µl)	transfection mix/ dish (ml)	cell seeding number	cell seeding density (cells/ml)	cell susp. volume (ml)
6	21	5	490	10	490	10	1	700 000	175 000	4
10	58	10	980	20	980	20	2	1 933 000	245 000	8
15	152	20	2460	40	2460	40	5	5 065 000	340 000	15

Table 2: volume for triplicate + excess (+ 1 vol)

plate type	surface area (cm^2)	final volume single well (µl)	Optimem for siPOOL dilution (µl)	siPOOL pre-dilution stock **0,05** or **0,15** µM (µl)	Optimem for RNAiMax dilution (µl)	RNAiMax (µl)	total transfection mix (µl)	transfection mix/ well (µl)
6	9	2000	840	160	984	16	2000	500
24	1,8	500	160	40	196	4	400	100
96	0,3	100	32	8	39,2	0,8	80	20
384	0,1	25	8	2	9,6	0,4	20	5

Protocol (derive pipetting volumes from table 1 and 2)

1. ***siPOOL dilution:*** Pipette Opti-MEM for siPOOL dilution (col 4) in a reaction tube and add siPOOL pre-dilution stock (col 5) . Mix well by vortexing or pipetting up and down. Spin down droplets

2. ***RNAiMAX dilution:*** Pipette Opti-MEM for RNAiMax dilution (col 6) in a reaction tube and add RNAiMax. (col 7). Mix well by vortexing or pipetting up and down. Spin down droplets. (An RNAiMax dilution mastermix [wells + excess volume] for all transfection reactions is recommended.)

3. ***Transfection mix*:** combine siPOOL dilution (step 1) with RNAiMax dilution (step 2) in a 1:1 ratio and mix well by vortexing or pipetting up and down. Spin down droplets.
   Incubate 5 min at room temperature (Reaction is stable for at least 30 min).

4. Transfer transfection mix to the bottom of cell culture plate or dish. For 384-well plates it a quick spin is recommended.

5. Add cell suspension (col 11) to transfection reaction. For plates and large wells, mix gently by pipetting. For 96 and 384well plates, simply adding the cell suspension is sufficient. Do not shake or vortex. To avoid edge effects in 96- or 384-well plates, let cells settle at RT for 30 min before transferring plates to incubator.

For the analysis of target gene silencing by qPCR, harvest cells 24h after transfection for RNA extraction. For different analyses (e. g. functional assays), extended incubation periods will frequently be necessary. Make sure to reduce cell seeding density according to the doubling time of the cells used.