Versatile use of transfection reagents for short double-stranded RNAs and DNA plasmids in human cells

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Introduction:

The development of RNA-based technologies in cell biology, such as RNA-mediated post-transcriptional gene silencing using double-stranded short interfering (si)RNAs, has represented a breakthrough in studies of mammalian cells. Since their discovery (see 1-2), an ever growing number of studies have used siRNAs not only to silence individual genes and dissect their function, but also in large genome-wide screenings using siRNA libraries to identify functional connections in genetic networks (3) and in the development of potential therapeutic applications (4).
These studies often require the cotransfection of siRNAs together with DNA plasmids encoding either a siRNA-resistant version of that same gene to assess the specificity of gene silencing, or a gene thought to be functionally related to it for the purpose of functional complementation assays (see for example 5-6).
Cotransfection assays however often involve laborious procedures, which may involve changing the culture medium to enable the sequential use of different reagents for optimal transfection of the RNA and DNA components. When a single transfection reagent is used, it is often found that single reagents may work preferentially for one type of nucleic acid and less effectively for the other. Here we have assayed the recently developed K2 reagent to assess its performance in single DNA or RNA transfection and in DNA/RNA co-transfection assays.

Materials and Methods:

Cell cultures: Experiments were performed in the HeLa cell line (ATCC-CCL-2), plating $8x10^5$ cells in 60 mm-wells in 5 ml (final volume) of complete D-MEM medium supplemented with 10% foetal bovine serum. For immunofluorescence (IF) assays cells were grown directly on sterile, coverslips in the culture well. Cells were grown at 37^0C in a 5% CO2 atmosphere.
Plasmid: pEGFP-N1 (4,7 Kb) was from Clontech.
RNA oligonucleotides: Double-stranded RANBP1-specific siRNAs (5'-GGAGCGAGGCACUGGUGAC-3') were validated in previous studies (7).
Transfection reagents: In all protocols using the K2 Transfection System (K2 transfection reagent & K2 Multiplier) Biontex Laboratories GmbH), 2 hours before transfections the K2 Multiplier was added to cell cultures (50 µl Multiplier: 5 ml of culture medium). The K2 transfection reagent was assayed for both single, and concomitant, DNA and RNA transfection. For comparison Lipofectamine 2000 (Invitrogen) was used in plasmid transfection and plasmid plus RNA co-tranfection; Oligofectamine (Invitrogen) was used for siRNA transfection alone. All mixes of nucleic acid plus transfection reagents were prepared in OptiMEM (Gibco). The ratio of plasmid to transfection reagent was as recommended by the manufacturers and volumes are indicated in Table 1.
DNA transfection protocol: Cells were transfected with pEGFP plasmid using either K2 Transfection System or Lipofectamine 2000. Transfection mixes were incubated for 20 minutes at

room T° before adding to the cell cultures. After 6 hours (Lipofectamine 2000) or 24 hours (K2) from transfection, the medium was replaced with fresh medium. Cells were harvested after 48 hours from the start of transfection.

RNA transfection: Cells were transfected with RANBP1-specific siRNAs 24 hours after seeding, using either K2 or Oligofectamine. Transfection mixes were incubated for 20 minutes at room T° before adding to the cell cultures. The medium was replaced with fresh medium 24 hours after transfection and cells were harvested after further 24 hours.

DNA and RNA co-transfection: Cells were simultaneously transfected with RANBP1-specific siRNAs and pEGFP DNA 24 hours after seeding under the conditions listed in Table 1. Transfection mixes were incubated for 20 minutes at room T° before addition to the cell cultures. The medium was replaced with fresh medium 6 hours (Lipofectamine 2000) or 24 hours (K2 Transfection System) after transfection. Cells were harvested after 48 hours from transfection.

Microscopy: Cells grown on sterile coverslips were fixed in 3.7% para-formaldehyde (PFA), then permeabilised in 0.1% Triton-X100–PBS and processed for immunofluorescence (IF) using goat anti-RANBP1 antibody (C-19 Santa Cruz Biothecnology) (primary antibody) followed by donkey anti-goat Ig-FITC (Jackson Immunoresearch) or donkey anti-goat TRITC (Jackson Immunoresearch) as secondary antibodies. Cells were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.1 μg/ ml) and mounted in Vectashield medium (Vector Laboratories). Slides were observed under a Nikon Eclipse 90i microscope equipped with a Qicam Fast 1394 CCD camera (QImaging). Images were acquired using Nis-Elements AR 3.2 (Nikon).

Western Immunoblotting: Whole cell extracts were prepared from transfected HeLa samples by lysing in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 1 mM EGTA, 0.25% sodium deoxycholate) supplemented with protease and phosphatase inhibitors. Proteins were resolved through SDS-PAGE (12%) and transferred onto nitrocellulose membranes (Protran BA83, Whatman). 40 μg of extract per lane were loaded. Membrane-bound proteins were stained with 0.2 % Ponceau S in 0.3% tricarboxylic acid solution, then washed in 0.3 % tricarboxylic acid solution. Blocking and incubations with antibodies were performed at room T° in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% low-fat milk. The following primary antibodies were used: goat anti-RANBP1 (C-19, Santa Cruz Biotechnology); rabbit anti-GFP (Abcam 6556); goat anti-actin (I-19, Santa Cruz Biotechnology). Secondary antibodies were: donkey anti-goat HRP and goat anti-rabbit HRP (both from Santa Cruz Biotechnology). Signals were visualized using the enhanced chemoluminescence detection protocol (ECL plus, Amersham Biosciences). The relative protein abundance was estimated by calculating the ratio [(protein signal intensity - background signal) / (normalising housekeeping protein signal intensity – background signal)] after densitometric scanning of the bands using Photoshop CS6 13.0.

Results:

Our chosen transfection reporters were a) the EGFP plasmid, a widely used DNA cloning vector easily detectable as a transfection reporter, due to the green emission of its fluorescent EGFP tag, and b) short double-stranded RNA oligonucleotides specifically targeting the RANBP1 gene, widely expressed in all cells: this enables an easy measurement of the efficiency of siRNA transfection by quantifying the loss of RANBP1 protein in transfected cells (7). This interference typically causes chromosome mis-segregation phenotypes in mitotic cells, which are easy to recognise and cannot be confused with any non-specific effect of the transfection reagents (7-9). We then measured to efficiency of transfection of DNA, siRNAs or both together, in HeLa cells.

a) DNA transfection: After 48 hours of transfection of pEGFP plasmid in HeLa cell cultures, we find that the K2 Transfection System performed very effectively in terms of transfection efficiency, as evidenced by measuring the signal intensity of the pEGFP-encoded GFP product by both Western immunoblotting (Fig.1, left panel; EGFP-specific signals are normalized to actin) and by fluorescence assays (Fig.1, right panel), by counting EGFP-expressing cells over the total number of DAPI-stained cells. Obviously, detection of protein abundance by Western blot entails an intermediate step in the antibody: antigen recognition which may "correct" the measured signal intensity, whereas the EGFP fluorescence intensity directly measures the protein abundance. Single-cell analysis under a fluorescence microscope using the same exposure time depicted “highly expressing”, when the EGFP signal was fully saturated, or “low expressing” when the signal was below saturation but still evident in the transfected cells (Fig.2). The K2 Transfection System increased the frequency of both classes compared to Lipofectamine.

b) RNA transfection: To assess the efficiency of the K2 Transfection System in siRNA transfection assays, we used siRNAs targeting the RANBP1 gene transcript and, 48 hours after transfection, measured the residual RANBP1 protein signal in interfered cultures. These assays showed that K2 is an effective reagent for transfection of siRNAs (Fig. 3). Western blot protein analysis (Fig.3, left panel) showed that the RANBP1 signal (normalized to actin) was substantially reduced in cultures transfected with the K2 Transfection System compared to non-interfered controls, and was also lower than in cultures transfected with Oligofectamine. In IF assays most cells displayed very faint residual RANBP1 signals, if any (Fig.3, right panel). IF analysis also demonstrated that the cell morphology was well preserved in transfected samples (600 analyzed cells per condition) (Fig.4).

c) DNA and RNA co-transfection: We also simultaneously co-transfected EGFP plasmid and RANBP1-specific siRNAs, testing two ratios of nuclei acid: reagent volumes for the K2 Transfection System (see table 1), and lipofectamine for comparison, then evaluated the protein abundance for both the transfected GFP and the residual interfered RANBP1, in Western assays. Transfected samples were also visualized under the green (EGFP) and red (secondary antibody to anti-RANBP1) channels by fluorescence microscopy. Western blot analysis (Fig.5, left panel) revealed a low signal for RANBP1, and a high signal for EGFP protein, in K2-transfected cultures under both tested ratios (both signal intensities were normalized to actin). IF assays confirmed high pEGFP expression and, concomitantly, effective siRNA-dependent interference in K2-transfected cultures. Images of samples transfected with K2 transfection reagent in 1:4 ratio show higher transfection signals at the single cell level compared to Lipofectamine 2000 or to K2 in 1:2 ratio, but occasionally some fluorescently stained spots were seen with this high ratio. Thus, it is advisable to adjust the ratio of K2 reagent volume to the number of plated cells, depending on the particular experiment, so as to eliminate the formation of such spots while achieving satisfactory levels of interference at the single cell level.

Conclusion / summary:

In the present assays, we have transfected HeLa cells using K2 Transfection System and adapted the transfection reagent volume to the specific transfection application, i.e. for either DNA, or short RNAs, or both simultaneously. In general, the K2 Transfection System performs better than other commonly used commercial systems in all three applications. In siRNA transfection, the product of the interfered gene was barely visible (if at all) by Western immunoblotting. By IF, many cells showed only barely visible residual signals and many appeared to have been fully depleted of the interfered protein. Transfected cultures generally retained a well-preserved morphology, highly

suitable for high-resolution analysis of the cellular phenotypes arising in interfered cells. In DNA/RNA co-transfection, K2 performed better than other reagents. Fluorescence analysis of individual cells showed highly efficient induction of plasmid-encoded protein and simultaneous depletion of the siRNA target. Our results suggest that the experimental protocol can be successfully optimized to the specific requirements of the experiments and indicate that the K2 Transfection System can be employed as a highly effective and versatile reagent with many potential applications.

References:

1. Meister G, and Tuschl T. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature 431:343-9.

2. Fire AZ. 2007. Gene silencing by double-stranded RNA. Cell Death Differ. 14:1998-2012.

4. Mohr SE, Smith JA, Shamu CE, Neumüller RA, and Perrimon N. 2014. RNAi screening comes of age: improved techniques and complementary approaches. Nat Rev Mol Cell Biol. 15:591-600.

4. Vaishnaw AK, Gollob J, Gamba-Vitalo C, Hutabarat R, Sah D, Meyers R, de Fougerolles T, and Maraganore J. 2010. A status report on RNAi therapeutics. Silence. 1:14.

5. Roscioli E, Di Francesco L, Bolognesi A, Giubettini M, Orlando S, Harel A, Schininà ME, and Lavia P. 2012. Importin-β negatively regulates multiple aspects of mitosis including RANGAP1 recruitment to kinetochores. J Cell Biol 196:435-50.

6. Asteriti IA, Giubettini M, Lavia P, and Guarguaglini G. 2011. Aurora-A inactivation causes mitotic spindle pole fragmentation by unbalancing microtubule-generated forces. Mol Cancer 10:131

7. Tedeschi A, Ciciarello M, Mangiacasale R, Roscioli E, Rensen WM, and Lavia P. 2007. RANBP1 localizes a subset of mitotic regulatory factors on spindle microtubules and regulates chromosome segregation in human cells. J Cell Sci 120:3748-61.

8. Li HY, Ng W P, Wong CH, Iglesias PA, and Zheng Y. 2007. Coordination of chromosome alignment and mitotic progression by the chromosome-based Ran signal. Cell Cycle 6, 1886-1895.

9. Rensen WM, Roscioli E, Tedeschi A, Mangiacasale R, Ciciarello M, Di Gioia SA, and Lavia P. 2009. RANBP1 downregulation sensitizes cancer cells to taxol in a caspase-3-dependent manner. Oncogene 28:1748-58.

Appendix: Tables and/or figures:

Table 1. Tested conditions for transfection of $8x10^5$ HeLa cells (6x 60mm well plates)

Nucleic acid: reagent (v/v) ratio[a]	Nucleic acid volume (µl)		Reagent volume (µl)			
	DNA	RNA	LIPO	OLIGO	K2	
1:4	6	/	/	/	24	DNA only
1:1	6	/	6	/	/	
1:2	/	4	/	/	8	RNA only
1:4	/	4	/	16	/	
1:2	6	4	/	/	20	DNA+RNA
1:4	6	4	/	/	40	
1:1	6	4	10	/	/	

a. The ratio of volume of nucleic acid : volume of transfection reagent was chosen based on the manufactureres' instruction

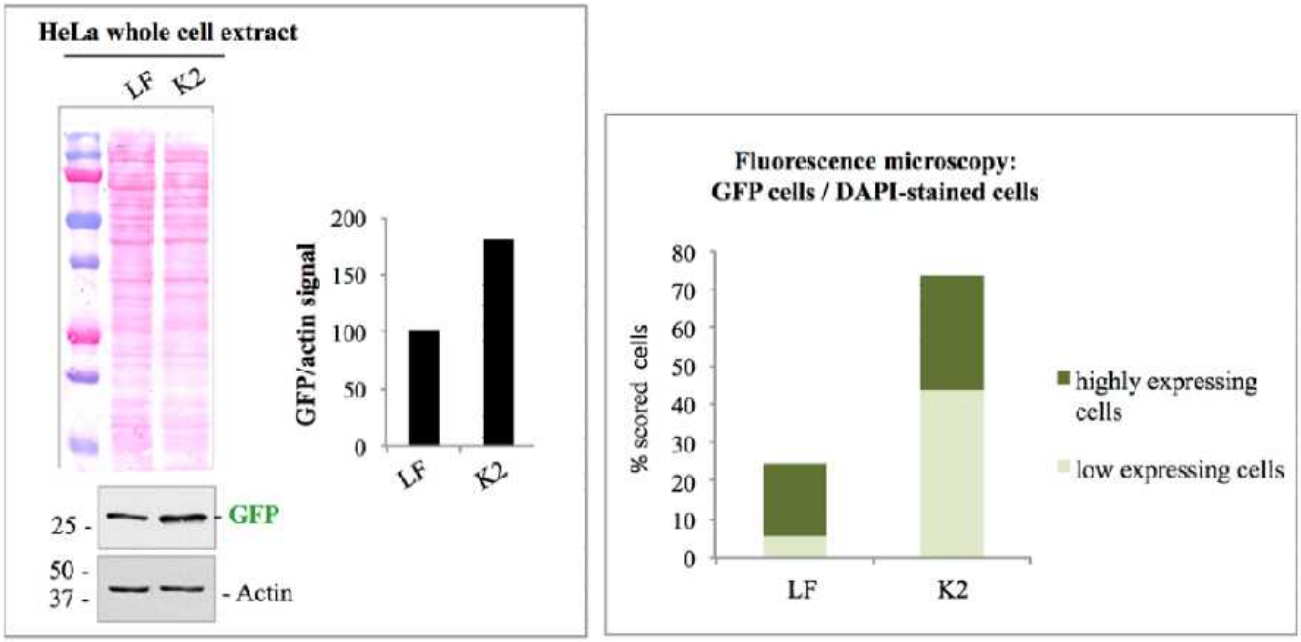

Figure 1. Quantitative results from Western immunoblotting (left panel; 40 µg extract were loaded in each lane) and from fluorescence microscopy scoring of EGFP-positive cells (right panel) to assess the efficiency of DNA plasmid transfection using either Lipofectamine (LF) or K2 Transfection System (600 counted cells per sample).

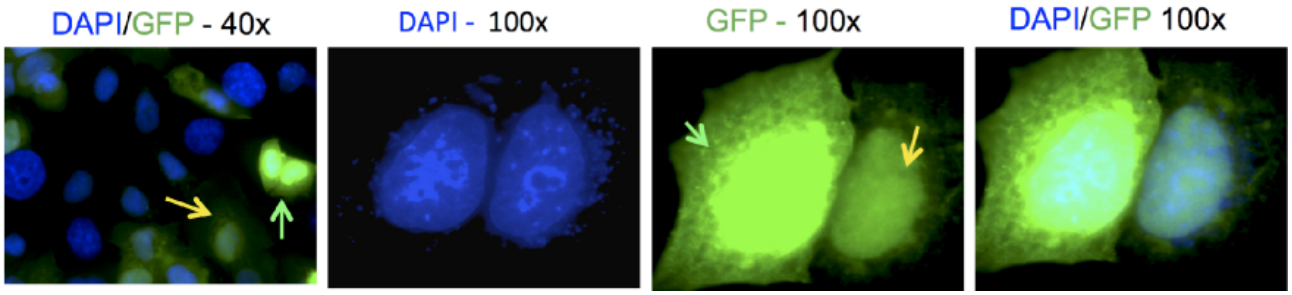

Figure 2. Fluorescence microscopy assays of pEGFP-transfected cultures using K2 reagent. The leftmost panel shows a field taken under a 40x objective; the 100x panels depict examples of low (yellow arrows) and highly expressing cells (green arrows).

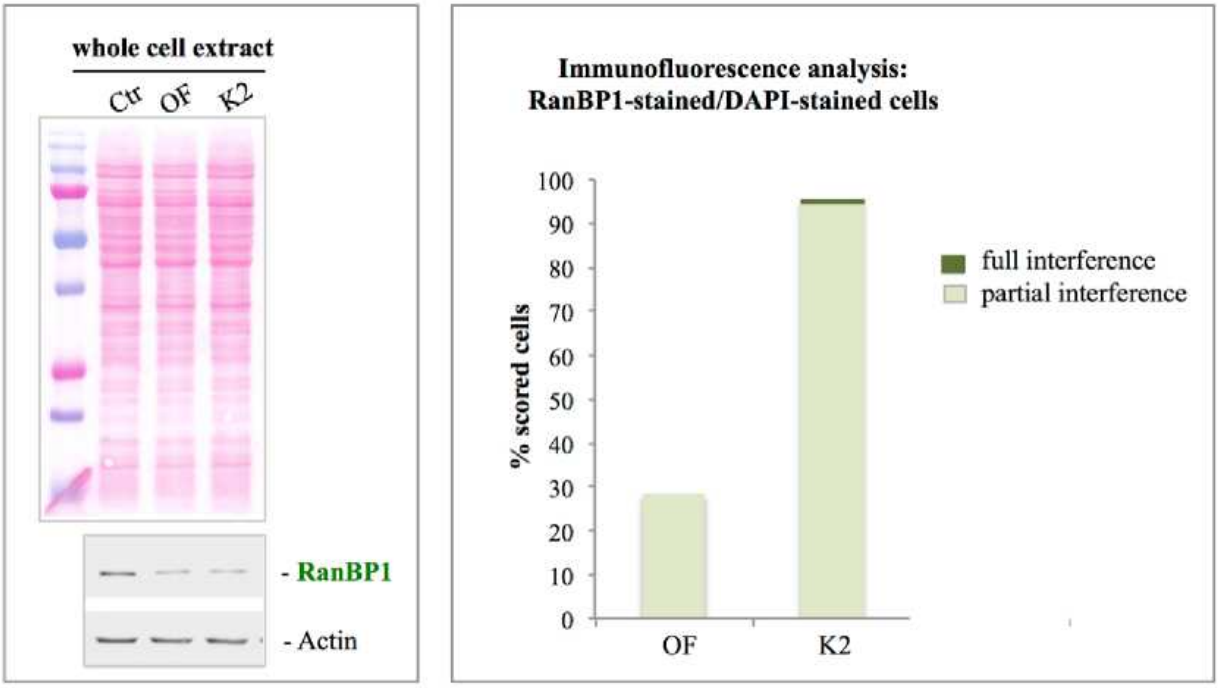

Figure 3. Quantitative analysis of the efficiency of transfection of RANBP1-specific siRNAs using K2 Transfection System or Oligofectamine (OF) by Western immunoblotting (left panel) of the residual RANBP1 protein compared to non-interfered control cultures (Ctr). The right panel shows the frequency of cells expressing faint (partial interference) or no (full interfrence) residual RANBP1 signals by immunoflurescence microscopy.

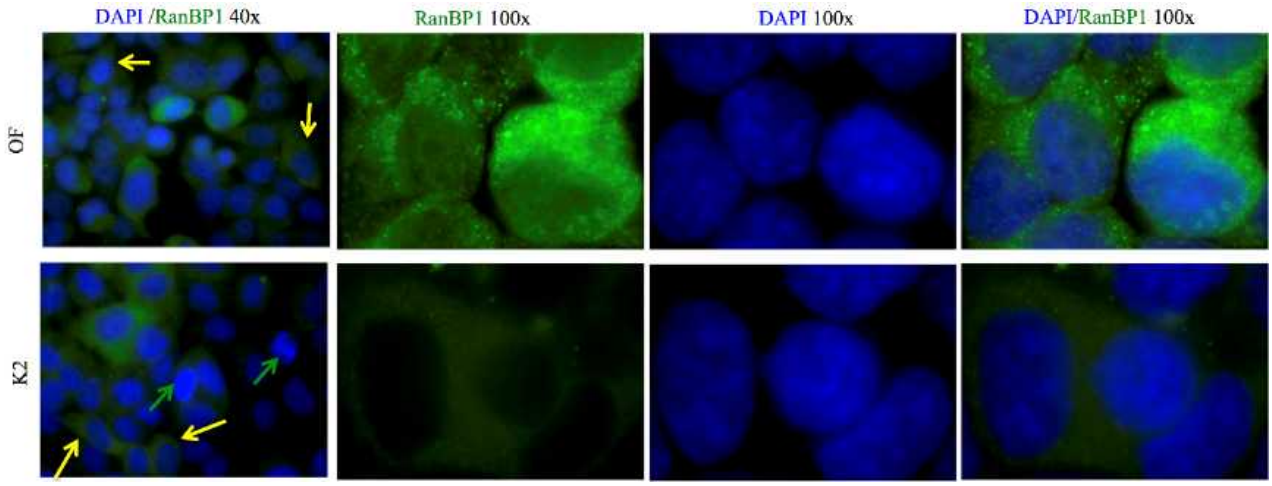

Figure 4. Examples of fields from cell cultures transfected with siRNAs targeting the RANBP1 protein using either K2 reagent or Oligofectamine (OF) after IF processing. Green and yellow arrows in the merge (DAPI + RANBP1) panels point respectively to fully interfered (no signal) and partially interfered cells (faint residual signal) for the RANBP1 protein target.

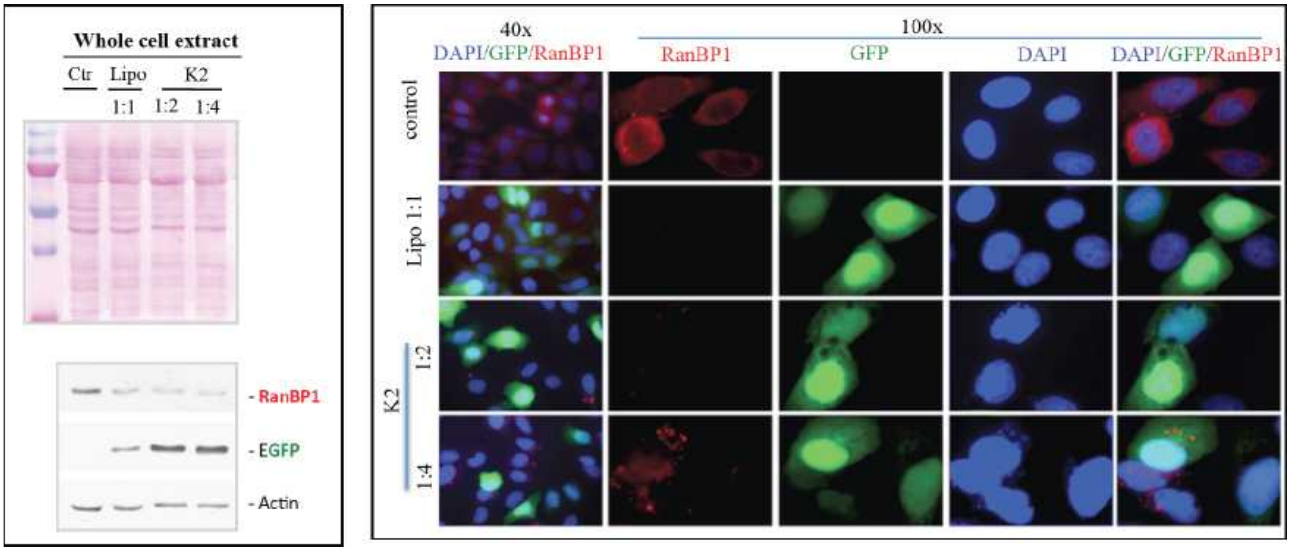

Figure 5. Evaluation of the efficiency of cotransfection of DNA and siRNAs using the K2 Transfection System or Lipofectamine (v/v ratios of nuclei acids to reagent are indicated). Left panel: Western immunoblotting shows the efficiency of K2 in co-transfection of DNA (revealed by EGFP product abundance) and RNA siRNAs (evidenced by RANBP1 protein decrease). Note that both the increase in EGFP, reflecting DNA transfection, and the decrease in RANBP1, reflecting siRNA transfection, are more effective in K2-transfected samples compared to Lipofectamine. Right panel: IF assays of transfected cultures.