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(57) **Abrégé/Abstract:**

The invention relates to a composition for inhibiting the expression of a target gene in a eukaryotic cell by RNA interference, the composition comprising a genetic construct and the target gene being introduced into the eukaryotic cell by means of said construct, and the construct having a first target gene and a first siRNA tag. The invention also relates to a composition for inhibiting the expression of one or more target genes in a eukaryotic cell by RNA interference. The composition comprises at least two genetic constructs and the target genes are introduced into the eukaryotic cell by means of said constructs, a) the first construct containing a first target gene and a first siRNA tag and b) the second construct containing a second target gene and a second siRNA tag, and the first and the second siRNA tag having different nucleic acid sequences. The invention finally relates to a method for inhibiting the expression of a target gene in a eukaryotic cell. Said method comprises the following steps: a) providing at least one eukaryotic cell, said cell being capable of RNA interference, b) transfecting the cells with a composition according to the invention and c) introducing at least one siRNA that is complementary to a siRNA tag of a transfected construct to inhibit the expression of the target gene that forms a transcription unit together with the siRNA tag.



Abstract

The invention relates to a composition for inhibiting the expression of a target gene in a eukaryotic cell by RNA interference, the composition comprising a genetic construct and the target gene being introduced into the eukaryotic cell
5 by means of said construct, and the construct having a first target gene and a first siRNA tag. The invention also relates to a composition for inhibiting the expression of one or more target genes in a eukaryotic cell by RNA interference. The composition comprises at least two genetic constructs and the target genes are introduced into the eukaryotic cell by means of said constructs, a) the first
10 construct containing a first target gene and a first siRNA tag and b) the second construct containing a second target gene and a second siRNA tag, and the first and the second siRNA tag having different nucleic acid sequences. The invention finally relates to a method for inhibiting the expression of a target gene in a eukaryotic cell. Said method comprises the following steps: a) providing at least
15 one eukaryotic cell, said cell being capable of RNA interference, b) transfecting the cells with a composition according to the invention and c) introducing at least one siRNA that is complementary to a siRNA tag of a transfected construct to inhibit the expression of the target gene that forms a transcription unit together with the siRNA tag.

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RNA interference tags

The present invention relates to a composition for inhibiting the expression of one or more target genes
5 in a eukaryotic cell by means of RNA interference, and to the use of appropriate compositions and to a method for inhibiting the expression of one or more target genes in a eukaryotic cell by means of RNA interference.

10

The phenomenon known from the literature as "RNA interference" (RNAi) is based on the fact that short RNA molecules (siRNA, small interfering RNA) can interact with messenger RNA (mRNA) in the cell
15 (Literature: Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C., Nature Feb 19, 1998; 391 (6669): 744-745). A complex mechanism, which is controlled by enzymes, results in breakdown (degradation) of the mRNA, thus inhibiting the
20 expression of the mRNA and therefore also protein expression (called gene silencing, i.e. switching off a gene or gene inactivation).

Besides siRNA, further small RNA species have been
25 discovered, for example the micro-RNAs (miRNA) or short hairpin RNAs (shRNA), which are likewise able to inhibit protein expression by related mechanisms.

WO 02/44321 discloses the introduction of siRNA into
30 cells in order to inhibit the expression of a gene which occurs naturally in the cells. WO 02/44321 further discloses the introduction of a foreign target gene into a cell. Expression of the introduced target gene can be inhibited by additional introduction of
35 siRNA, which is always derived from the sequence of the introduced target gene, in the expression.

It has emerged that not every sequence is equally suitable for the RNA interference mechanism. Some

criteria for the composition of an efficient siRNA have been identified. Reynolds et al. published a selected criteria in Nat. Biotechnol. Mar. 2004, 22(3): 326-330. Electronic publication took place on February 1, 2004
5 with the title "*Rational siRNA design for RNA interference*".

The conventional practice is to derive specific siRNAs individually for each gene to be inhibited from the
10 sequence thereof. Such a procedure is time-consuming and of low efficiency because the derived siRNAs frequently prove to be unable to inhibit gene expression satisfactorily only when tested. In addition, each individual siRNA sequence entails the
15 risk of having its own nonspecific gene regulation profiles, the so-called off-target effects.

Since the prior art discloses exclusively compositions and methods in which each siRNA is derived individually
20 and in an elaborate manner directly from the gene to be inhibited, the object of the present invention is to make it possible to inhibit simply, efficiently and universally the expression of target genes introduced into the cell by means of RNA interference ("RNAi").

25 The present invention is therefore to be seen in that the composition of the invention comprises a construct, and the target gene is introduced by means of this construct into a eukaryotic cell, where the construct
30 includes at least one first target gene and at least one associated so-called "siRNA tag". An siRNA tag is defined herein as a genetic element which forms a transcription unit with the target gene and is specifically recognized by a complementary siRNA. The
35 gene constructs containing the siRNA tag are thus subjected to RNA interference or another gene-inhibitory process. It is preferred according to the invention to use siRNA tags known to be able to bring

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about particularly potently an RNA interference in a particular cell and ideally cause minimal off-target effects.

The present invention allows in particular also simultaneous, but mutually independent, regulation of a plurality of introduced target genes by means of specifically designed siRNAs and their associated target sequences.

In accordance with one aspect of the present invention, there is provided a composition for inhibiting the expression of a target gene in a eukaryotic cell by means of RNA interference, wherein the composition comprises a genetic construct, and the target gene is introduced by means of this construct into the eukaryotic cell, wherein the construct includes a first target gene and a first siRNA tag, and wherein the first siRNA tag consists of the sequence SEQ ID NO: 1, the sequence SEQ ID NO: 2 or the sequence SEQ ID NO: 3.

In accordance with another aspect of the present invention, there is provided a composition for inhibiting the expression of one or more target genes in a eukaryotic cell by means of RNA interference, wherein the composition comprises at least two genetic constructs, and the target genes are introduced by means of these constructs into the eukaryotic cell, wherein a) the first construct comprises a first target gene and a first siRNA tag, and b) the second construct comprises a second target gene and a second siRNA tag, where the first and the second siRNA tags differ in nucleic acid sequence, and wherein the first and the second siRNA tags consist of the sequence SEQ ID NO: 1, the sequence SEQ ID NO: 2 or the sequence SEQ ID NO: 3.

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In accordance with yet another aspect of the present invention, there is provided a use of a composition as described herein for inhibiting the expression of at least one target gene in a eukaryotic cell.

- 5 In accordance with a further aspect of the present invention, there is provided a method for inhibiting the expression of a target gene in at least one eukaryotic cell, comprising: a) providing at least one eukaryotic cell capable of RNA interference, b) transfecting the eukaryotic cell with a
10 composition as described herein, and c) introducing into the eukaryotic cell at least one siRNA which is complementary to an siRNA tag of a transfected construct.

Further advantageous aspects, details and embodiments of the invention are described hereinbelow.

- 15 The composition of the invention is thus suitable for inhibiting the expression (e.g. the ectopic expression) of one or more target genes in a eukaryotic cell by means of RNA interference and also relates to the use of corresponding compositions, and a method for inhibiting the expression of one

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or more target genes in a eukaryotic cell by means of RNA interference.

Definitions

siRNA means in the context of the present invention all RNA
5 species capable of RNA interference, i.e. are able to bring
about inhibition of the expression of a transcript. The
inhibition normally takes place by sequence-specific
recognition of an siRNA tag by the siRNA. An siRNA preferably
has a 100% homology with the associated siRNA tag. However,
10 examples in which a lower degree of homology may be sufficient
for a certain inhibiting effect of an siRNAs are also known.
This phenomenon may also be based on translation

inhibition, as described also for the mode of action of the so-called micro-RNAs (miRNAs).

siRNA tags are sequences which are complementary to an
5 siRNA. They form a transcription unit with the target
gene and are located in the 5'-non-coding or in the 3'-
non-coding region of the transcript, and/or they
overlap with the coding region of the transcript. The
fact that the siRNA tags are independent sequences
10 means that they have the advantage of being universally
employable. An siRNA tag may also be a constituent of
an artificial or natural promoter/enhancer/silencer.
The gene silencing mediated in this way is known to
those skilled in the relevant art under the heading
15 "transcriptional inhibition".

A target gene means a nucleic acid sequence which codes
for a functional RNA (for example a tRNA, rRNA etc.) or
a protein, the expression or translation of which is to
20 be inhibited by means of siRNA through breakdown of the
transcript.

A composition means in the context of the invention an
aqueous solution comprising one or more different
25 recombinant nucleic acids. The solution preferably
comprises further constituents which make transfection
of the nucleic acid possible. Corresponding
compositions are sufficiently well known to the person
skilled in the art from the prior art.

30 Many biological questions cannot be meaningfully
answered by stating absolute values. It is often
considerably more informative for example to compare
the biological effects of an expression of genes
35 directly. Particularly suitable for this purpose is a
composition of the invention for inhibiting the
expression of a target gene or a plurality of target
genes in a eukaryotic cell by means of RNA

interference, which is characterized in that it comprises at least two genetic constructs, and the target genes are introduced by means of these constructs into the eukaryotic cell, where (a) the
5 first construct includes a first target gene and a first siRNA tag, and (b) the second construct includes a second target gene and a second siRNA tag, where the first and the second siRNA tag are different from one another.

10

It is possible through the use of two different siRNA tags to modulate the two gene expressions which are to be compared. For this purpose, two different target genes are employed on the two constructs used.

15

It is further advantageous to use the same target gene on two constructs in order in this way to obtain information on dose effects for the gene and for the siRNA.

20

A further possibility is to study two alleles of the same target gene in a cell by two constructs by means of the composition of the invention. The different siRNA tags make it possible to modulate the expression
25 of the two alleles individually and thus obtain information about the functions of the different alleles, e.g. dominant negative forms and wild-type variants or else various splice variants.

30 The composition of the invention is not confined to one or two constructs. It is possible to add a third construct, a fourth construct and any number of further constructs which, besides a target gene, always comprise an siRNA tag. This makes it possible to
35 investigate complex interactions of genes and, by introducing siRNAs, inhibit the expression of individual target genes or groups of target genes in a

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modulating way, in order thus to obtain pointers to the effect of the target genes or else the cellular genes.

The target genes can be fused to a marker gene for simple monitoring of the expression thereof. It is particularly advantageous to fuse different target genes to different marker genes, so that fusion proteins are expressed, making it possible to detect the individual target genes specifically. Suitable marker genes for fusion from the group of known marker genes are: (enhanced) green fluorescent protein and derivatives thereof ((E)GFP; YFP; CFP), also dsRed, Myc tag, E tag, FLAG tag, Glu-Glu tag, GST tag, HA tag, His tag, HSV tag, luciferase, MBP, protein C tag, S tag, T7 tag, V5 tag, VSV-g tag, avidin/streptavidin/strep tag, thioredoxin, His-patch thioredoxin, β -galactosidase, chloramphenicol acetyltransferase, cellulose binding domains (CBDs), chitin binding domain, staphylococcal protein A, streptococcal protein G, neo, hyg, pac, zeo, gpt, ble, dhfr, hpt and npt II. Further marker genes for fusion making detection of the target gene possible can likewise be used.

Marker genes from the group of fluorescence-generating proteins are preferably used, because detection thereof is not complicated. An advantage of this type of detection is that determination of the living cell is possible by non-invasive fluorescence measurement or fluorescence microscopy. It is particularly preferred for two distinguishable marker genes to code for different fluorescence-generating proteins which generate fluorescence differing in wavelength.

The composition of the invention allows studies on different eukaryotic cells. The cells may be for example of animal origin. It is moreover possible for the cells to remain in the animal *in vivo*, to be taken from an animal, to embody an animal unicellular

organism or else to be derived from a cell culture with animal cells. The animal cells may be for example cells derived from a mammal (called mammalian cells).

5 On the other hand, the cells may also be of vegetable origin. The cells may remain in a plant *in vivo*, be taken from a plant, embody a plant unicellular organism or else be derived from a cell culture with plant cells.

10

The cell may furthermore be a fungal cell. These cells of mycotic origin include all fungi capable of RNA interference. Yeasts for example are known as model organism from the prior art. It is known from the literature that, for example, *Saccharomyces spec.* is not capable of RNA interference. By contrast, it is known that, for example, the fission yeast *Schizosaccharomyces pombe* is amenable to RNA interference.

20

The sequences selected according to the invention as siRNA tag either are not themselves a constituent of the transcript to be inhibited, or overlap only partly therewith. It has surprisingly been found that the siRNA tag need not be a constituent of the coding sequence; on the contrary, it is sufficient for efficient RNA interference that the siRNA tag is merely in fact a constituent of the transcript, meaning that it forms a transcription unit with the target gene.

30

Sequences advantageously used as siRNA tags are those which do not occur naturally in the cell to be transfected. They may, of course, also be sequences which are not, or currently not yet, listed in the available sequence databases. A further possibility is also to use sequences which have no correspondence in nature, and these are therefore referred to as artificial (or synthetic) sequences.

35

It is possible and preferable to avoid an interaction of the siRNAs directed against siRNA tags with gene products of the target cell to be transfected by deriving the sequence of the siRNA tag and thus of the siRNA from nucleic acid sequence segments which do not themselves occur in the target cell to be transfected. It is preferred for this purpose in particular for the siRNA tags of the invention to be derived from the genome of prokaryotes or archae-bacteria. These nucleic acid sequences are particularly preferably derived from the genome of archae-bacteria, for example from the genera *Thermotoga* (e.g. *Thermotoga maritima*, etc.), *Methanosarcina* (e.g. *Methanosarcina mazei*, etc.), *Pyrococcus* (e.g. *P. furiosus*, *P. horikoshii*, *P. woesei*, etc.), *Halobacterium* (e.g. *H. cutirubrum*, *H. denitrificans*, *H. halobium*, etc.), *Methanobolus* (e.g. *M. bombayensis*, *M. oregonensis*, *M. sicilae*, etc.), *Sulfolobus* (e.g. *S. acidocaldarius*, *S. brierleyi*, *S. hakonensis*, etc.) or *Acidilobus* (e.g. *A. aceticus*). Further corresponding archae genera are known to a person skilled in the art.

The siRNA tags of the invention very particularly preferably have the sequences indicated in table 1 and shown in context once more in figure 2, although the use of siRNA tags of the invention is not confined thereto. Table 1 thus shows an overview of the sequences of the three siRNA tags used herein by way of example, and of the associated siRNAs in each case.

Table 1

Sequence name	siRNA sequence	siRNA tag sequence
Sequence 274	5'-GGGUAUCGACGAUUACAAAUU-3' 3'-GUCCCAUAGCUGCUAAUGUUU-5'	CAGGGTATCGACGATTACAAA [SEQ1]
Sequence 1248	5'-GCGUUGAAAUAGCGUACAAATT-3' 3'-dTTCGCAACUUUAUCGCAUGUU-5'	AAGCGTTGAAATAGCGTACAA [SEQ2]
Sequence 2904	5'-GGAUACGCGGGACGUUUAAUU-3' 3'-GACCUAUGCGCCUGCAAUUU-5'	CTGGATACGCGGGACGTTTAA [SEQ3]

The siRNA tag of the composition of the invention may be located either 3'-downstream or 5'-upstream of the coding region of the mRNA whose expression is to be inhibited. As in the case of the siRNA tag located 3'-
5 downstream and in the case of that located 5'-upstream it is possible for the siRNA tag to overlap with the sequence of the coding region. It is worthwhile for certain questions for the position of the siRNA tag to be located in an intron of the unspliced mRNA, for
10 example in order to investigate further the selective mechanisms of the RNA interference itself.

In order to facilitate purification and also detection of the protein encoded by the target gene, the target
15 gene is preferably fused to a peptide tag for protein purification. For example, the His tag is used for fusion to the target genes coding for proteins, because this tag is small and consists for example of 6 histidine amino acids. Purification via nickel-NTA
20 columns is possible without complications. These nickel-NTA columns are commercially available. The composition which comprises a tag for protein purification is compatible with fusion products of target gene and marker gene. Besides the His tag, it is
25 possible to use many other tags for protein purification, for example HA tags, ERK tags, GFP and related fusion tags, Myc tags, FLAG tags, GST tags, Strep tags, β -Gal tags, MBP tag and other tags are likewise suitable.

30 The cells are transfected according to the invention with a construct or a plurality of constructs. It is possible in this connection for each construct to be a constituent of its own vector, or two or more
35 constructs can be present on one vector. It is ensured thereby that the two or more constructs are introduced into the cell in a fixed stoichiometric ratio. Plasmids are particularly suitable vectors for the constructs.

It is also possible to employ transposons or viral vectors or other autonomous nucleic acids as vectors. In the simplest case, a vector is dispensed with, and the construct is part of an amplified nucleic acid, for example part of a PCR product.

The composition which comprises at least one construct is used according to the invention to make it possible to inhibit the expression of an introduced target gene in a cell. The cell is transfected with the composition for this purpose. At the same time, previously or subsequently, the cell is transfected with an siRNA which interacts with the siRNA tag of the transcript of the construct and brings about inhibition of expression of the target gene by means of RNA interference.

In an alternative embodiment, the eukaryotic cell into which the target gene is to be introduced may also be in the form of a lysate.

The method for inhibiting the expression of a target gene in at least one eukaryotic cell comprises the following steps according to the invention:

- (a) provision of at least one eukaryotic cell, where the cell is capable of RNA interference,
- (b) transfection of the at least one eukaryotic cell with at least one composition of the invention, and
- (c) introduction of at least one siRNA which is complementary to an siRNA tag of a transfected construct,

whereby expression of the target gene is inhibited.

Various possibilities are available for introducing the transcript of the target gene according to the invention into the cell. On the one hand, the cell can be transfected directly with a construct of the invention in the form of an RNA, for example of an

mRNA, itself. On the other hand, the cell can also be transfected with a nucleic acid amplification product, for example PCR product, or nucleic acid fragment, in which case these have a promoter which brings about transcription of the mRNA of the target gene in the cell. The cell is preferably transfected with a vector, however, in which case the vector comprises a promoter which is active in the cell and brings about transcription of the target gene.

10

If the cell is transfected with a PCR product or a vector which comprise a promoter, in one embodiment the promoter is under the control of an effector. The effector-controlled promoter makes further control of expression possible.

15

The sequence of the siRNA tag employed according to the invention preferably comprises a sequence which is not known from eukaryotes. siRNA tags of the invention can therefore be derived from prokaryotes or represent artificial sequences which have no correspondence with known sequences from databases. The fact that the siRNA tags are not a constituent of the sequence of the target genes means that they are employable universally. A significant advantage is that the siRNA tags can be cloned at the level of DNA, in front of or behind each sequence coding for an mRNA or other functional sequence such as, for example, tRNA, rRNA, etc. Each siRNA tag thus represents a cassette which may be for example a component of a cloning vector.

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A further advantage of the invention is that it is possible to accumulate experience about which siRNA tags bring about RNA interference particularly potently in which cells. Precisely these siRNA tags can then be employed repeatedly for different target genes.

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In the drawing:

figures 1A and 1B show an exemplary overview of the function and use of an siRNA tag of the invention;

5

figures 2A and 2B show exemplary DNA cassettes comprising siRNA tag sequences and suitable for cloning into a plasmid;

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figures 3A to 3C show exemplary immunofluorescence images of pEGFP constructs employed with siRNA tag, in each case without and with specific siRNA; and

15

figure 4 shows an example of a selective gene silencing by cotransfection of two expression plasmids comprising constructs of the invention (here with sequences coding for the fluorescent proteins EGFP and DsRed) with different siRNA tags and the respective siRNAs.

20

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Fig. 1A shows a fundamental scheme of the principle by which an siRNA leads, via a complementary siRNA tag fused to the target gene ("gene of interest"), to a reduction in the expression of the complete construct.

30

Fig. 1B represents by way of example the detailed location of an siRNA tag in the 3' region of a gene construct coding for a protein. In this case, the siRNA tag was introduced between translation STOP codon signals and a poly-A signal in the 3'-untranslated region (3'UTR). An siRNA tag can in this case be a component of a larger DNA construct which comprises further functional genetic elements (protein tag for purification or detection of a protein, STOP codon

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sequences, poly-A signal sequences, etc.) and is cloned as transcription unit into the construct.

Three different DNA cassettes which, besides the siRNA tag sequence, also comprise further properties for cloning and subsequent expression of the recombinant products were designed in each case specifically for the three siRNAs validated by way of example (see fig. 2). These DNA cassettes have cohesive ends for ligation into the intended vectors. A sequence coding for a His tag is present in the DNA cassettes in fig. 2A for later detection of fusion proteins. By contrast, introduction of a His tag was dispensed with in the DNA cassettes depicted in fig. 2B. The triple stop signal, and a polyadenylation signal, supplement the properties of the DNA cassette which are necessary for correct expression of the later gene construct.

Fig. 3 shows siRNA tag-mediated gene silencing of pEGFP constructs (plasmids coding for EGFP). HeLa S3 cells transfected with pEGFP constructs which comprise either the siRNA tag sequence No. 247, 1248 or 2904 (SEQ ID 1, 2 or 3, see table 1), with or without the respectively complementary siRNAs, were used. Fig. 3A and fig. 3B are on the one hand fluorescence and transillumination photographs of the same section excited with light of the stated wavelength, and on the other hand phase contrast images for control purposes. The indicated clones are without His tag (fig. 3A) or with His tag (fig. 3B). Fig. 3C shows the immunological detection of the expression of constructs with His tag by means of detection of the His tag from lysates of transfected cells. When there is simultaneous cotransfection of the complementary siRNAs, gene silencing of the fusion proteins takes place. Cells transfected with pEGFP vector, and nontransfected cells, were used as controls.

Fig. 4 finally shows selective gene silencing by cotransfection of two expression plasmids with different siRNA tags and the respective siRNAs. Fluorescence and transillumination photographs of HeLa
5 cells transfected with pDsRed 274, pEGFP 1248 and siRNAs of sequences 274 and 1248 (SEQ ID 1 and 2, see table 1). The images represent identical sections which had been excited with light of the wavelength indicated in each case.

10

The present invention describes an approach in which tag-specific siRNA molecules are employed for the specific gene inactivation (gene silencing) of transcripts without the need to design and prepare for
15 an individual transcript, e.g. an mRNA or another functional RNA such as, for example, a tRNA, rRNA, etc., an siRNA specifically aimed thereat. The principle underlying this approach can be described as follows.

20

The DNA fragment coding for the functional RNA to be investigated can be cloned in a eukaryotic expression plasmid in order for example to make recombinant expression of an encoded protein possible or to
25 discover the effects of a functional RNA. Fusion of a double-stranded DNA which comprises an siRNA tag for a specific siRNA in the reading frame of the functional RNA to be investigated makes gene inactivation of the functional RNA possible through cotransfection of this
30 modified vector together with the specific siRNA. The double-stranded DNA molecule may, besides the recognition site for the siRNA (siRNA tag), include further specific properties such as, for example, coding sequences for short protein tags (e.g. His tag,
35 Strep tag), STOP codons, polyadenylation signals or overhangs generated by restriction enzymes for direct cloning of the double-stranded molecule. This is depicted diagrammatically in figure 1.

The siRNA tags to be incorporated into the genetic construct are preferably derived from prokaryotic, particularly preferably from archae-bacterial gene sequences, which have a minimal, preferably no, 5
homology with eukaryotic gene sequences. This minimizes the risk of the siRNA directed against the siRNA tag causing nonspecific effects. Prokaryotic gene sequences have proved to be particularly suitable for the siRNA
10 tags of the invention, especially sequences from archae, for example from the genera *Thermotoga* (e.g. *Thermotoga maritima*, etc.), *Methanosarcina* (e.g. *Methanosarcina mazei*, etc.), *Pyrococcus* (e.g. *P. furiosus*, *P. horikoshii*, *P. woesei*, etc.),
15 *Halobacterium* (e.g. *H. cutirubrum*, *H. denitrificans*, *H. halobium*, etc.), *Methanlobus* (e.g. *M. bombayensis*, *M. oregonensis*, *M. siciliae*, etc.), *Sulfolobus* (e.g. *S. acidocaldarius*, *S. brierleyi*, *S. hakonensis*, etc.) or *Acidilobus* (e.g. *A. aceticus*). Further corresponding
20 archae genera are known to a person skilled in the art. There are various bioinformatics methods for checking whether a particular prokaryotic sequence has homologies with a previously known eukaryotic sequence, such as, for example, a modified Smith-Waterman
25 algorithm, which was employed in the present case.

For the experiments in the context of the present invention, three siRNAs were selected by way of example, which on the one hand offered a very high
30 probability of arriving at the desired results, and which on the other hand showed according to the Smith-Waterman algorithm the smallest agreement with known eukaryotic genes. The function of these siRNAs was investigated by fusing synthetic, double-stranded (ds)
35 DNA molecules which possess an appropriately complementary siRNA binding site (siRNA tag) to the 3' end of the EGFP expression cassette of an EGFP reporter plasmid (pEGFP-C1).

Three different DNA cassettes which, besides the siRNA tag sequence, also include further properties for cloning and subsequent expression of the recombinant products (see figure 2) were designed for each of the three siRNA molecules validated by way of example in the context of the present invention (see table 1). These DNA cassettes have cohesive ends for ligation into the intended vectors. A sequence coding for a His tag is present for later detection of the fusion proteins for the DNA cassettes in figure 2A. On the other hand, no His tag was introduced in the DNA cassettes depicted in figure 2B. The triple STOP codon, and a polyadenylation signal supplement the properties of the DNA cassette which are necessary for correct expression of the later gene construct.

The DNA cassettes shown (see figure 2) were then cloned into the pEGFP-C1 expression plasmid which had been linearized using the restriction enzymes EcoRI and BamHI.

Recombinant clones were characterized by sequencing both the insert itself and the vector in the region of the boundaries of the insert.

After the molecular characterization of the recombinant pEGFP plasmid which expresses the RNAs with the siRNA tags, these constructs can be used for functional analysis of the appropriate siRNAs.

In a first experiment, all the constructs were used in transient transfection experiments in order to analyze the EGFP expression of each clone as indicator of the correct transcription and translation of the modified expression cassette. In order to check this HeLa S3 cells were transfected with the individual recombinant plasmids pEGFP-274 (coding for EGFP-siRNA tag

SEQ ID 1), pEGFP-1248 (coding for EGFP-siRNA tag
SEQ ID 2), and pEGFP-2904 (coding for EGFP-siRNA tag
SEQ ID 3), and the EGFP expression was determined by
fluorescence microscopy 48 hours after the
5 transfection. Cotransfection of the DNA constructs
together with the appropriate siRNA directed against
the respective siRNA tag made functional
characterization thereof possible. 48 hours after the
cotransfection, the cells transfected with the DNA
10 constructs and the specific siRNA showed a significant
reduction in EGFP expression compared with the cells
transfected with the plasmid alone (see figure 3a).

In the case of the genetic constructs which code for a
15 C-terminal His tag fusion, correct expression of the
EGFP-His fusion protein was investigated by Western
blotting. HeLa S3 cells were transfected with the
plasmids pEGFP-274-hs (coding for EGFP-His tag-siRNA
tag SEQ ID 1), pEGFP-1248-hs (coding for EGFP-His tag-
20 siRNA tag SEQ ID 2), pEGFP-2904-hs (coding for EGFP-His
tag-siRNA tag SEQ ID 3) or pEGFP-C1 (initial plasmid
without insert). Cotransfection of the DNA constructs
together with the appropriate siRNA directed against
the respective siRNA tag also made functional
25 characterization thereof possible in this case.
48 hours after the cotransfection, the cells
transfected with the DNA constructs and the specific
siRNA showed a significant reduction in EGFP expression
compared with the cells transfected with the plasmid
30 alone (see figure 3b). In a further part of the
experiment, the cells were lysed 48 hours after the
transfection, and the proteins were extracted and
investigated by Western blotting using Penta-His
monoclonal antibodies specific for the His tag to find
35 whether they express the His tag. The EGFP-His fusion
protein (with a molecular weight of about 28 kDa) was
detectable only in cells transfected with recombinant
EGFP constructs, whereas the fusion protein did not

occur in nontransfected cells and in pEGFP-C1-transfected cells. Cotransfections of the individual pEGFP constructs with the siRNA directed against the respective siRNA tag led in each case to an efficient
5 gene silencing effect (concerning this, see the Western blot shown in figure 3C).

One of the most important advantages of the system of the invention which employs tag-specific siRNA is the
10 possibility of being able to use two or more siRNA tag-expressing constructs which are located on one or more vectors, for example plasmids, together with the appropriately functionally validated siRNAs. This system makes it possible for a plurality of recombinant
15 genes to be coexpressed, and for each of these components to be switched off specifically by cotransfection with the appropriate siRNA.

An important experiment used to investigate the
20 cotransfection of two siRNA tag-reporter constructs together with the respective tag-specific siRNAs directed at the expressed gene is depicted by way of example in figure 4. In order to investigate the gene silencing of one or simultaneously both plasmid
25 constructs in parallel-transfected cells, HeLa cells were cotransfected with the plasmids pDsRed 274 (codes for DsRed-siRNA tag SEQ ID 1) and pEGFP 1248 (codes for EGFP-siRNA tag SEQ ID 2). In separate approaches, either a nonspecific control siRNA or one of the
30 respectively specific tag-siRNAs (sequence 274, specific for siRNA tag SEQ ID 1, or sequence 1248, specific for siRNA tag SEQ ID 2) were transfected in addition with these two plasmids. In a further approach, both specific siRNAs were present together
35 with the plasmids in the transfection mixture. It was possible by these experiments to demonstrate the specificity of the siRNA employed for the respective siRNA tag, which represents the basis for differential

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expression control in experiments. The cotransfections were analyzed with a fluorescence microscope after incubation for 48 hours.

5 As shown in figure 4, the reporter genes of both plasmid constructs are expressed in a cell despite cotransfection of a non-functional control siRNA (column A). The HeLa cells transfected with both plasmids show, even with simultaneous transfection of a
10 control siRNA, parallel expression of the fluorescent proteins. On selective inhibition of DsRed expression by the siRNA with sequence 274 (specific for the siRNA tag SEQ ID 1, see table 1), a large decrease in the fluorescence signal is evident; expression of the EGFP
15 protein is meanwhile not impaired (column B). Conversely, selective inhibition of the EGFP protein by the siRNA with sequence 1248 (specific for the siRNA tag SEQ ID 2, see table 1) is shown, without a reduction in the DsRed signal in this case (column C).
20 Finally cotransfection of both plasmids with the respective specific siRNAs inhibits the expression of both reporter genes so strongly that virtually no fluorescence signals are now emitted by the cells (column D).

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 52362-22 SEQ 11-SEP-09 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

<110> Bielke, Wolfgang
Hahn, Peter

19a

<120> RNA Interference Tags

<130> QIA-18895

<140> Not yet assigned

<150> PCT/EP2007/053459

<151> 2007-04-10

<160> 6

<170> PatentIn version 3.3

<210> 1

<211> 21

<212> DNA

<213> Artificial

<220>

<223> siRNA-Tag Sequence

<400> 1

cagggtatcg acgattacaa a

21

<210> 2

<211> 21

<212> DNA

<213> Artificial

<220>

<223> siRNA-Tag Sequence

<400> 2

aagcgttgaa atagcgtaca a

21

<210> 3

<211> 21

<212> DNA

<213> Artificial

<220>

<223> siRNA-Tag Sequence

<400> 3

ctggatacgc gggacgttta a

21

<210> 4

<211> 19

<212> RNA

<213> Artificial

<220>

<223> siRNA sequence with a UU overhang at the 3'-end of the sense strand and a GU-overhang at 3'-end of the anti-sense strand

19b

<400> 4
ggguaucgac gauuacaaa

19

<210> 5
<211> 19
<212> RNA
<213> Artificial

<220>
<223> siRNA sequence with a dTT- overhang at the 3'-end of the sense
strand and the 3'-end of the anti-sense strand

<400> 5
gcguugaaau agcguacaa

19

<210> 6
<211> 19
<212> RNA
<213> Artificial

<220>
<223> siRNA sequence with a UU overhang at the 3'-end of the sense
strand and a GA-overhang at 3'-end of the anti-sense strand

<400> 6
ggauacgcgg gacguuuaa

19

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CLAIMS:

1. A composition for inhibiting the expression of a target gene in a eukaryotic cell by means of RNA interference, wherein the composition comprises a genetic construct, and the target gene is introduced by means of this construct into the eukaryotic cell, wherein the construct includes a first target gene and a first siRNA tag, and

wherein the first siRNA tag consists of the sequence SEQ ID NO: 1, the sequence SEQ ID NO: 2 or the sequence SEQ ID NO: 3.

2. A composition for inhibiting the expression of one or more target genes in a eukaryotic cell by means of RNA interference, wherein the composition comprises at least two genetic constructs, and the target genes are introduced by means of these constructs into the eukaryotic cell, wherein

a) the first construct comprises a first target gene and a first siRNA tag, and

b) the second construct comprises a second target gene and a second siRNA tag,

where the first and the second siRNA tags differ in nucleic acid sequence, and

wherein the first and the second siRNA tags consist of the sequence SEQ ID NO: 1, the sequence SEQ ID NO: 2 or the sequence SEQ ID NO: 3.

3. The composition as claimed in claim 2, wherein the first target gene and the second target gene differ in nucleic acid sequence.

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4. The composition as claimed in either of claims 2 and 3, wherein the first target gene represents a first allele and the second target gene represents a second allele of the same gene.

5 5. The composition as claimed in claim 2, wherein the first target gene and the second target gene are identical in nucleic acid sequence.

6. The composition as claimed in any one of claims 2 to 5, wherein the composition comprises a third construct,
10 where the third construct includes a third target gene and a third siRNA tag, wherein the sequence of the third siRNA tag differs from the sequences of the first and the second siRNA tag, and

wherein the third siRNA tag consists of the sequence
15 SEQ ID NO: 1, the sequence SEQ ID NO: 2 or the sequence SEQ ID NO: 3.

7. The composition as claimed in claim 6, wherein the composition comprises a fourth construct which includes a fourth target gene and a fourth siRNA tag, wherein the sequence
20 of the fourth siRNA tag differs from the sequences of the first, second and third siRNA tag.

8. The composition as claimed in any one of claims 1 to 7, wherein at least one of the target genes is fused to a marker gene, thereby providing a fused gene which makes it
25 possible to detect the expression of a fusion protein expressed by the fused gene.

9. The composition as claimed in any one of claims 2 to 8, wherein at least two of the target genes are fused to a

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marker gene which makes it possible to detect the expression of the fusion protein expressed by the fused gene.

10. The composition as claimed in claim 8 or 9, wherein the marker gene is selected from the group of marker genes
5 consisting of green fluorescent protein and derivatives thereof, dsRed, Myc tag, E tag, FLAG tag, Glu-Glu tag, GST tag, HA tag, His tag, HSV tag, luciferase, MBP, protein C tag, S tag, T7 tag, V5 tag, VSV-g tag, avidin/streptavidin/strep tag, thioredoxin, His-patch thioredoxin, β -galactosidase,
10 chloramphenicol acetyltransferase, cellulose binding domains (CBDs), chitin binding domain, staphylococcal protein A, streptococcal protein G, neo, hyg, pac, zeo, gpt, ble, dhfr, hpt and npt II.

11. The composition of claim 10, wherein the derivatives
15 of green fluorescent protein are GFP, EGFP, YFP and CFP.

12. The composition as claimed in any one of claims 8 to 11, wherein the first marker gene and the second marker gene code for fluorescence-generating proteins.

13. The composition as claimed in any one of claims 1
20 to 12, wherein the eukaryotic cell is of animal origin.

14. The composition as claimed in claim 13, wherein the eukaryotic cell is a mammalian cell.

15. The composition as claimed in any one of claims 1 to 12, wherein the eukaryotic cell is of vegetable origin.

25 16. The composition as claimed in any one of claims 1 to 12, wherein the eukaryotic cell is of mycotic origin.

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17. The composition as claimed in claim 16, wherein the eukaryotic cell is a yeast cell capable of RNA interference.

18. The composition as claimed in any one of claims 1 to 17, wherein the eukaryotic cell is in the form of a lysate.

5 19. The composition as claimed in any one of claims 1 to 18, wherein the at least one siRNA tag is derived from a sequence which is not a constituent of the sequence of the target gene.

10 20. The composition as claimed in any one of claims 1 to 19, wherein the siRNA tag is derived from an artificial nucleic acid sequence.

21. The composition as claimed in any one of claims 1-20, wherein the siRNA tag is derived from a nucleic acid sequence not occurring in the target cell.

15 22. The composition as claimed in any one of claims 1-21, wherein the at least one siRNA tag is derived from the nucleic acid sequence of organisms not capable of RNA interference.

20 23. The composition as claimed in any one of claims 1 to 22, wherein siRNA tag is a constituent of a promoter, enhancer or silencer.

25 24. The composition as claimed in any one of claims 1 to 23, wherein the at least one siRNA tag is located downstream or upstream of the target gene or overlaps with the target gene, and wherein the siRNA tag forms a coherent transcription unit with the target gene.

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25. The composition as claimed in any one of claims 1 to 24, wherein siRNA tag is located in an intron of the target gene or overlaps with an intron.

26. The composition as claimed in any one of claims 1 to 25, wherein at least one construct includes a tag fused to the target gene for protein purification.

27. The composition as claimed in claim 26, wherein the protein tag for protein purification is selected from the group consisting of His tags, HA tags, ERK tags, GFP and related fusion tags, Myc tags, FLAG tags, GST tags, Strep tags, β -Gal tags and MBP tag.

28. The composition as claimed in any one of claims 1 to 27, wherein the at least one construct is located in a vector.

29. The composition as claimed in any of claims 2 to 27, wherein two of the constructs are located in two different vectors.

30. The composition as claimed in any one of claims 2 to 27, wherein at least two of the constructs are located in one vector.

31. The composition as claimed in any one of claims 28 to 29, wherein at least one vector is a plasmid.

32. The composition as claimed in any one of claims 28 to 30, wherein at least one vector is a transposon.

33. The composition as claimed in any one of claims 28 to 30, wherein at least one vector is a virus.

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34. The composition as claimed in any one of claims 1 to 27, wherein the at least one construct is a constituent of a PCR product.

35. A use of the composition as claimed in any one of
5 claims 1 to 33 for inhibiting the expression of at least one target gene in a eukaryotic cell.

36. An *in vitro* method for inhibiting the expression of a target gene in at least one eukaryotic cell, comprising:

10 a) providing at least one eukaryotic cell capable of RNA interference,

b) transfecting the eukaryotic cell with a composition as claimed in any one of claims 1 to 34, and

15 c) introducing into the eukaryotic cell at least one siRNA which is complementary to an siRNA tag of the transfected construct, wherein the siRNA binds to the siRNA tag, thereby inhibiting the expression of the target gene.

37. The method as claimed in claim 36, wherein the eukaryotic cell is in the form of a lysate.

20 38. The method as claimed in claim 36 or 37, wherein the siRNA is introduced into the cell in the form of a nucleic acid encoding the siRNA, and wherein synthesis of the siRNA is under the control of a promoter.

39. The method as claimed in claim 38, wherein the promoter is under the control of an effector.

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40. The method as claimed in claim 39, wherein the effector is transiently applied in order to bring about time-controlled synthesis of the siRNA.

41. The method as claimed in any one of claims 36 to 40,
5 wherein the siRNA comprises the complement of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

42. The method as claimed in any one of claims 36 to 41, wherein the inhibition of the target genes is controlled by sequential introduction of siRNA.

10 43. The method as claimed in claim 42, wherein the use of the composition is for use in transient generation of different expression patterns in a cell or plurality of cells.

44. The method as claimed in any one of claims 36 to 43,
15 wherein the expression of at least one of the target genes is reduced by at least 40%.

45. The method as claimed in claim 44, wherein the expression of at least one of the target genes is reduced by at least 70%.

46. The method as claimed in claim 45, wherein the
20 expression of at least one of the target genes is reduced by at least 90%.

47. The method as claimed in any one of claims 36 to 46, wherein the degree of inhibition of at least one of the target genes is determined by means of quantifying the signal of a
25 fused marker gene.

48. The method as claimed in any one of claims 36 to 47, wherein the transfection of the cell takes place transiently.

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49. The method as claimed in any one of claims 36 to 48, wherein the cell is stably transfected.

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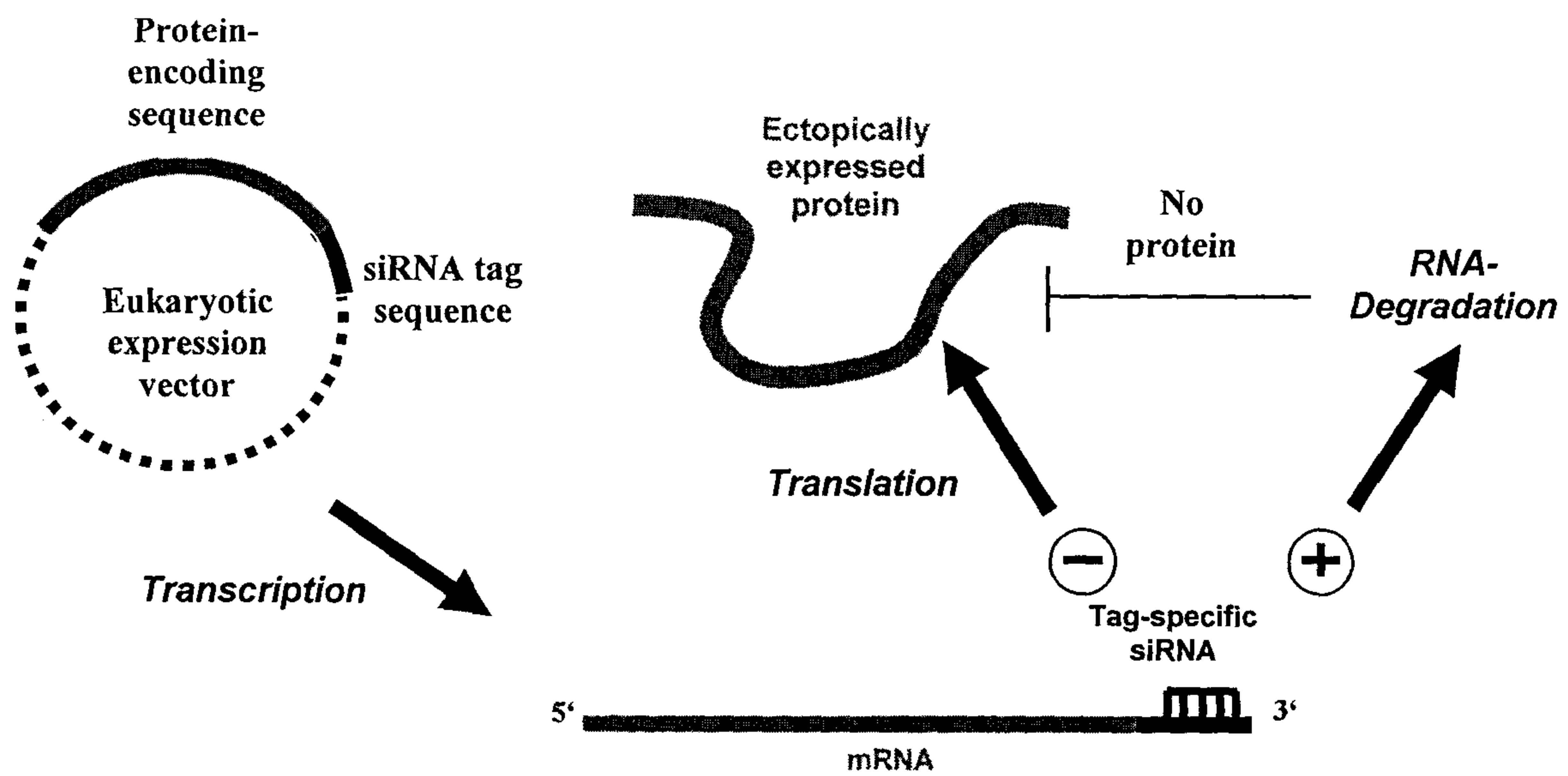


FIG. 1A

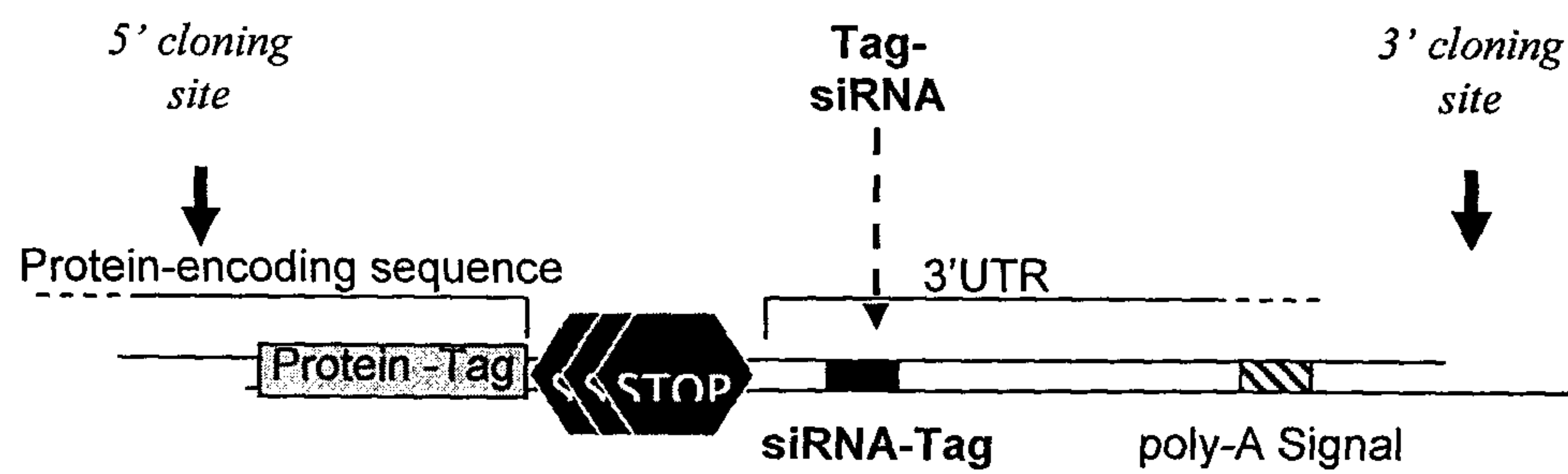


FIG. 1B

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5' cloning site		(His) Protein tag	Stop-Codons	siRNA-Tags	Poly-A signal	3' cloning site
Oligo: 274-6						
274-hs fwd: 5'	AATTCCCACCACCATCACCATCACCATCACC	ATAGTAATGACAGGGTATCGACGATTACAAATAATAAAG	3'			
274-hs rev: 3'	GGGTGGTGGTAGTGGTAGTGGTAGTGATCATTACT	GTCCCATAGCTGCTAATGTTTATTATTTCCTAG	5'			
Oligo: 1248-6						
1248-hs fwd 5'	AATTCCCACCACCATCACCATCACCATCACC	ATAGTAATGAAGCGTTGAAATAGCGTACAAATAATAAAG	3'			
1248-hs rev 3'	GGGTGGTGGTAGTGGTAGTGGTAGTGATCATTACT	TTCGCAACTTTATCGCATGTTATTATTTCCTAG	5'			
Oligo: 2904-6						
2904-hs fwd 5'	AATTCCCACCACCATCACCATCACCATCACC	ATAGTAATGACTGGATACGCGGGACGTTTAAATAATAAAG	3'			
2904-hs rev 3'	GGGTGGTGGTAGTGGTAGTGGTAGTGATCATTACT	GACCTATGCGCCCTGCAAATTATTATTTCCTAG	5'			

FIG. 2A

5'cloning site		Stop Codons	siRNA tags	poly A signal	3'cloning site
Oligo: 274-4					
274 fwd: 5'	AATTCCCTAGTAATGACAGGGTATCGACGATTACAAATAATAAAG	3'			
274 rev: 3'	GGATCATTACTGTCCCATAGCTGCTAATGTTTATTATTTCCTAG	5'			
Oligo: 1248-4					
1248 fwd 5'	AATTCCCTAGTAATGAAGCGTTGAAATAGCGTACAAATAATAAAG	3'			
1248 rev 3'	GGATCATTACTTTCGCAACTTTATCGCATGTTATTATTTCCTAG	5'			
Oligo: 2904-4					
2904 fwd 5'	AATTCCCTAGTAATGACTGGATACGCGGGACGTTTAAATAATAAAG	3'			
2904 rev 3'	GGATCATTACTGACCTATGCGCCCTGCAAATTATTATTTCCTAG	5'			

FIG. 2B

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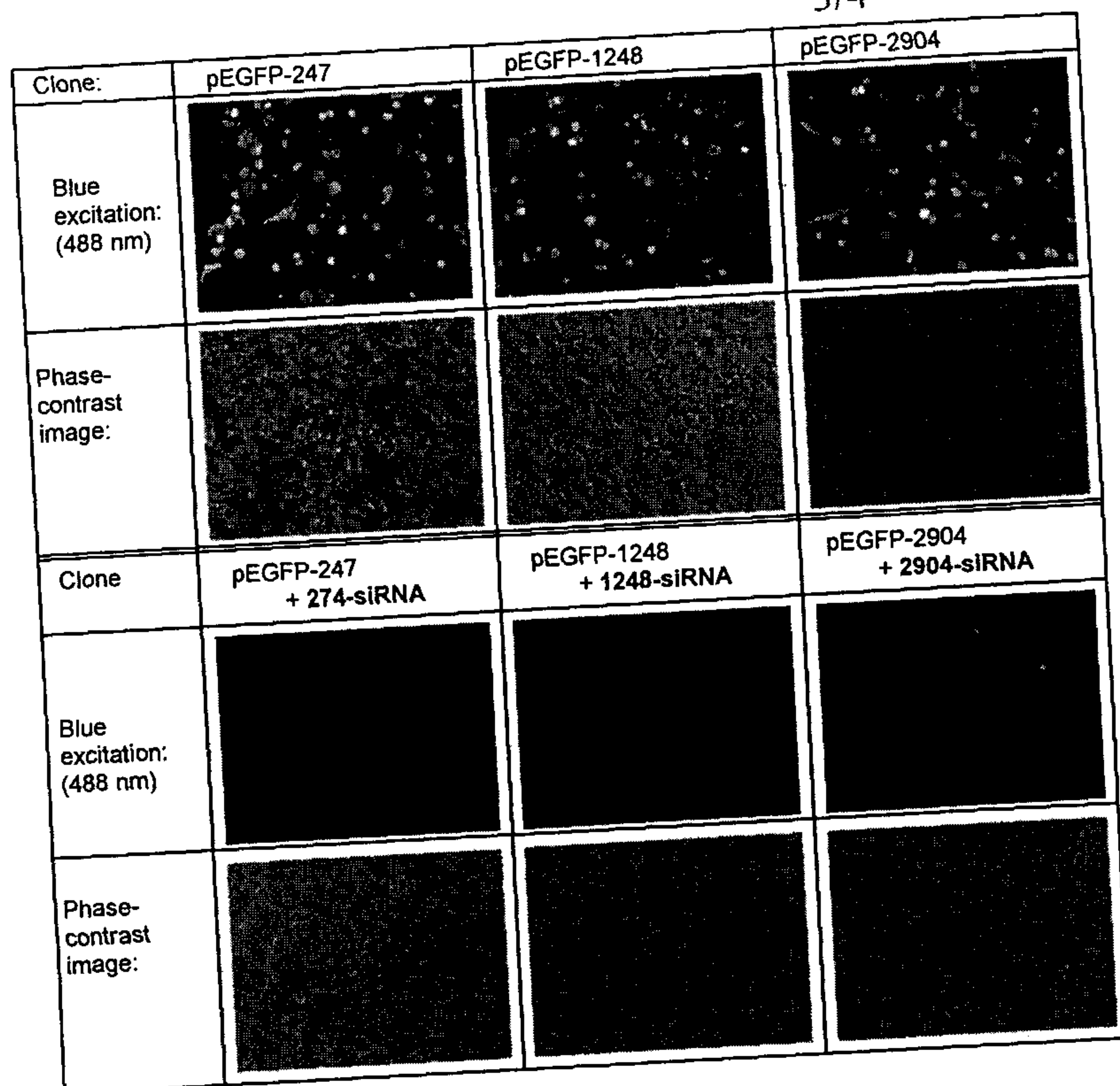


FIG. 3A

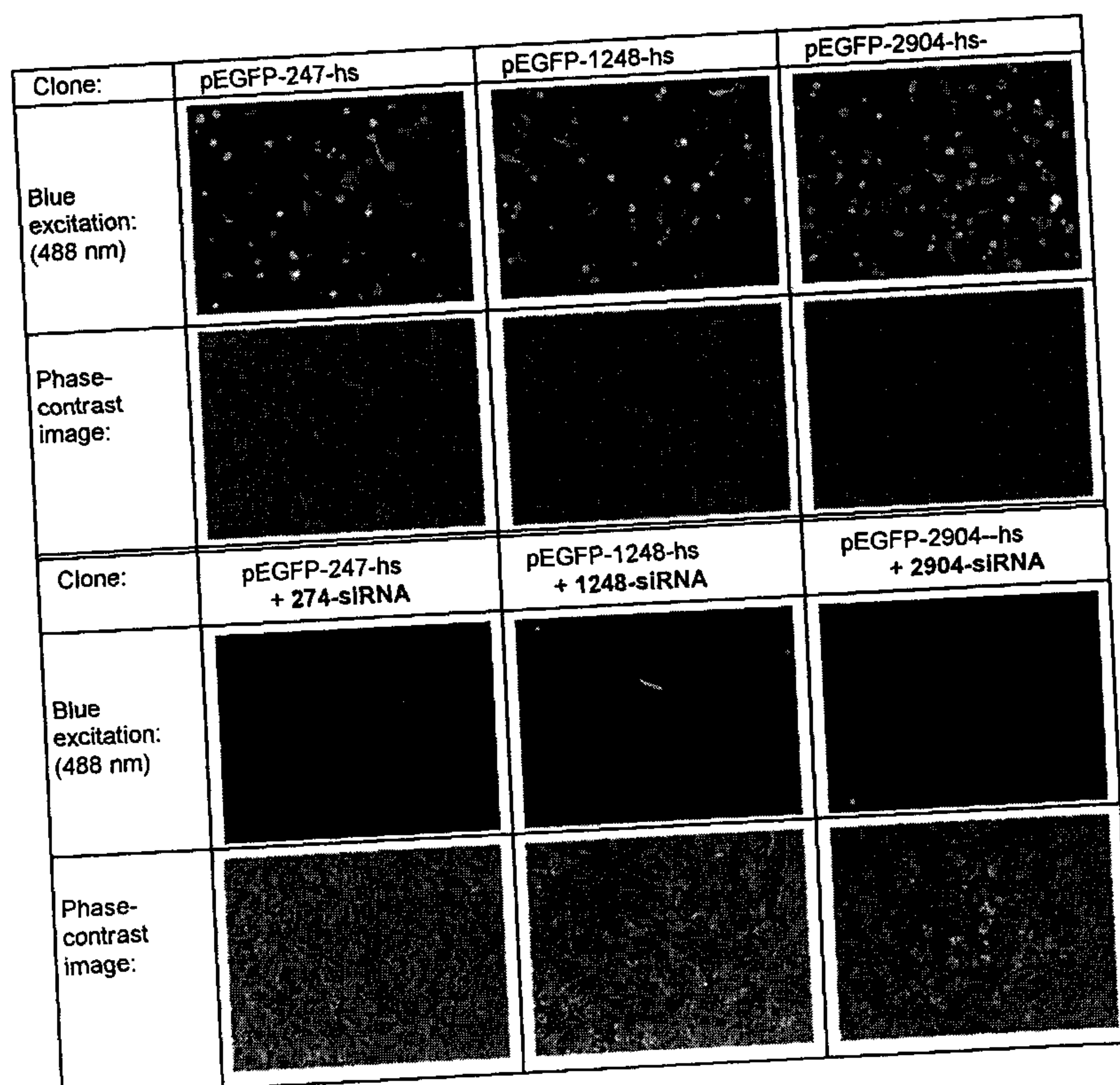


FIG. 3B

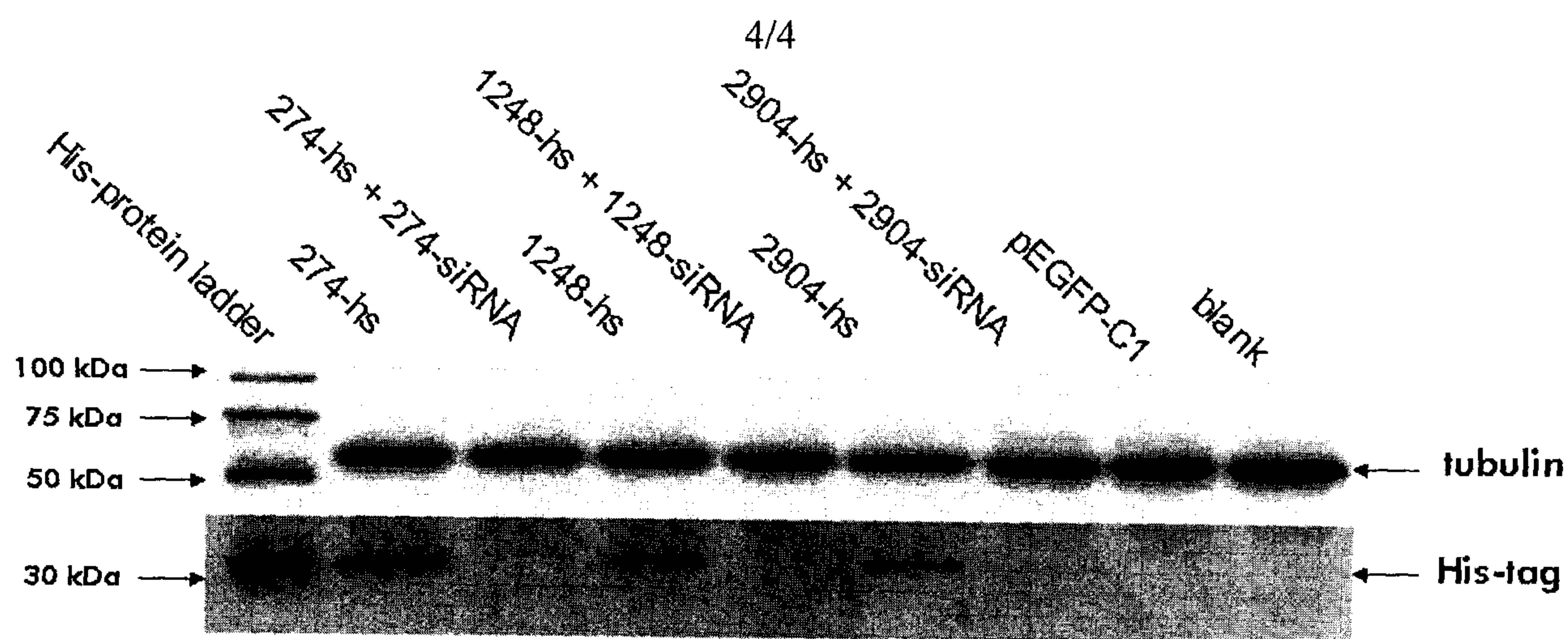


FIG. 3C

	Column A	Column B	Column C	Column D
	pEGFP 1248 + pDsRed 274 + control-siRNA	pEGFP 1248 + pDsRed 274 + 274-siRNA	pEGFP 1248 + pDsRed 274 + 1248-siRNA	pEGFP 1248 + pDsRed 274 + 274-siRNA +. 1248 siRNA
Blue excitation: (488 nm)				
Green excitation: (558 nm)				
Super- imposed fluorescences				
Phase- contrast image:				

FIG. 4