APPENDIX

Inhibition of gene function in mammalian cells using shorthairpin RNA (shRNA)

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Inhibition of gene function in mammalian cells using short-hairpin RNA (shRNA)

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Summary

RNAi is now the preferred method for silencing gene expression in a variety of systems. In this chapter we describe the procedure for applying short-hairpin RNA (shRNA) to study gene function. Detailed descriptions of target site selection, shRNA construction, shRNA transfection and target knockdown validation are included.

1 Introduction:

Gene silencing by antisense technology is now being used as a powerful molecular tool to study gene functions in living organisms. The antisense agents bind to target messenger RNA (mRNA), thus inactivating the target gene expression. The inhibitory effects on protein production from the corresponding gene may result in phenotypic changes. Thereby, the function of the gene can be understood. To date, there are a number of antisense molecules that can affect efficient post-transcriptional gene silencing. They include antisense oligonucleotides (ON), antisense 'third-generation' nucleic acid analogues (peptide nucleic acid -PNA, locked nucleic acid - LNA or morpholinos), ribozymes, small-interfering RNAs (siRNAs), short-hairpin RNAs (shRNAs) and microRNAs (miRNAs). These antisense molecules cause specific gene inhibitory effects through different mechanisms. In this chapter, we demonstrate the characteristics of gene silencing using RNAi-based short-hairpin RNA (shRNA) technology.

RNAi is a highly conserved gene silencing mechanism that plays an important role in regulation of gene expression. In addition, the RNAi system is important in protecting the host cell from viral infections and invasion by mobile genetic elements (Obbard *et al.*, 2008).

The RNAi pathway takes place in the cytoplasm and can be subdivided into two phases; an initiation and an effector phase. In the initiation phase, long dsRNAs are cleaved by the RNase III-like nuclease Dicer to produce 21-23 nt duplex RNAs, called small interfering RNAs (siRNAs). During the effector phase, the siRNA molecule is incorporated into the RNA-induced silencing complex (RISC), where an ATP-dependent RNA helicase activity unwinds the duplex. The siRNA strand which is antisense to the target RNA (guide strand) is incorporated into RISC, while the complementary passenger strand is destroyed. The guide strand permits highly sequence-specific recognition of the complementary mRNA, which is then cleaved by Argonaute 2, a component of RISC. This results in inhibition of protein synthesis from the mRNA (Shrivastava and Srivastava, 2008).

Unlike miRNA and other long dsRNAs, shRNAs transcribed from exogenously introduced DNA do not require Drosha processing in the nucleus. These tight hairpin turn RNA structures are transported directly to the cytoplasm via exportin-5 were they are cleaved by Dicer into siRNA molecules, which then follow the RNAi pathway for gene silencing (Yi *et al.*, 2003).

RNAi is now a well-established method for high-throughput analysis as well as for functional studies in vitro, including mammalian cells (Scherr and Eder, 2007). Two different methods are commonly used to deliver siRNA molecules for gene silencing in mammalian cell lines; 1) synthetic siRNAs (Watts *et al.*, 2008) and 2) RNA polymerase III transcribed shRNAs from plasmids or viral vectors (Wang *et al.*, 2008;Walchli and Sioud, 2008).

Plasmid vector based shRNA expression is a low-cost and easy-to-perform method for studying gene function in mammalian cells. In addition, this strategy offers the advantage of inducible siRNA expression in the case where gene silencing is expected to have deleterious effects on the target cell (Henriksen *et al.*, 2007).

2 Materials

2.1 Oligonucleotides and plasmids used for cloning

1 Oligonucleotides:

Name:	Sequence (5'-3'):	Description:
ON 3	GTTTTCCCAGTCACGACGTTGTA	M13 forward sequencing primer
ON10	CGGGATCCAAAAAAGGTCTGGGTCCTTGCAGACCACG CCCGACCAAGCTTCGCCGGGCATGATCTGCAAGAACC CAGACCGGTGTTTCGTCCTTTCCACAA	Reverse antiMYCN-27 shRNA cloning primer. Contain BamHI
ON11	CGGGATCCAAAAAAGCTAGTGCTCCTCGGCCTAGAAG GAGTAGCAAGCTTCCCACTCCCTCCAGGCCGAGGAGC ACCAGCGGTGTTTCGTCCTTTCCACAA	Reverse antiMYCN-1291 shRNA cloning primer. Contain BamHI
ON19	CGGGATCCAAAAAAGAATCACTCAGAGTGTCCCCTCC GGAAGTGAAGCTTGACCTCCGGAGAGGACACCCTGA GCGATTCGGTGTTTCGTCCTTTCCACAA	Reverse antiMYCN-760 shRNA cloning primer. Contain BamHI
ON20	CGGGATCCAAAAAAGTTCTTGAGACACACAGCGATGG TAAATGGAAGCTTGCATTCACCATCACTGTGCGTCCC AAGAACGGTGTTTCGTCCTTTCCACAA	Reverse antiMYCN-887 shRNA cloning primer. Contain BamHI
ON22	ATAAGAATGCGGCCGCAAGGTCGGGCAGGAAGAGGG CC	U6 forward primer. Contain NotI.
ON51	CGGGATCCAAAAAAGAGCGTTCGGAGCTGATGGCCAT AAATACGAAGCTTGGTACTTATGACCACCAACTCCGA ACGCTCGGTGTTTCGTCCTTTCCACAA	Reverse cloning primer for SCR shRNA. Contain BamHI
ON 56	ATTTGGGTCGCGGTTCTTG	UBC forward QPCR primer
ON 57	TGCCTTGACATTCTCGATGGT	UBC reverse QPCR primer
ON 87	CACCCTGAGCGATTCAGATGA	MYCN forward QPCR primer
ON 89	CCGGGACCCAGGGCT	MYCN reverse QPCR primer
ON 58	GCAGCTACTCCTCCAGCTCT	NFL forward QPCR primer
ON 59	ACTTGAGGTCGTTGCTGATG	NFL reverse QPCR primer
ON 60	TCCAGCCCAGAGACACTGATT	NPY forward QPCR primer
ON 61	AGGGTCTTCAAGCCGAGTTCT	NPY reverse QPCR primer
ON 96	AAGTTCTACGGTGACGAGGAG	CRT forward QPCR primer
ON 97	GTCGATGTTCTGCTCATGTTTC	CRT reverse QPCR primer
ON 100	AGATCCCGGAGTTGGAAAAC	c-MYC forward QPCR primer
ON 101	AGCTTTTGCTCCTCTGCTTG	c-MYC reverse QPCR primer
ON 170	TCACCCACACTGTGCCCATCTACGA	β-actin forward QPCR primer
ON 171	CAGCGGAACCGCTCATTGCCAATGG	β-actin reverse QPCR primer
ON 176	TGACACTGGCAAAACAATGCA	HPRT1 forward QPCR primer
ON 177	GGTCCTTTTCACCAGCAAGCT	HPRT1 reverse QPCR primer
ON 304	CGAGAGCGAGCGGATGA	CHGB forward QPCR primer
ON 305	GGCGTGTCTTCACTTCTTCAGA	CHGB reverse QPCR primer

.

2 Plasmids:

pSHAG-Ff1 (Paddison *et al.*, 2002) encodes an U6-driven anti-luciferase (anti-luc) shRNA homologous to nucleotides 1340-1368 of the coding sequence of the firefly luciferase gene (NCBI accession number U47296).

2.2 Cell culture and transfection

1 Neuroblastoma cell line SK-N-BE(2) – MYCN amplified.

2 6-well multiwell culture plates (Falcon)

3 RPMI-1640 supplemented with 10% fetal bovine serum (FBS).

4 Phosphate-buffered saline (PBS)

5 Trypsin solution – 0,25 % trypsin and 0,05% ethylenediamine tetraacetic acid (EDTA) in PBS , pH 7,5

6 Cells were maintained in a humidified 37^{o}C incubator with 5% CO_2, supplied with fresh complete medium every 3 days, and subcultured before confluence was reached.

7 Lipofectamin2000 (Invitrogen)

2.3 Cellular protein isolation and Western blot analysis

1 Tropix lysis buffer, Protease inhibitor (Roche), DTT

2 XCell SureLock^{TM} Mini-Cell (Invitrogen)

3 NuPAGE Novex 4-12% Bis-Tris Gel – 1,0mm x 10 well (Invitrogen)

4 NuPAGE LDS Sample Buffer (4x) (Invitrogen)

5 Markers. MagicMarkTMXP Western standard (Invitrogen) as a protein size marker. Kaleidoscope Prestained Standard (Bio-RAD) for visualisation of size during electrophoresis and protein transfer efficiency from gel to membrane during blotting.

6 Methanol

7 Running buffer; NuPAGE MOPS SDS Running Buffer (20x) (Invitrogen)

8 XCell II^{TM} Blot Module (Invitrogen)

9 Immobilon-FL PVDF transfer membrane (Millipore)

10 Whatman 3MM chromatography paper

11 Transfer buffer: NuPAGE Transfer Buffer (20x) (Invitrogen)

12 Blocking buffer: Odyssey$^{®}$ Blocking buffer (LI-COR Biosciences)

13 Primary Ab:
anti-N-myc mouse mAb (Calbiochem)
anti-actin rabbit pAb (Sigma)

14 Secondary Ab.
Alexa Fluor® 680 conjugated goat anti-mouse IgG (Invitrogen)
IRDye800CW conjugated goat anti-rabbit IgG (Rockland)

15 PBST ; PBS containing 0,1 % Tween-20

16 Odyssey Infrared Imaging System (LI-COR)

2.4 Total RNA isolation and cDNA synthesis

1 RNeasy Plus Mini Kit (Qiagen)

2 QIAshredder™ (Qiagen)

3 Superscript™ III reverse transcriptase (Invitrogen)

4 Oligo-dT_{20} primer

5 dNTPs

6 RNase inhibitor

7 Thermocycler

2.5 Real-time RT-PCR analysis

1 Power SYBR Green PCR Master Mix (Applied Biosystems)

2 MicroAmp Optical 96-well Reaction Plate (Applied Biosystems)

3 MicroAmp Optical Adhesive Film (Applied Biosystems)

4 7300 Real Time PCR System (Applied Biosystems)

5 7300 System Sequence Detection Software v1.4 (Applied Biosystems)

6 qBase (http://medgen.ugent.be/qbase/)

3 Methods

This section gives a detailed description of the design and cloning of anti-MYCN shRNA constructs. A detailed procedure for cell transfection and evaluation of shRNA treated neuroblastoma cells, using confocal microscopy, Western blot and real-time RT-PCR analysis, is also described.

3.1 Selection of anti-MYCN shRNA target sites

There are many factors affecting shRNA efficiency. Among the most important factors are both shRNA and target mRNA structures. Several software programmes for siRNA target prediction have been developed, but no single standard exists for predicting the best siRNA target sequence (Li and Cha, 2007). Functional studies are required to evaluate the efficiencies of any shRNA constructed.

We chose 4 different target sites in the MYCN cDNA (GeneBank accession NM_005378) sequence (figure 1A). Two sites (antiMYCN-27 and antiMYCN- 1291) were picked at random. The other two target sites (antiMYCN-760 and antiMYCN-887) and a scrambled shRNA sequence were picked using Genescript siRNA Target Finder and Genescript siRNA Sequence Scrambler (http://www.genscript.com/tools.html), respectively (Wang and Mu, 2004).

All antiMYCN shRNA sequence candidates were BLASTed (NCBI database) to ensure that only the MYCN mRNA was targeted.

3.2 Designing reverse primers for anti-MYCN shRNA cloning

The PCR-based cloning strategy used to construct the U6 expressed shRNAs from the pSHAG plasmid, requires a reverse primer containing the complete shRNA sequence.

The following steps describe how ON-20, the reverse primer for amplifying antiMYCN-887, was designed:

1 Pick a 29 nt target sequence which ends with a C from the MYCN cDNA sequence:

5'–**CATTCACCATCACTGTGCGTCCCAAGAAC**–3'

2 Reverse complement the target sequence to create the 'antisense' strand:

5'–**GTTCTTGGGACGCACAGTGATGGTGAATG**–3'

3 Add a HindIII-containing 'loop' sequence to the 3'end:

5'–GTTCTTGGGACGCACAGTGATGGTGAATG**CAAGCUUC**–3'

4 Add the reverse complement of the 'antisense' sequence to the 3'end of the 'loop' sequence:

5'–GTTCTTGGGACGCACAGTGATGGTGAATGCAAGCUUC**CATTCACCATCACTGTGCGTCCCAAGAAC**–3'

This sequence represents the shRNA molecule (see figure 1b).

5 Change 4 nucleotides in the 'sense' strand to create G-U basepairs in the shRNA stem sequence. **Note 1**:

5' -GTTCTTGGGACGCACAGTGATGGTGAATGCAAGCUUCCATT**T**ACCATC**G**CTGTG**T**GTC**T**CAAGAAC – 3'

6 Add 6 thymidines to create the RNA polymerase III transcription termination sequence:

5’ – GTTCTTGGGACGCACAGTGATGG /……../CCATCGCTGTGTGTCTCAAGAAC**TTTTTT** – 3’

7 Reverse complement the sequence:

5’ –**AAAAAAGTTCTTGAGACACACAGCGATGG**/……../**CCATCACTGTGCGTCCCAAGAAC** – 3’

8 Add a 21 nt downstream U6 promoter binding sequence (GGTGTTTCGTCCTTTCCACAA) to the 3’ end:

5’ –AAAAAAGTTCTTGAGAC/……../CCATCACTGTGCGTCCCAAGAAC**GGTGTTTCGTCCTTTCCACAA** – 3’

9 Add a BamHI restriction enzyme site to the 5’ end for cloning purposes to finish the reverse cloning primer ON-20 (see section 2.1):

5’ –**CGGGATCC**AAAAAAGTTCTTGAGAC/……../CCATCACTGTGCGTCCCAAGAACGGTGTTTCGTCCTTTCCACAA – 3’

3.3 Construction of anti-MYCN shRNA expressing plasmids

Plasmids containing different anti-MYCN shRNA sequences, expressed from a U6 promoter, are made using a PCR-based strategy. In this strategy, 272 bp of the U6 promoter from pSHAG-Ff1 is amplified using a NotI-containing U6 forward primer (ON22) in combination with different BamHI-containing reverse primers including the complete anti-MYCN shRNA sequences. **Note 2**.

PCR products are digested with NotI/BamHI and purified from agarose gels, before ligation into NotI/BamHI digested pSHAG-Ff1 plasmids. Reverse primers ON10, ON11, ON19, ON20 and ON51 are used to construct plasmids pantiMYCN-27, pantiMYCN-1291, pantiMYCN-760, pantiMYCN-887 and pScr-shRNA, respectively. Numbers in plasmid names indicates the first position of the shRNA target recognition site in the MYCN cDNA sequence. All plasmid constructs are verified by DNA sequencing using ON-3.

3.4 Transient transfection of anti-MYCN shRNAs into a MYCN-amplified neuroblastoma cell line

1 Day 1: Seed $1.3x10^5$ SK-N-BE(2) cells into each well of a 6-well tissue culture plate.

2 Day 2: Cells are transfected with 3 μg plasmids pantiMYCN-27, pantiMYCN-1291, pantiMYCN-760, pantiMYCN-887 or pScr-shRNA using Lipofectamin2000 (4 μl) in a total of 2 ml media in each well according to the manufacturers protocol.

3 Day 5: Isolate total cellular RNA and protein extracts.

Transfection efficiencies typically vary between 50-80%.

3.5 Total cellular protein isolation

1 Wash cells with 2 ml PBS, make sure to remove all supernatant.

2 Add trypsin solution.

3 When cells detach from the culture dish (few minutes at room temperature), add 1,0 ml RPMI1640 with 10% serum, resuspend and transfer to 1,5 ml Eppendorf tubes.

4 Wash cells once in 0,5 ml PBS.

5 Resuspend cells in 40 µl Tropix lysis buffer containing protease inhibitor and 1 mM DTT **Note 3**.

6 Leave on ice for 5 minutes. Sentrifuge at top speed for 5 minutes. Collect the supernatants containing total cellular proteins in fresh tubes.

7 Measure total protein concentrations.

3.6 Western immunoblot analysis

To investigate the effect of anti-MYCN shRNA knockdown at the protein level, we use western immunoblot analysis. The XCell SureLock^{TM} Mini-Cell and XCell II^{TM} Blot Module (Invitrogen) is used to resolve the proteins by Bis-Tris polyacrylamide gel electrophoresis and to transfer the resolved proteins from the gel to a membrane support, respectively. The protocols were done according to the specifications of the producer and briefly include the following steps:

Separation of proteins by Bis-Tris polyacrylamide gel electrophoresis:

1 Assemble the electrophoresis chamber with a 4-12 % Bis-Tris Gel and running buffer.

2 Load 25 µg total protein in sample buffer preheated for 10 min at 70^{o}C to each sample well (total 20 µl). Include MagicMark (1 µl) and Kaleidoscope (7µl) markers in separate wells.

3 Run the gel at constant 200 volt for 1 hour.

Transfer of resolved protein from the gel to a membrane support:

4 Prepare the membrane by briefly soaking it for 10 sec in 100% methanol, 5 sec in water and store it in transfer buffer until used

5 Assemble the gel/blot sandwich from the cathode core in the following order: 2x blotting pads, 1 Whatman 3MM filter paper, gel, membrane, 1x Whatman 3MM filter paper and 3x blotting pads. **Note 4.**

6 Assemble the Mini-Cell Blot Module with the gel/blot sandwich.

7 Electroblot at 30 volts for 1 hour. The kaleidoscope marker colours should be transferred to the membrane.

Processing of the blot for detection of specific proteins with an antibody:

8 Wash membrane in PBS for 5 min.

9 Block membrane in blocking buffer for 1 hour.

10 Add primary antibodies anti-N-myc (1:400) and anti-actin (1:1000) diluted in blocking buffer containing 0,01% SDS and 0,01% Tween-20. Incubate over night at 4^{o}C. **Note 5.**

11 Wash membrane 4 x 5 min at room temperature in PBST.

12 Add secondary antibodies diluted 1:5000 in blocking buffer containing 0,01% SDS and 0,01% Tween-20. Incubate 1 hour at room temperature. Cover in aluminium to protect from light.

13 Wash membrane 4 x 5 min at room temperature in PBST. Protect membrane from light.

14 Scan membrane on an infrared imaging system to develop the final western immunoblott.

Results:
Western immunoblot analysis was used to evaluate the effect of different anti-MYCN shRNA constructs. As can be seen from figure 2, the MYCN protein levels are reduced to different degrees with the tested shRNAs. aMN-887 is the most efficient anti-MYCN shRNA. β-actin protein levels remain unaffected by the different transfections.
These results show that the knockdown effect by shRNAs at the RNA level is reflected at the MYCN protein level.

3.7 Total RNA isolation

We use the RNeasy Plus Mini Kit to isolate total RNA samples. This kit includes gDNA Eliminator Mini Spin Columns for efficient DNA removal and do not require additional DNase treatment. Procedures are according to that recommended by the manufacturer; and include the following steps:

1 Cells from one transfected well of a 6-well culture dish are disrupted by addition of 350 µl of Buffer RLT Plus

2 Homogenize cell lysates using QIAshredder^{TM}. **Note 6.**

3 Closely follow steps 4-12 in the Qiagen protocol for purification of total RNA from animal cells. Samples are usually eluted in 35 µl RNase-free water both in step 11 and 12.

3.8 cDNA synthesis

Superscript^{TM} III reverse transcriptase is used to reverse transcribe total RNA to cDNA.

1 For each RNA sample prepare the following in a 0,5 µl microcentrifuge tube;

Component:	Volume:
Oligo-dT_{20} primer (50 µM) **Note 7.**	1 µl
dNTP (2,5 mM each)	1 µl
$MgCl_2$ (25 mM)	1 µl
1,4-2,0 µg total RNA **Note 8.**	x µl
RNase-free water	(13 – x) µl
TOTAL:	13 µl

2 Incubate at 65 ^{o}C for 5 minutes, then on ice for 1 minute.

3 Add the following to RNA-containing solution from step 1.

Component:	Volume:
(RNA-containing solution from step1)	(13 µl)
5 x First Strand buffer	4 µl
DTT (0,1 M)	1 µl
RNase inhibitor	1 µl
SuperScript™ III **Note 9.**	1 µl
TOTAL:	20 µl

4 Incubate in a thermocycler;

Temperature:	Time (minutes):
50 ^{o}C	60
70 ^{o}C	15
4 ^{o}C	until PCR setup is ready

3.9 Real-time PCR analysis

1 Prepare stocks of master reaction mixes. Each reaction includes the following:

Component:	Volume:
Nuclease-free water	6,5 µl
2 x SYBR Green Master Mix	12,5 µl
cDNA template (30 x diluted)	5,0 µl
Primer mix (5 µm each primer) **Note 10.**	1,0 µl
TOTAL:	25 µl

2 Add 25 µl of the reaction mix to each well of an optical 96-well reaction plate.

3 Seal the plate with adhesive film. Make sure all edges are properly sealed.

4 Spin the reaction plate at 1600 rpm for 1 minute.

5 Start the PCR software on the real-time PCR system and assign each well with the correct sample / control

6 Insert the reaction plate and execute the PCR with the following program:

Pre-incubation:

Temperature:	Time:
50 °C	2 min.
95 °C	10 min.

Amplification (40 cycles):

Temperature:	Time:
95 °C	15 sec.
60 °C	1 min.

Melting curve:

Temperature:	Time:
95 °C	15 sec.
60 °C	1 min.
95 °C	15 sec..
60 °C	15 sec.

7 Calculate relative gene expressions. **Note 11**.

Results:

To measure the direct effect of different anti-MYCN shRNAs on MYCN mRNA, we use real-time RT-PCR analysis. Figure 3a shows relative expression levels of MYCN mRNA. The reductions in MYCN mRNA levels are consistent with the decrease of MYCN protein observed on western immunoblots. aMN-887 is the most efficient shRNA showing a 70 % reduction of MYCN mRNA in transient transfection experiments. To support the observed MYCN knockdown effects, we quantified c-MYC mRNA levels in the transfected cells using quantitative real-time RT-PCR. Previous studies have shown that there is a reverse correlation of MYCN and c-MYC expression in neuroblastoma cells (Breit and Schwab, 1989;Westermann *et al.*, 2008). Figure 3b shows a close reverse correlation between MYCN mRNA and c-MYC mRNA expression, supporting the knockdown efficiencies of the tested anti-MYCN shRNAs. Based on the measured CT values in these experiments, MYCN mRNA levels exceed c-MYC mRNA levels by a factor of 10^4 in the MYCN-amplified neuroblastoma cell line SK-N-BE(2).

In addition, to indirectly verify MYCN downregulation upon anti-MYCN shRNA treatment, we used real-time RT-PCR to confirm neuronal differentiation observed by immunostaining confocal microscopy (data not shown). We chose a panel of 4 neuronal differentiation markers, Neuropeptid Y –NPY (Jalava *et al.*, 1992), Calreticulin - CRT (Johnson *et al.*, 1998; Hsu *et al.*,

2005), Chromogranin B – CHGB (Jogi *et al.*, 2004), Neurofilament L – NFL (Breen and Anderton, 1991) and investigated mRNA levels in aMN-887 treated neuroblastoma cells. All markers were significantly increased in anti-MYCN shRNA treated cells (figure 4), confirming the observed neuronal differentiation.
Hypoxanthine phosphoribosyltransferase 1 (HPRT1), β-actin and Ubiquitin C (UBC) were used as housekeeping genes in all real-time PCR experiments. **Note 12.**

4 Notes

1 The G-U basepairs added are not central for the efficiency of the shRNA, but they are thought to stabilize the shRNA molecule (Paddison *et al.*, 2004). Furthermore, the wobble basepairs aid sequencing the shRNA construct and is also believed to reduce unwanted immunostimulation.

2 When using very long primers (the reverse primer including the entire shRNA construct) in PCR, it is recommended to add DMSO to the PCR reaction mix. We use 2 µl DMSO in a 50 µl PCR reaction mix.

3 Lysis of cells can be done directly in the well, but this requires larger volume of lysis buffer, and thus results in lower concentration of protein in the final solution. This might cause problems since there is only room for ca 20 µl sample in each well of the western gel.

4 Blotting pads, Whatman paper and membrane should be soaked thoroughly in transfer buffer before assemble. Avoid air bubbles.

5 We perform primary antibody staining in 50 ml centrifuge tubes to reduce the amounts of blocking buffer and antibody (total 5 ml). Membranes are rolled in oven mesh sheets to ensure complete exposure to the antibody solution.

6 Passing cell lysates 5 times through a RNase-free 20-gauge needle works as well.

7 Random hexamer primer works as well.

8 If RNA concentration is below the amount required to get 1,4 µg in 10 µl, we use Microcon Ultracel YM-100 filters (Millipore) to up-concentrate the RNA.

9 A No-RT control reaction, lacking reverse transcriptase, is always used as a control for DNA removal. Using primer sets located within an exon sequence (ex: UBC) will give PCR amplification whenever DNA is present in this reaction.

10 If the same cDNA template is being analyzed for multiple genes, it is more efficient to add primers separately. Mix the other components of the master reaction mix in the desired magnitude (number of paralleles multiplied with number of genes being analyzed) Apply 1 µl of primer mix to the well intended for its respective genes. Then add 24 µl of the reaction mix to the wells.

11 We use qBase v1.3.5 to calculate relative expression levels with 2-3 housekeeping genes (Hellemans *et al.*, 2007).

12 There is not one single housekeeping gene suitable for all experiments. It is recommended to test the consistency of expression of several housekeeping genes in your

own experiments. We have used the Human Endogenous Control Gene Panel (tataa Biocenter) to find the housekeeping genes most suitable for our experimental setup. We always use 2-4 different housekeeping genes in each real-time PCR experiment.

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Legends to figures:

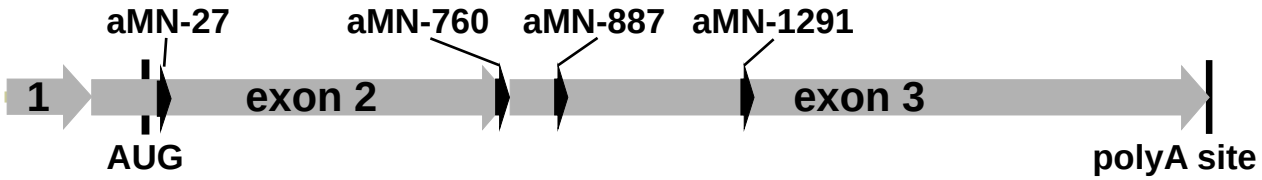
Figure 1. A. Schematic representation of MYCN cDNA. Exons are shown as grey arrows. Localization of antiMYCN shRNA target-sites are indicated by black arrowheads. **B.** Sequence and secondary structure representation of antiMYCN-887 (aMN-887) shRNA.

Figure 2. Western immunoblotting analysis of MYCN in antiMYCN shRNA transfected SK-N-BE(2) cells. Knockdown effects from 4 different antiMYCN shRNAs (aMN-27, aMN-760, aMN-887 and aMN-1291) were compared. aMN-887 is the most efficient antiMYCN shRNA.

Figure 3. Real-time RT-PCR analysis of MYCN (**A**) and c-myc (**B**) mRNA levels in shRNA transfected SK-N-BE(2) cells. Knockdown effects from 4 different antiMYCN shRNAs (aMN-27, aMN-760, aMN-887 and aMN-1291) were compared. aMN-887 is the most efficient antiMYCN shRNA, showing approximately 70 % reduction in MYCN mRNA compared to the scrambled control (SCR). A close reverse correlation is observed between MYCN mRNA and c-MYC mRNA expression levels.

Figure 4. Real-time RT-PCR analysis of known neuronal differentiation markers, neuropeptid Y, neurofilament L, chromogranin B and calreticulin, in shRNA transfected SK-N-BE(2) cells. All markers show increased expression when transfected with an antiMYCN shRNA (aMN-887) compared to a scrambled control shRNA (SCR).

A



MYCN cDNA (NM005378)

B

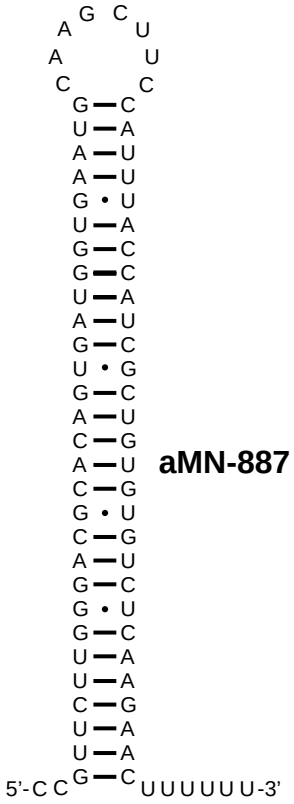


figure 1

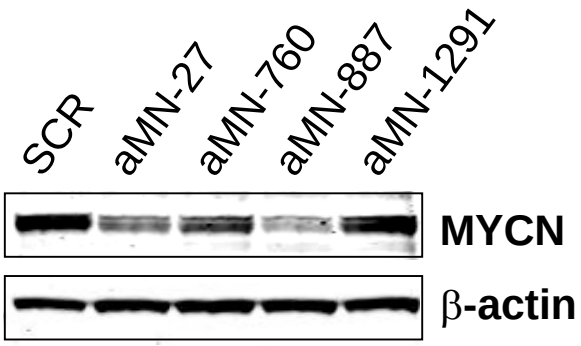
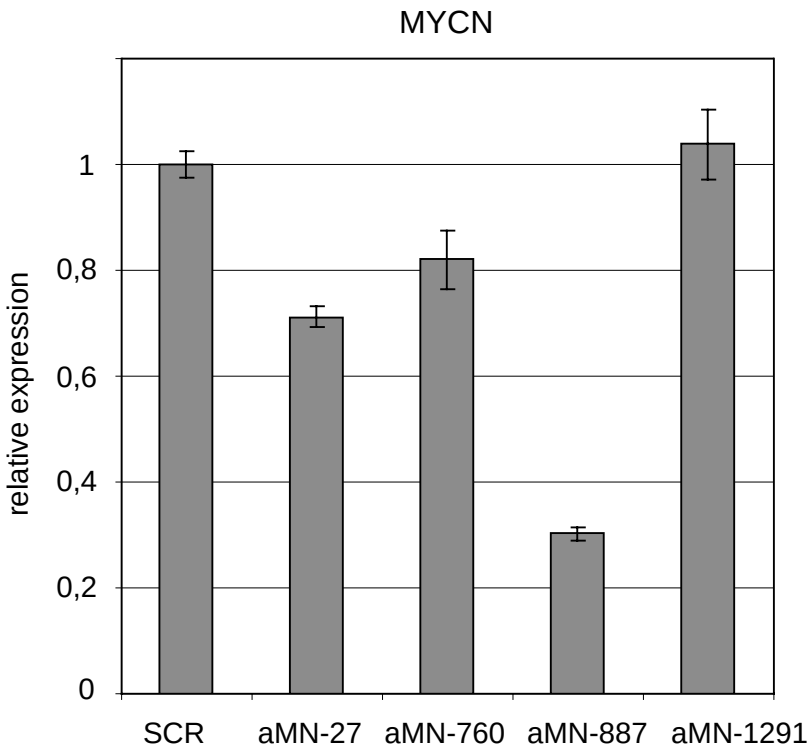


figure 2

A


MYCN
relative expression
1
0,8
0,6
0,4
0,2
0
SCR
aMN-27
aMN-760
aMN-887
aMN-1291

B

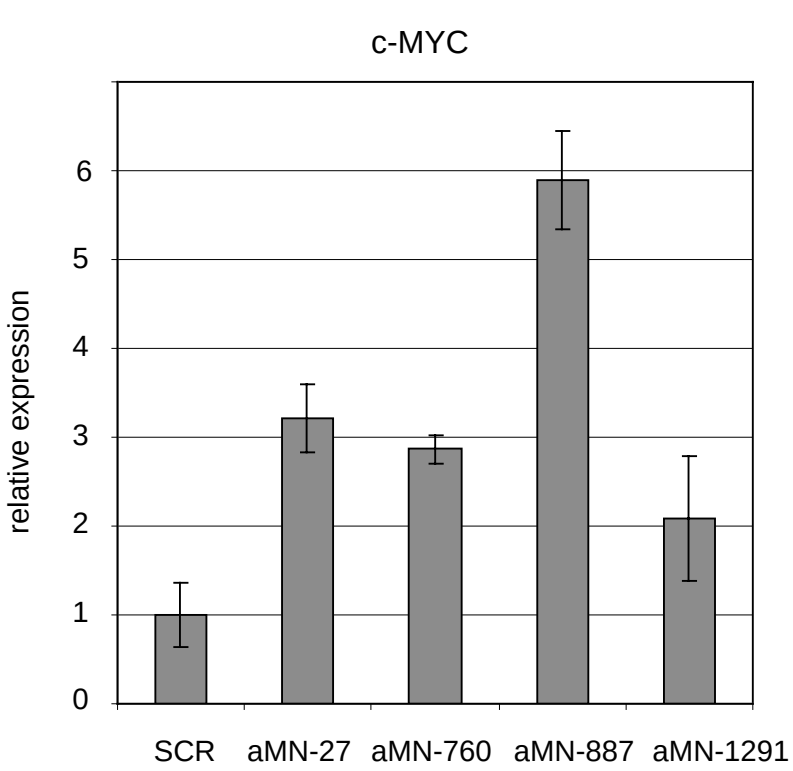

c-MYC
relative expression
6
5
4
3
2
1
0
SCR
aMN-27
aMN-760
aMN-887
aMN-1291

figure 3

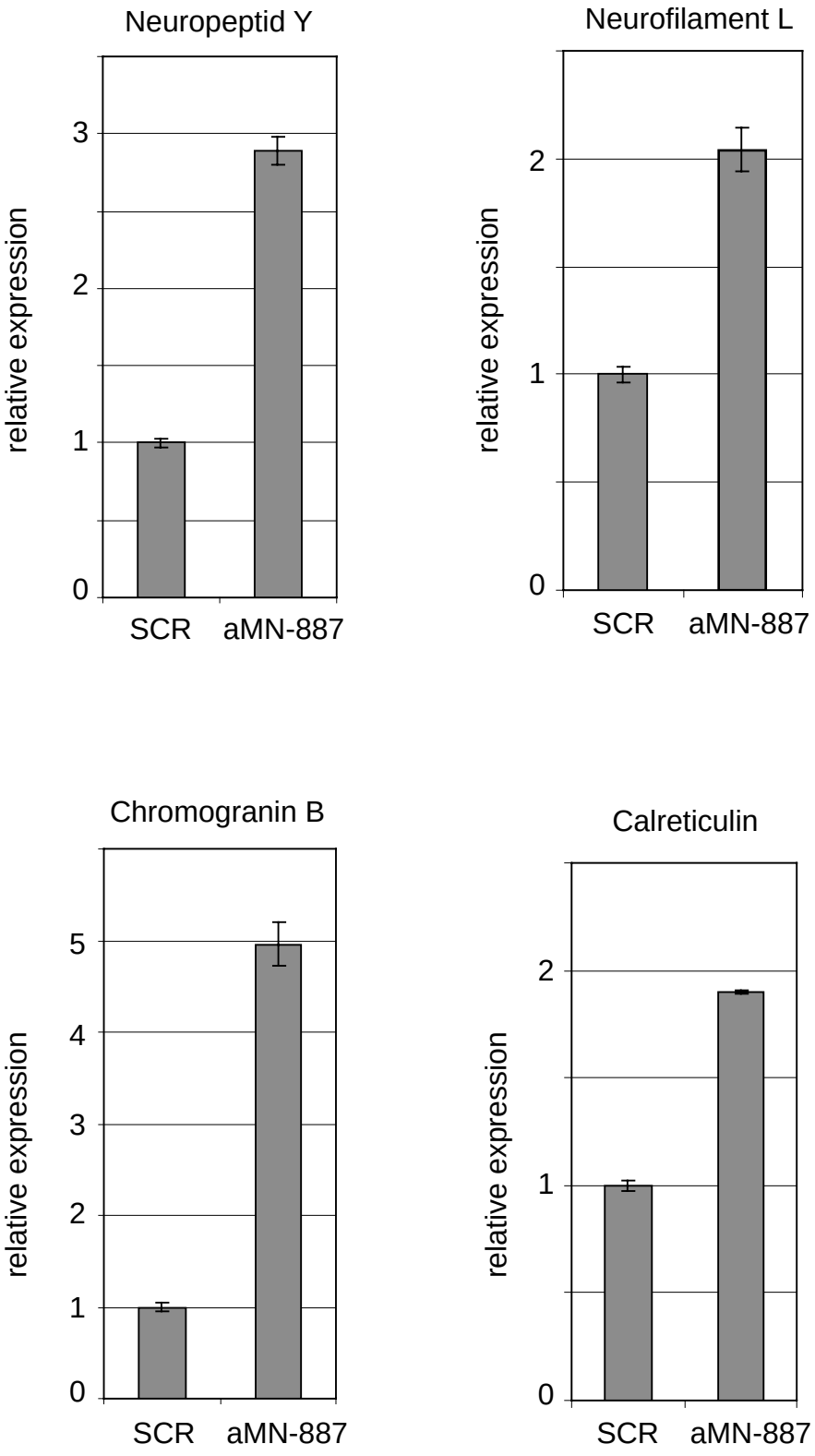

Neuropeptid Y
relative expression
0
1
2
3
SCR
aMN-887
Neurofilament L
relative expression
0
1
2
SCR
aMN-887
Chromogranin B
relative expression
0
1
2
3
4
5
SCR
aMN-887
Calreticulin
relative expression
0
1
2
SCR
aMN-887

figure 4