upstate
cell signaling solutions
a Serologicals Company

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Certificate of Analysis

pKD-FAK-v1

(mammalian FAK siRNA expression plasmid)
Catalog # 62-174
Lot # 29095

Product Description: Transfection grade mammalian expression plasmid containing human FAK sequence that when expressed forms a short-hairpin RNA (siRNA), which gets processed into a FAK siRNA. The expression of the siRNA is under the control of the human H1 RNA polymerase III promoter and is terminated as illustrated below.

Gene Target GenBank Accession Numbers: NM_005607; NM_153831.

Species of Gene Target: Human.

Storage and Stability: Stable for 5 years at either 4°C or -20°C from date of shipment.

Formulation: **5μg** affinity purified DNA eluted and packaged aseptically in **50μl** of 10mM Tris-HCl, pH 8.0, 1mM EDTA. The final concentration is 100ng/μl.

Guarantee: This product is guaranteed to knockdown the FAK mRNA by at least 70% (as compared to pKD-NegCon-v1) when transfected into HeLa cells following the protocol stated on page 4.

Related Products (with catalog number):
pKD-NegCon-v1 (62-002)
pKD-FAK-v4 (62-175)
FAK Polyclonal Antibody (06-543)
FAK Monoclonal Antibody (05-182)

Aliases: PTK2; FADK; FAK1; pp125FAK.

Terms of Use

Opening and use of this product means that the purchaser agrees with the following conditions: The pKD family of plasmids may not be freely distributed, resold, modified for resale or used to manufacture commercial products without prior written approval from Upstate USA, Inc. If you do not agree with these conditions, please return product to Upstate USA, Inc. for a full refund.

FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Quality Control Testing

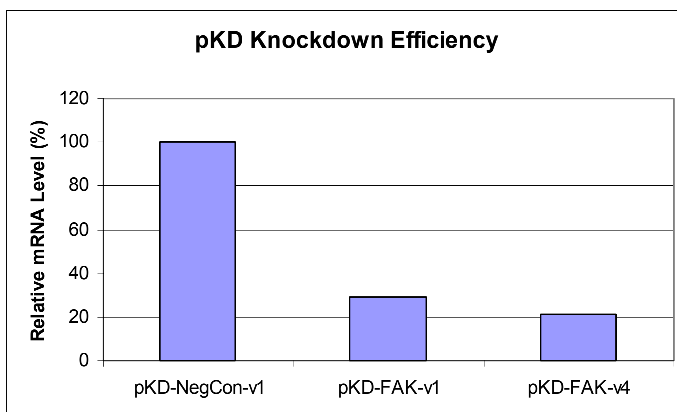
Restriction Enzyme Digest: This lot of DNA was cut with *Eco*RI and *Spe*I, which generated the expected fragments.

Plasmid Transfection and Quantitative RT-PCR Analysis: Representative data shown below.

Q-RT-PCR Analysis: The efficiency of the pKD-FAK-v1 plasmid to knockdown mRNA levels was measured by quantitative RT-PCR using Taqman™ probes. Briefly, HeLa cells were transfected (see protocol page 4) with the pKD-FAK-v1 plasmid and RNA was harvested 48 hours post-transfection. The reduction in FAK mRNA expression was determined by comparison to RNA harvested from cells transfected with the negative control plasmid (pKD-NegCon-v1). Results were normalized by measuring GAPDH expression levels.

Sequencing: The siRNA insert was verified by automated, PCR-based, dideoxy chain-terminating sequencing on the template plasmid.

Purity: The OD_{260}/OD_{280} for this lot of DNA is 1.78.



pKD shRNA family
3099 bp

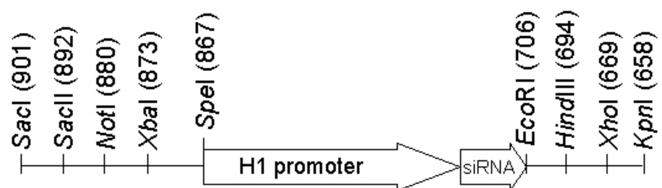
f1 (+) ori
PvuI (504)
PvuII (533)
KpnI (658)
XhoI (669)
PvuI (680)
HindIII (694)
EcoRI (706)
shRNA sequence
H1 Promoter
SpeI (867)
XbaI (873)
NotI (880)
SacII (892)
SacI (901)
PvuII (1119)
pUC ori
ApaLI (1609)
amp R
PvuI (2558)
ApaLI (2855)

Plasmid Information:

Locations of Plasmid Features:

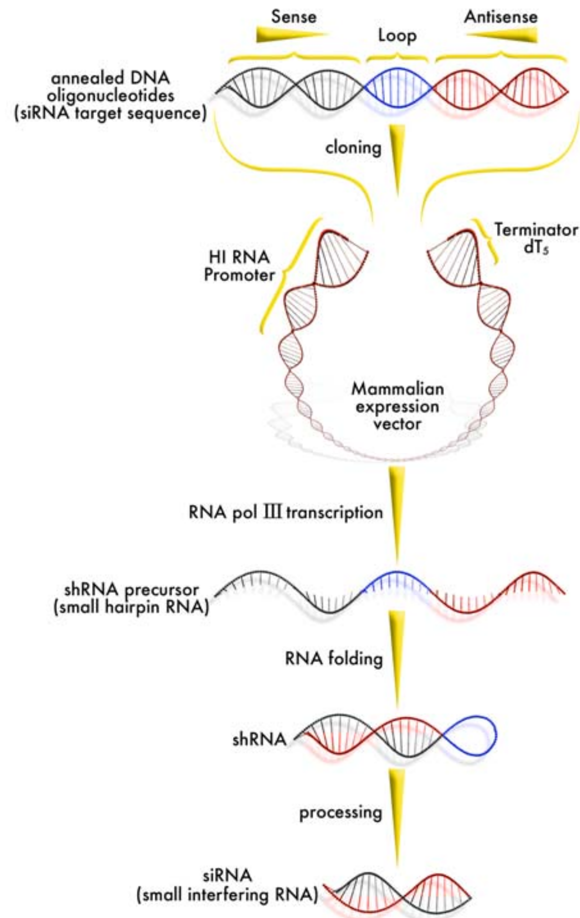
H1 RNA Pol III promoter:	nt 766 to 866
siRNA insert:	nt 717 to 765
Terminator sequence:	nt 711 to 716
f1 (+) Ori:	nt 138 to 444
pUC Ori:	nt 1208 to 1875
Amp resistance gene:	nt 2026 to 2883

Schematic Map of pKD siRNA Expression Plasmids:



pKD Design and siRNA Production

The pKD-FAK-v1 plasmid was constructed such that when it is transfected into mammalian tissue culture cells, the cloned sequence gets transcribed and processed into a functional siRNA molecule. The figure below graphically represents the concept of the pKD vector and the design and cloning of the double stranded DNA oligonucleotides.



Schematic of pKD design and concept. A double-stranded, annealed DNA oligonucleotide is generated that corresponds to the target gene mRNA sequence such that the target gene sense sequence is represented 5' of its antisense and is separated by a 9 base pair "loop" region. This oligo is then cloned into an expression vector that uses the human H1, RNA polymerase III-based promoter to express the cloned sequence. The transcription is terminated by a dT_5 sequence immediately 3' of the cloned oligos. The RNA transcript is then able to fold onto itself as the sense and antisense regions are able to base-pair. The 9 nucleotide "loop" region allows for the short hairpin RNA (siRNA) to form. Cellular ribonucleases process the siRNA into a functional, short interfering RNA (siRNA).

The siRNA target sequence is 21 nucleotides in length and was designed using a highly advanced search algorithm. The sequence contains perfect complementarity to the FAK gene target but contains minimal or no homology to other sequences within the genome. The siRNA target sequence was utilized to design the DNA oligonucleotides that were cloned into the pKD vector. The DNA oligonucleotides contain the sense strand (or target sequence) followed by a loop sequence and then the anti-sense strand. Additional nucleotides were added to the 3' end for cloning purposes. The oligonucleotides were cloned immediately downstream of the H1 RNA promoter and just upstream of the poly dT transcription termination sequence. The plasmid was then verified to be correct by restriction digest and sequence analysis. Finally, the pKD-FAK-v1 plasmid was functionally assayed and showed the ability to knockdown at least 70% of the FAK mRNA target by quantitative RT-PCR analysis.

pKD Plasmid Transfection Protocol

Note: The following protocol has been optimized for transfecting HeLa cells in a 96 well plate. For all other cell lines and plate formats, it is highly recommended that you optimize your transfection conditions because plating density and cell type greatly influence transfection efficiency. In addition, other cell lines may require a different transfection reagent.

Cell Plating

1. Plate HeLa cells (2K/well in 90µl) in DMEM (Mediatech Catalog # 10-013CM) supplemented with 10% FBS and 1% Penicillin-Streptomycin in 96-well TC plate format.
2. Incubate 24 hours at 37°C and 5% CO_2 to achieve 50-80% confluency before plasmid transfection.

Transfection Complex

3. In a small sterile tube, combine the following in the prescribed order, as the order of addition of components to the complex mixture is important:

Serum-free DMEM	9.425µl
FuGene6 Transfection Reagent (Roche Catalog # 1 814 443)	0.075µl
pKD Plasmid DNA (0.1mg/ml)	0.5µl
Total	10µl

NOTE: A mastermix of the serum-free DMEM and FuGene6 can be made based on the number of samples to be tested and then aliquoted into tubes prior to DNA addition. The FuGene6 MUST be added directly into the serum-free DMEM media. Do **not** let FuGene6 touch any plastic other than the pipette tip!

4. Mix tube contents by gently tapping. Do not vortex!
5. Incubate at room temperature for a minimum of 15 minutes and not more than 45 minutes.

Plasmid Transfection

6. Do **not** remove old media.
7. Add 10µl of Transfection Complex to each individual well while gently rocking the plate.
8. Incubate cells at 37°C and 5% CO_2 for 24-48 hours before harvesting for RNA and 72-96 hours before harvesting for protein.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a HeLa cell lysate preparation from pKD plasmid transfected cells (cell lysis buffer: 2X Laemmli sample buffer - 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue) and transfer the proteins to PVDF. Wash the blotted PVDF twice with water.
2. Block the blotted membrane in freshly prepared 3% nonfat dry milk (Catalog # 20-200) in PBS with 0.05% Tween®-20 (PBST-MLK) for 20 minutes at room temperature with constant agitation.
3. Incubate the membrane with primary antibody diluted in freshly prepared PBST-MLK overnight with agitation at 4°C.
4. Wash the membrane twice with water.
5. Incubate the membrane in the corresponding secondary antibody diluted in PBST-MLK for 1.5 hours with agitation at room temperature.
6. Wash the PVDF twice with water.
7. Wash the PVDF in PBS-0.05% Tween®-20 for 3-5 minutes.
8. Rinse the PVDF in 4-5 changes of water.
9. Use Visualizer™ Western Blot Detection Kit (Catalog # 64-201 or 64-202) for chemiluminescent protein detection.