



cell signaling solutions

## Certificate of Analysis

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### pKD-TRAF3-v4

(mammalian TRAF3 siRNA expression plasmid)

Catalog # 62-058

Lot # 28975

**Product Description:** Transfection grade mammalian expression plasmid containing human TRAF3 sequence that when expressed forms a short-hairpin RNA (shRNA), which gets processed into a TRAF3 siRNA. The expression of the shRNA is under the control of the human H1 RNA polymerase III promoter and is terminated as illustrated.

**Gene Target GenBank Accession Numbers:**  
NM\_003300, NM\_145725, NM\_145726.

**Species of Gene Target:** Human.

**Alternate Nomenclature:** LAP1, CAP-1, CRAF1, and CD40bp.

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

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

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

**Related Products (with Catalog Number):**

pKD (62-001)

pKD-NegCon-v1 (62-002)

pKD-TRAF3-v2 (62-036)

### 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

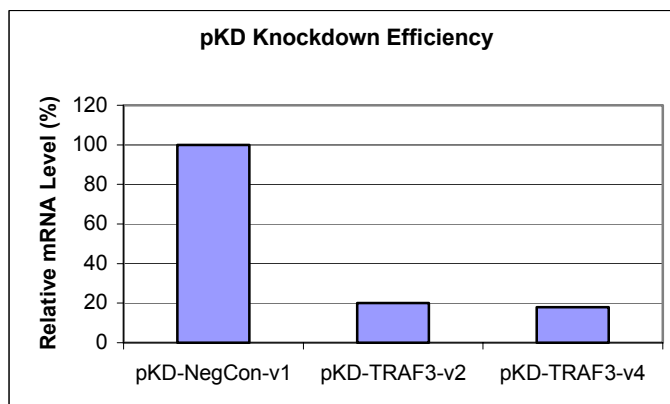
**Restriction Enzyme Digest:** This lot of DNA was cut with *EcoRI* and *SpeI*, which generated the expected fragments.

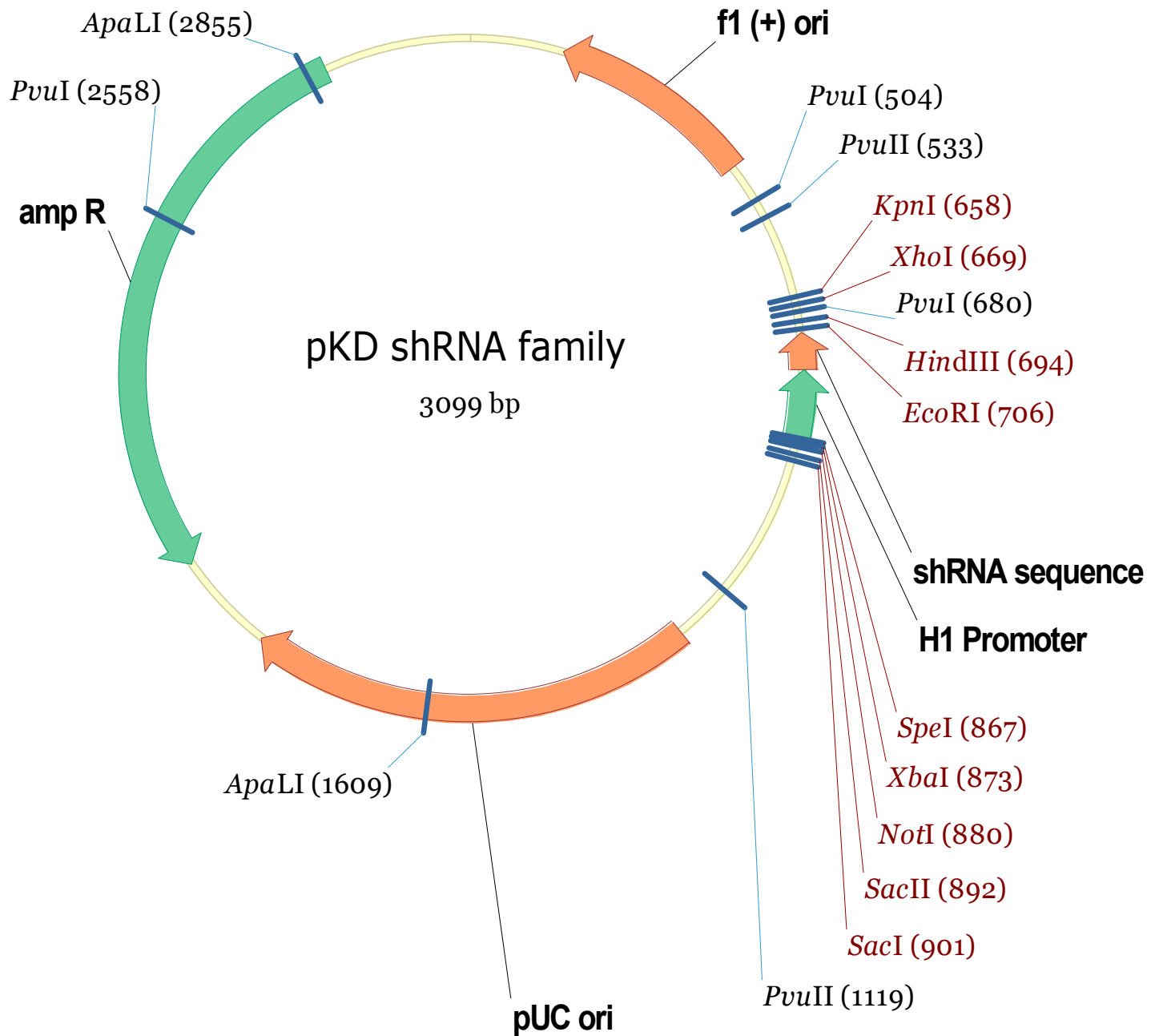
**Plasmid Transfection and Quantitative RT-PCR Analysis:** Representative data shown below.

**Q-RT-PCR Analysis:** The efficiency of the pKD 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 gene specific pKD plasmid and RNA was harvested 48 hours post-transfection. The reduction in 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 shRNA insert was verified by automated, PCR-based, dideoxy chain-terminating sequencing on the template plasmid.

**Purity:** The OD<sub>260</sub>/OD<sub>280</sub> for this lot DNA is 1.89.



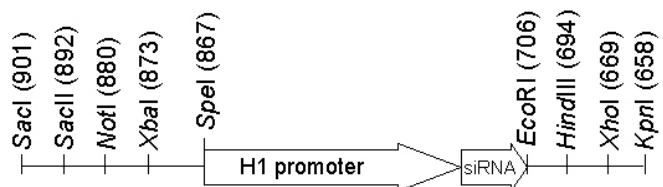


**Plasmid Information:**

**Locations of Plasmid Features:**

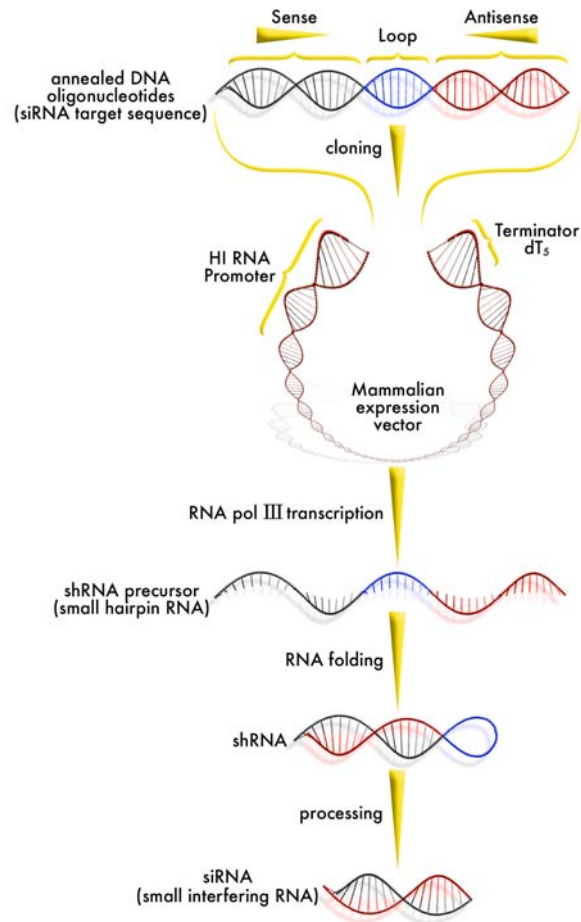
H1 RNA Pol III promoter:	nt 766 to 866
shRNA insert:	nt 717 to 765
Terminator sequence:	nt 711 to 716
f1 (+) Ori:	nt 138 to 444
pUC Ori:	nt 1208 to 1875
Amp resistance gene:	nt 2026 to 2883

**Schematic Map of pKD shRNA Expression Plasmids:**



### siRNA Oligonucleotide Design and Cloning into pKD

The vector, pKD, is designed to receive double stranded DNA oligonucleotides so that when the resulting plasmid is transfected into mammalian tissue culture cells, the cloned sequence gets transcribed and processed into a siRNA. The figure below graphically represents the concept of the pKD vector and the design 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 8 base pair "loop" region. This oligo is then cloned into an expression vector that uses the human HI, RNA polymerase III-based promoter to express the cloned sequence. The transcription is terminated by a dT<sub>5</sub> sequence immediately 3' of the cloned oligos. The transcript is then able to fold onto itself as the sense and antisense regions are able to base-pair. The 8 nucleotide "loop" region allows for the short hairpin RNA (shRNA) to form. Cellular ribonucleases process the shRNA into a functional, short interfering RNA (siRNA).

The DNA oligonucleotides are designed with the first 22 nucleotides being the sequence used in the siRNA for the target gene following the Tuschl rules: AAN19 (see Elbashir, S.M., *et al.*, *Methods* **26**: 199-213, 2002, or <http://www.rockefeller.edu/labheads/tuschl/sirna.html>.) Immediately 3' to these 21 bases are 8 random bases that will serve as a "loop". Immediately downstream of the loop is the antisense sequence of the first 22 nucleotides. This is then followed by the dT<sub>5</sub> terminator. Additional nucleotides are added to both the 5' and 3' ends for cloning.

## **siRNA 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 90 $\mu$ l) 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<sub>2</sub> 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 (Mediatech Catalog # 10-013CM)	9.425 $\mu$ l
FuGene6 Transfection Reagent (Roche Catalog # 1 814 443)	0.075 $\mu$ l
siRNA Plasmid DNA (0.1mg/ml)	0.5 $\mu$ l
Total	<u>10<math>\mu</math>l</u>

**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 10 $\mu$ l of Transfection Complex to each individual well while gently swirling the plate.
8. Incubate cells at 37°C and 5% CO<sub>2</sub> 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<sup>®</sup>-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<sup>®</sup>-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.