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CT26.WT CRISPR Transfection Kit (for Colon Carcinoma Cells, CRL-2638)

Catalog No. 2134 Size: 1.5 ml

Contents and Shipping:
CT26.WT CRISPR Transfection Kit includes CT26.WT CRISPR Transfection Reagent (1.5 ml), Transfection Enhancer (0.5 ml), and Complex Condenser (0.5 ml). CT26.WT CRISPR Transfection Reagent is supplied in liquid form at a concentration of 1 mg/ml, shipped at ambient temperature.

Description:
Proprietary formulation reagent optimized for CRISPR/Cas9 genetic modification procedures.

CT26.WT Cell Line Description:
The CT26.WT cell line was established from BALB/c mice chemically induced and cloned with the retroviral vector LXSN, comprising the lacZ gene. CT26.WT cells display a fibroblast cell morphology and have known to be highly tumorigenic. The CT26.WT cell line is vital for colorectal carcinoma research and is commonly employed as a model for immunotherapy protocols testing. CT26.WT is a great transfection host used for immune response studies as well as for the development of colorectal cancer therapeutics.

Storage:
Store reagent at 4ºC upon receipt. If stored properly, reagent is stable for 6 months.

Intended Use:
For *in vitro* use only.

Required materials (not included in the kit):
- Cas9 NLS protein
- crRNA and tracrRNA (for manual sgRNA formation)
- sgRNA

* Altogen Biosystems do not provide Cas9 protein, tracrRNA, or crRNA.
**These components of CRISPR/Cas9 gene editing systems must be obtained separately.

SDS:
SDS documents are available online at altogen.com

To Place an Order:
Both domestic (USA) and international orders can be placed online at altogen.com

Transfection Resource: altogen.com/transfection-resource

CRISPR/Cas9

Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR) refer to short, palindromic segments of viral DNA that are stored by bacteria. When a secondary viral infection occurs, a nuclease guided by RNA from the stored CRISPR locates and cleaves the viral DNA. The nuclease (Cas) is used for gene editing experiments by selecting the sequence that it targets. When double stranded DNA is cut, several repair mechanisms are initiated by the cell. The non-homologous end joining (NHEJ) pathway puts back together the cleaved DNA, which can result in gene knockout as a result of being cut off from the main strand. This may also result in frameshift mutations and accidental insertions of nearby DNA. The homology directed repair (HDR) pathway uses nearby strands of DNA that overlap the hanging ends to precisely repair the DNA. HDR can be used to introduce genes into DNA by flagging the desired introduced gene with segments that overlap the cleaved ends of cellular DNA, resulting in a segment known as a repair template.

Cas9 Functionality

Cas9 is a DNA-binding nuclease that introduces nicks in double-stranded DNA. Before Cas9 can cut cellular DNA at a desired region, it must be paired with a guiding sequence of RNA used by the protein to locate the target region. This sequence of RNA (crRNA) must be designed and isolated for each specific gene-editing experiment. The crRNA isn't directly connected to the Cas9 protein though, rather the crRNA binds at one end to another sequence – tracrRNA – forming a hairpin structure to which the Cas9 protein binds. The tracrRNA is specific to the Cas9 protein, and must first anneal to the crRNA before being attached to Cas9. Together, the hybridized crRNA and tracrRNA are referred to as sgRNA (single-guide RNA). The sgRNA then binds to the Cas9 protein, forming a ribonucleoprotein (RNP) complex that enters the cell during transfection. The Cas9 protein cuts DNA locally three nucleotides upstream of a short sequence called the protospacer adjacent motif (PAM). The PAM sequence differs for different Cas proteins, but for Cas9 the PAM sequence is of the form NGG, where N can be any other nucleotide. This sequence is found fairly often in DNA, and the Cas9 protein will repeatedly bind to various PAM sites in search of a region matching the crRNA. Once a site that exactly matches the crRNA sequence is found, the Cas9 protein cleaves double-stranded DNA exactly three nucleotides upstream of the PAM sequence. For Eukaryotic cells, the Cas9 protein must have an added nuclear localization sequence (NLS) that will allow it to enter the nucleus of the cell.

Designing crRNA

crRNA design involves several important factors: length, specific segments, and overlap with tracrRNA. The Cas9 can only bind to a short crRNA sequence. The crRNA must have a 19-20 nucleotide region corresponding to the target DNA sequence of interest (20 nucleotides length is commonly used to target a specific region without any off-target effects).

crRNA must be designed to meet a few particular functions of the Cas9 protein:

- The segment of targeted cellular DNA with the crRNA sequence must have a PAM sequence.
- Cas9 protein will cleave the double-stranded cellular DNA three nucleotides upstream of the PAM sequence. Do not include the PAM sequence in the crRNA.

The crRNA must bind to the tracrRNA sequence before being coupled with the Cas9 protein. It is important to know what sequence the corresponding tracrRNA has, so that the crRNA is designed to have ~15nt overlap with the sequence. This overlap is necessary for proper formation of the RNP complex.

Gene Knockouts and Gene Insertions

To knockout a target gene from a cell genome, two crRNA sequences should be designed that flag the desired gene on both sides. Combine the RNP's formed from both crRNA sequences when transfecting cells, as the DNA must be cleaved at both ends of the gene for NHEJ to knockout the targeted sequence.
To insert a gene into a host cell genome, generate a repair template encoding the construct of interest that will be delivered into target cells during transfection. When designing a repair template, it is important to ensure that it cannot be targeted by the RNP complex (with selected crRNA). The repair template should include the desired gene at the location where the Cas9 cleaves double stranded DNA to prevent further cleavage. The repair template should extend 40-60 base pairs on either side of the targeted cut site so that the HDR pathway can incorporate it into the DNA during repair.

CRISPR/Cas9 Transfection Protocol (for 24 well plate):

1. Culture CT26.WT cells to around 70% confluence in complete growth medium.
2. Plate 10,000 - 15,000 CT26.WT cells per well in 0.5 ml of serum-free growth medium 12–24 hours prior to transfection.
3. If you already have pre-formed sgRNA complexes, proceed to step 4, if you have crRNA and tracrRNA in separate solutions, proceed to step 3a.
    a. Combine crRNA and tracrRNA in a 1:1 molar ratio.
    b. Heat to 90ºC.
    c. Cool solution on ice for 30 minutes.
4. Mix sgRNA with Cas9 NLS protein in a 2:1 molar ratio.
5. Incubate RNP complexes for 30 minutes at room temperature.
6. Mix RNP complexes with CT26.WT CRISPR transfection reagent. For Cas9 protein with an NLS sequence and attached sgRNA, combine 40 µl serum-free medium, 5.5 µl transfection reagent, and 750 ng of RNP complexes.
    a. If using a repair template, add 50 ng during this step. The repair template must also be encapsulated to ensure high transfection efficiency.
    b. If using higher amount of repair template, add higher amount of transfection reagent proportionally (1 µl for every 50 ng of DNA).
7. Incubate complexes for additional 30 minutes at room temperature.
8. Optional: add 2 µl of Complex Condenser to reduce the size of the encapsulated RNP complexes, however this may increase cell toxicity.
9. Add encapsulated RNP complexes to the 24 well plate with cultured cells. Test for target gene expression and or lack thereof in 36-72 hours.

*Note that for Eukaryotic cells the efficiency is expected to be 10-15%.

Scaling Up or Down Transfections:

Culture Vessel Surface Area (cm^2)	Volume of Growth Medium (ml)	Transfection Reagent (µl)	Complex Condenser (µl)	Transfection Enhancer (µl)
96-well, 0.3 cm x cm	0.12	1.5	0.3	0.3
24-well, 2 cm x cm	0.5	5.5	2	2
12-well, 4 cm x cm	1	12	4	4
6-well, 10 cm x cm	3	35	12	12
60-mm, 20 cm x cm	5	60	20	20
10-cm, 60 cm x cm	15	180	60	60

Optimizing Transfection:

To obtain the highest transfection efficiency, optimize transfection conditions by varying CT26.WT cell density and amount of transfection reagent. High passage of CT26.WT cells and use of antibiotics (or growth factors) may require using larger volumes of CT26.WT transfection reagent per reaction.

Certificate of Analysis:
Transfection reagent was tested *in vitro* and passed QC. Transfection reagent was tested for absence of nuclease contamination and microbial contamination. Rev. 04/29/2025.

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