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Product Information

Custom CRISPR Plant Products

Catalog Number **CRISPRPL**

Product Description

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins are the components of the adaptive immune systems in bacteria and archaea for defense against infectious bacteriophages and plasmids. Among diverse CRISPR-Cas systems, type II CRISPR-Cas9 systems have been harnessed for gene editing in eukaryotic organisms including mammalians and plants. CRISPR-Cas9 consists of a Cas9 protein, a CRISPR RNA (crRNA), and a trans-activating crRNA (tracrRNA). In gene editing applications, crRNA and tracrRNA are often fused into a single guide RNA (sgRNA) (Figure 1). The ribonucleoprotein invades the target with crRNA guide sequence by forming a 20-bp RNA/DNA hybrid and displacing the opposite DNA strand after it encounters a protospacer adjacent motif (PAM), such as NGG. Cas9 endonuclease subsequently cleaves the complementary DNA strand (target strand) with a HNH nuclease domain and the displaced DNA strand (non-target strand) with a RuvC-like nuclease domain to create a double strand break (DSB). The repair of the DSB by host cell via non-homologous end joining (NHEJ) or homology directed repair (HDR) pathways can be utilized to create gene knockout or introduce a specific genetic modification through homologous recombination with a DNA donor.

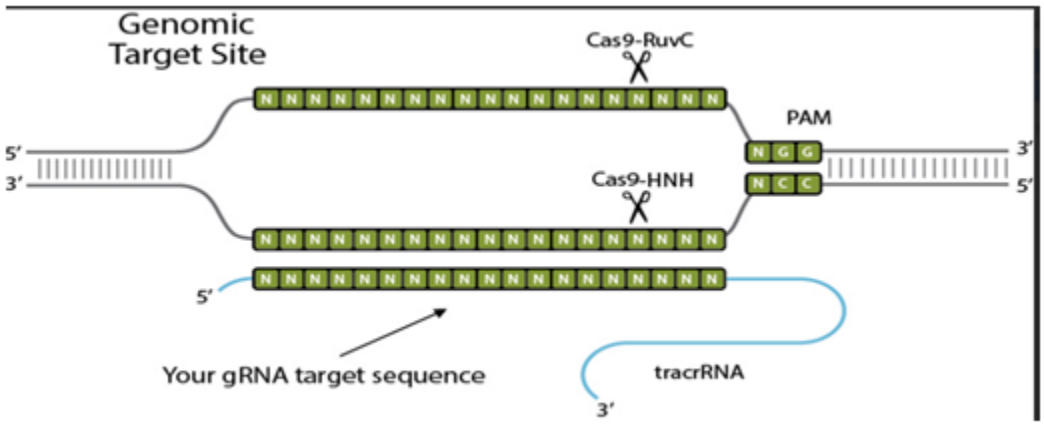


Figure 1. Schematic of a CRISPR/Cas-targeted double-strand break.

Sigma plant CRISPR-Cas9 products are intended for *Agrobacterium*-mediated plant transformation or biolistic microparticle bombardment or protoplast transformation. The products are based on the type IIA CRISPR-Cas9 derived from *Streptococcus pyogenes*. The native Cas9 coding sequence was codon optimized for expression in monocots and dicots, respectively. The monocot Cas9 constructs contain a monocot U6 promoter for sgRNA expression, and the dicot Cas9 constructs contain a dicot U6 promoter. The plant selection markers include hygromycin B resistance gene, neomycin phosphotransferase gene, and the bar gene (phosphinothricin acetyl transferase). The vector maps are shown in Figures 2 and 3.

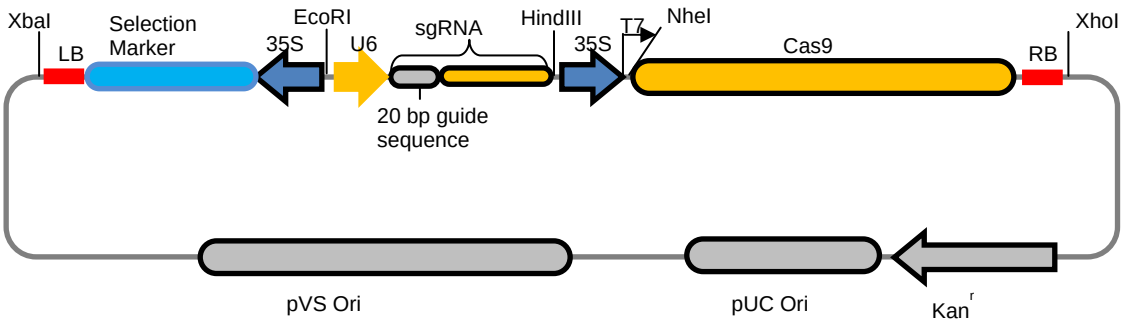


Figure 2. Schematic of CRISPR-Cas9 constructs for *Agrobacterium*-mediated plant transformation

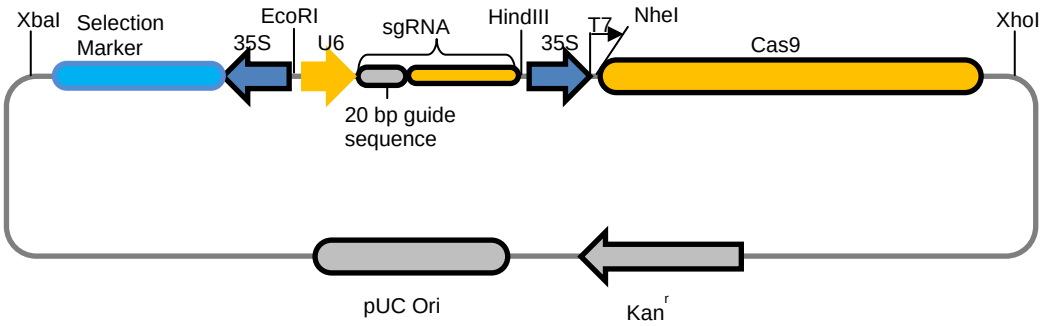


Figure 3. Schematic of CRISPR-Cas9 constructs for biolistics and protoplast plant transformation

Plant CRISPR-Cas9 Product List

Product No.	Transformation method	Cas9	sgRNA expression promoter	Selection marker
CRISPRPL	*Agrobacterium*	Monocot codon optimized	Monocot U6	Hygromycin
CRISPRPL	*Agrobacterium*	Monocot codon optimized	Monocot U6	Neomycin
CRISPRPL	*Agrobacterium*	Monocot codon optimized	Monocot U6	Bar
CRISPRPL	*Agrobacterium*	Dicot codon optimized	Dicot U6	Hygromycin
CRISPRPL	*Agrobacterium*	Dicot codon optimized	Dicot U6	Neomycin
CRISPRPL	*Agrobacterium*	Dicot codon optimized	Dicot U6	Bar
CRISPRPL	Biolistics/Protoplast	Monocot codon optimized	Monocot U6	Hygromycin
CRISPRPL	Biolistics/Protoplast	Monocot codon optimized	Monocot U6	Neomycin
CRISPRPL	Biolistics/Protoplast	Monocot codon optimized	Monocot U6	Bar
CRISPRPL	Biolistics/Protoplast	Dicot codon optimized	Dicot U6	Hygromycin
CRISPRPL	Biolistics/Protoplast	Dicot codon optimized	Dicot U6	Neomycin
CRISPRPL	Biolistics/Protoplast	Dicot codon optimized	Dicot U6	Bar

Precaution and Disclaimer
These products are for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage and Stability
Store CRISPR-Cas9 plasmid at -20^{o}C immediately upon arrival. Please avoid repeated freeze thawing of the plasmid. Practice aseptic technique to avoid DNase contamination of the components. Keep reagent vials and sample tubes closed when not in use.

Preparation of *Agrobacterium tumefaciens* Electro-competent Cells

1. Start a 1 mL culture in a 15-mL Falcon tube with a single colony of an *Agrobacterium tumefaciens* strain in LB medium, supplemented with appropriate antibiotics. Grow the culture overnight at 28^{o}C and 250 rpm.
2. Use the starter culture to inoculate a 20 mL culture in a 250-mL flask in LB medium, supplemented with appropriate antibiotics. Grow the culture overnight at 28^{o}C and 250 rpm.
3. Use the 20 mL overnight culture to start a 500 mL culture in a 2-L flask in LB medium without antibiotics. Grow the culture at 28^{o}C and 250 rpm to an OD_{600} of 0.5-1.0. Start the culture as early in the morning as possible.
4. Chill the culture on ice for 15-30 minutes. Precool centrifuge and bottles to 4^{o}C and prepare ice cold water and 10% glycerol.
5. Centrifuge the culture at 3,500 xg for 15 minutes at 4^{o}C.
6. Remove the supernatant and resuspend the cell pellet in 250 mL of ice cold water.
7. Centrifuge at 3,500 xg for 15 minutes at 4^{o}C.
8. Remove the supernatant and resuspend the cell pellet in 10 mL ice cold 10% glycerol. Transfer the cells into a 50-mL Falcon tube.
9. Centrifuge at 3,500 xg for 15 minutes at 4^{o}C. Repeat the ice cold 10% glycerol wash once.
10. Remove the supernatant and resuspend the cell pellet in 1.5 mL ice cold 10% glycerol.
11. Make 30-µL aliquots in 1.5 mL microcentrifuge tubes. Place the tubes in a cryo box and flash freeze in liquid nitrogen and store at -80^{o}C.

Transformation of *Agrobacteria tumefaciens* Electro-competent Cells with CRISPR-Cas9 plasmid using BioRad Gene Pulser Xcell

1. Dilute CRISPR-Cas9 plasmid to 15 ng/µL in water. Pre-chill cuvettes (0.1 cm, Biorad #1652089) at -20^{o}C for 20 minutes. Set Gene Pulser Xcell to the preset program for *Agrobacterium tumefaciens* (200 Ω, capacitance exender 250 µFD, capacitance 25 µFD).
2. Add 2 µL plasmid to 30 µL *Agrobacterium* competent cells in a 1.5 mL tube. Mix gently by flipping the tube.
3. Transfer the mixture into a pre-chilled cuvette by adding the cells to the side and then tapping down to eliminate air bubbles.
4. Snap cuvette into proper orientation. Press the pulse button to electroporate the sample.
5. Add 1 mL SOC medium to cuvette and pipette up and down to mix. Transfer the cells to a 15 mL Falcon tube (snap-cap tube).
6. Incubate at 28^{o}C and 250 rpm for 2-3 hr. Dilute cells 1:10 with SOS medium and plate 100 µL on a LB agar plate, supplemented with 25 µg/mL kanamycin and other appropriate antibiotics.
7. Incubate the plate at 28^{o}C for 2-3 days to obtain single colonies.

Clone Confirmation

1. Pick a single colony for culture in 2 mL LB medium, supplemented with 25 µg/mL kanamycin and other appropriate antibiotics. Incubate at 28°C with 250 rpm for 16-24 hours.
2. Transfer 50 µL of culture to a 1.5-mL tube and centrifuge at maximum speed for 5 minutes. Remove the supernatant. Add 50 µL of QuickExtract and mix briefly. Heat at 60°C for 15 minutes and then 95°C for 15 minutes. Chill the tube on ice for 2 minutes and centrifuge at maximum speed for 2 minutes. Use 2 µL of the extract for PCR amplification. Prepare glycerol stock with the remaining culture.

3. PCR screening

(1) Monocot CRISPR constructs:
Use the forward primer 5'- GACCAAGCCCGTTATTCTGAC-3' and the reverse primer 5'-AAGTCTGATGCAGCAAGCGAG-3' to amplify a monocot U6-sgRNA fragment (362 bp) to confirm that the selected *Agrobacterium* clones contain CRISPR plasmid. The PCR amplification conditions are: 95°C for 2 minutes for initial denaturation; 30 cycles of 95°C/30 seconds, 60°C/30 seconds, and 72°C/ 30 seconds; 1 cycle of 72°C/5 minutes.

Alternatively, design a sgRNA guide sequence-specific reverse primer to pair with the above forward primer to confirm the selected clones. The following is an example for a rice sgRNA guide sequence (highlighted in red) in a monocot CRISPR construct:

5'---CGCTTGCTGCATCAGACTTGGAGCTCCTGGTCCATCCACGGTTTTAGAGCTAGAAAT---3'

sgRNA guide sequence-specific reverse primer: 5'-CGTGGATGGACCAGGAGC-3' (Complementary to the underlined sequence).

(2) Dicot CRISPR-Cas9 constructs:
Use the forward primer 5'-ATCTCAAAATTCCGGCAGAAC-3' and the reverse primer 5'-CCATCCAATCACTACTTCGTCTC-3' to amplify a dicot U6-sgRNA fragment (305 bp) to confirm that the selected *Agrobacterium* clones contain CRISPR-Cas9 plasmid. The PCR amplification conditions are: 95°C for 2 minutes for initial denaturation; 30 cycles of 95°C/30 seconds, 60°C/30 seconds, and 72°C/ 30 seconds; 1 cycle of 72°C/5 minutes.

Alternatively, design a sgRNA guide sequence-specific reverse primer to pair with the above forward primer to confirm the selected clones. The following is an example for an *Arabidopsis* sgRNA guide sequence (highlighted in red) in a dicot CRISPR construct:

5'---TAGAGACGAAGTAGTGATTGAACCCTGAGGACATCCCATGTTTTAGAGCTAGAAAT---3'

sgRNA guide sequence-specific reverse primer: 5'-ACATGGGATGTCCTCAGGGTT-3' (Complementary to the underlined sequence).

Protoplast Transformation Protocol

1. Preparation of high quality plasmid DNA
   High quality DNA is a critical factor for achieving high transformation efficiency. Transform *E*. *coli* competent cells with CRISPR-Cas9 plasmid DNA and plate on kanamycin plates. Grow an overnight maxiprep culture with a single colony in LB medium, supplemented with kanamycin (25 µg/mL). Sigma HP Endo-free Maxiprep Kit (Cat. No. NA0400) is recommended for making high quality plasmid preparation. Reconstitute plasmid DNA in water at 2-3 µg/µL.

2. Reagent preparation

(1) Enzyme solution: 1% (wt/vol) cellulose Onozuka R10, 0.25% (wt/vol) macerozyme Onozuka R10, 0.4 M mannitol, 10 mM Ca_2Cl, 20 mM KCl, 0.1% BSA, and 20 mM MES, pH 5.7.

Prepare a buffer containing 0.4 M mannitol, 20 mM KCl, and 20 mM MES, pH 5.7. Preheat the buffer at 55^{o}C for 10 minutes. Add cellulose Onozuka R10 and macerozyme Onozuka R10 and vortex vigorously to dissolve. Heat the enzyme solution at 55^{o}C for 10 minutes to inactivate DNase and protease. Let the solution cool down to room temperature. Add Ca_2Cl to final concentration of 10 mM and BSA to final concentration of 0.1%. Filter through a 0.45 µm filter.

(2) PEG-calcium solution: 40% PEG 4000, 0.1 M Ca_2Cl, and 0.2 M mannitol.
Prepare the solution at least 1 hour before transformation to ensure the PEG is completely dissolved. Freshly prepared solution is recommended.

(3) Modified W5 solution: 154 mM NaCl, 125 mM Ca_2Cl, 5 mM KCl, 5 mM glucose, and 2 mM MES, pH 5.7.
Chill the solution on ice before use.

(4) Modified MMG solution: 0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES.

3. Protoplast preparation

(1) Cut plant tissues (leaves or other desirable tissues) to about 1-mm strips with a sharp blade and submerge the strips in 20 mL enzyme solution in a petri dish. Change the blade frequently to minimize cell damage from dull cutting edge. Infiltration the strips by vacuum for 30 minutes at dark. Continue the digestion for 4 hours or longer if necessary with gentle shaking (30 rpm). Filtrate released protoplasts through a 75 µm filter or screen pre-washed with water and equilibrated with modified W5 solution.

(2) Centrifuge protoplasts at 100 x g for 3 minutes at room temperature. Carefully remove the supernatant and wash the protoplasts with 20 mL of pre-chilled modified W5 solution.

(3) Centrifuge at 100 x g for 3 minutes at room temperature and remove the wash solution.

(4) Resuspend the protoplasts in 20 mL of pre-chilled modified W5 solution and incubated on ice for 30 minutes. Take 10 µL of the protoplasts and count with a hemocytometer during the incubation.

(5) Centrifuge the protoplasts at 100 x g for 3 minutes and remove the wash solution. Resuspend the protoplasts in modified MMG solution to a final concentration 2 to 5 x 10^5 cells/mL. Keep the cells at room temperature for immediate transformation.

4. Protoplast transformation

(1) Add 20 µL (20-40 µg) of plasmid DNA to a 5-mL culture tube at room temperature. Add 0.2 mL of cells to the tube and mix gently by tapping the tube with finger on the side.
(2) Add an equal volume (220 µL) of PEG-calcium solution and mix completely but gently by tapping the tube with finger on the side. Incubate at room temperature for 10 minutes.
(3) Add 3 mL of W5 solution and mix gently.
(4) Centrifuge at 100 x g for 3 minutes and remove the wash solution. Repeat the wash step for total of three times.
(5) After the final wash step, resuspend the protoplasts in 1 mL of W5 solution and transfer the protoplasts to a 6-well culture plate coated with 1% BSA. For regeneration, culture the transformed protoplasts in user-defined medium.
(6) Incubate the cells at room temperature for 2 days.

DNA Extraction from Protoplasts

1. Centrifuge protoplasts at maximum speed for 5 minutes and remove the supernatant. Add 100 µL of DNA extraction buffer (200 mM Tris, pH 8.0; 250 mM NaCl, 10 mM EDTA, pH 8.0; 0.1% SDS) and vortex briefly. Incubate at 60^{o}C for 20 minutes.
2. Add 100 µL of phenol/chloroform/isoamyl alcohol and vortex vigorously for 1 -2 minutes. Centrifuge at 4^{o}C with maximum speed for 10 minutes. Transfer the supernatant to a fresh 1.5-mL tube. Add two volumes (200 µL) of 100% ethanol and mix thoroughly.
3. Incubate at -20^{o}C for at least 30 minutes or overnight.
4. Centrifuge at 4^{o}C with maximum speed for 30 minutes. Carefully remove the supernatant. Wash with 200 µL of 70% ethanol.
5. Air-dry the pellet and resuspend in DNase-free water.

Target Cleavage Assay by Surveyor Nuclease (Cel-I)

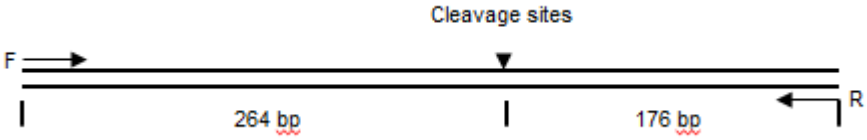
1. Prepare the genomic DNA from transformants
2. Design a pair of primers flanking the target site. The PCR fragment size may range from 250-500 bp.
3. Amplify the target region by PCR.
4. Re-anneal 10 µL of the PCR product in a thermo cycler with the following condition: 10 minutes, 95^{o}C; ramp down to 85^{o}C at -2^{o}C/s; ramp down to 25^{o}C at -0.1C/s; hold at 4^{o}C. Alternatively, purify the PCR product and re-anneal 200-500 ng of the purified PCR product in 1X PCR buffer.
5. After re-annealing, centrifuge the sample briefly and add 1 µL of Enhancer and 1 µL of Surveyor Nuclease. Digest the sample at 42^{o}C for 20 minutes.
6. Resolve the digestion sample on a 10% acrylamide gel.

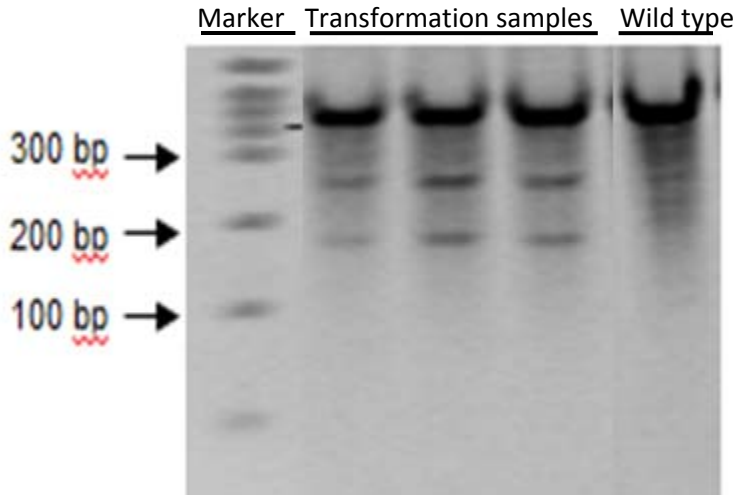
A Cel-I assay example is given below:

Arabidopsis GAPC-2
>gi|240254421:Arabidopsis thaliana chromosome 1
TATGTTGATTCTCTGTAAATTCGTTTTGACTGATTGTGTATATGGTATCGATTTGTTGCAGACATACATGTTTAAGTATGACAGTGTTC
ACGGTCAGTGGAAGCACCATGAGCTTAAGGTGAAGGATGACAAAACTCTTCTCTTCGGTGAGAAGCCAGTCACTGTTTTCGGCATCAGG
TACATAATCATGGATGATTTTATTATAGTGGTTCTCTGTTATGTTATTGAGAGTTGTGAAATTCTGATTTGTGGTGTTTTATTTTTGAA
G**GAACCCTGAGGACATCCCATGGG**GTGAGGCTGGAGCTGACTTTGTTGTTGAGTCTACTGGTGTCTTCACTGACAAAGACAAGGCTGCT
GCTCACTTGAAGGTTTGAACTTGGATCTTTTATATAATAGTCGAGAACTTGTTTATTTAAGATCTATATCATCATCATATTGTTTCATT
GTTATTCAGGGTGGTGCTAAAAAGGTTGTCATCTCTGCCCCAAGCAAAGATGCGCCCATGTTCGTTGTTGGTGTCAACGAGCACGAGTA
CAAGTCTGACCTTGACATTGTTTCCAACGCTAGTTGCACCACTAACTGCCTTGCTCCTCTTGCCAAGGTATCTTTTTCTGTTTCAACCC
AT

Gene: Arabidopsis GAPC-2
Target sequence and PAM: GAACCCTGAGGACATCCCATGGG
Forward primer: CGTTTTGACTGATTGTGTATATGGTATC
Reverse primer: CACCACCCTGAATAACAATGAA
PCR product: 440 bp
PCR condition: 98°C/2 minutes; 98°C/15 S, 60°C/30 S, 72°C/50 S; 34 cycles.

Cel-I digestion pattern:





Histochemical staining with X-Gluc for monitoring CRISPR-Cas9-GUS expression

Stock solutions

1 M sodium phosphate buffer, pH, 7.0: Prepare 1 M Na_2HPO_4 and 1M NaH_2PO_4 solutions. Mix 60 ml of 1 M Na_2HPO_4 solution with 40 ml of 1 M NaH_2PO_4 solution. Adjust pH to 7.0 with either solution. Sterilize by filtration or autoclave.

50 mM FerriCyanide ($K_3Fe(CN)_6$): Potassium ferricyanide(III) (K3Fe(CN)6, Sigma Cat. 702587.
Dissolve 82.3 mg in 5 ml water. Store at -20C. Discard if solution darkens.

0.1 M X-Gluc: Sigma Cat. B0522 or Gold Biotechnology Cat. G1281C
Dissolve 50 mg in 1 ml N,N-Dimethylformamide (N,N-DMF). Store at -20C or -80C.

Staining solution (Freshly prepared immediately prior to use)

Components	**Stock**	**Final**	**Volume per 10 ml**
1. Water			8.3 ml
2. Sodium phosphate, pH 7.0	1.0 M	100 mM	1.0 ml
3. EDTA (Sigma, pH 8.0)	0.5 M	10 mM	200 µl
4. Triton X-100	10%	0.1%	100 µl
5. $K_3Fe(CN)_6$	50 mM	1.0 mM	200 µl
6. X-Gluc	0.1 M	2.0 mM	200 µl

Staining protocol

1. Rinse seedlings or tissue briefly with water and immerse them in staining solution in a 50 ml conical tube or a 1.5 ml tube (for small tissue sample).
2. Vacuum infiltrate for 10 minutes. This step can be skipped for tender or tissue culture samples.
3. Incubate at 37^{o}C for 6 hours to overnight.
4. Remove staining solution and wash with several changes of 50% ethanol until tissue become clear. Store stained tissues in 50% ethanol.

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