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Precision gRNA Synthesis Kit

USER GUIDE

For the generation of full length gRNA (guide RNA ™) for use with CRISPR/Cas9-mediated genome editing

Catalog Number A29377

Publication Number MAN0014538

Revision D

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

Revision history: MAN0014538 D (English)

Revision	Date	Description
D	23 June 2025	• Updated to the current document template, with associated updates to the limited license information, warranty, trademarks, and logos. • The contents and storage table was updated to include additional instructions for storage of purification columns.
C.0	23 December 2022	• Changed dNTP concentration to 25 mM in Kit contents table. • Removed LULL and 568 and update to general LULL for single product. • Removed GeneArt™ from product name. • Updated products in Ordering information appendix. • Updated GeneArt™ CRISPR Search and Design Tool name to TrueDesign Genome Editor Software.
B.0	22 June 2016	Product name was updated.
A.0	15 September 2015	New document for the Precision gRNA Synthesis Kit.

The information in this guide is subject to change without notice.

Contents

1 Product information

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. SDSs are available from **thermofisher.com/support**.

Product description

The Precision gRNA Synthesis Kit is designed to provide a fast workflow for generating full-length gRNA for use with *Streptococcus pyogenes* Cas9 (either protein or mRNA) in CRISPR/Cas9-mediated genome editing. Using a customer supplied forward and reverse overlapping oligonucleotides that contain the target DNA sequence (i.e., CRISPR sequence), the kit can be used to generate a gRNA DNA template containing a T7 promoter by PCR.

The features of the kit include:

- Fast assembly and synthesis of any gRNA target in as little as four hours including template assembly
- High yield (>10 ug) and concentration (>200 ng/uL) of gRNA

Technology overview

Overview of CRISPR/Cas9-mediated DNA cleavage

The CRISPR (clustered regularly interspaced short palindromic repeats) system is a prokaryotic adaptive immune system that uses the RNA-guided DNA nuclease Cas9 to silence viral nucleic acids, and it has been shown to function as a gene editing tool in various organisms including mammalian cells.

The CRISPR system consists of a short non-coding guide RNA (gRNA) made up of a target complementary CRISPR RNA (crRNA) and an auxiliary trans-activating crRNA (tracrRNA). The gRNA guides the Cas9 endonuclease to a specific genomic locus via base pairing between the crRNA sequence and the target sequence, and cleaves the DNA to create a double-strand break (see Figure 1).

In bacteria CRISPR loci are composed of a series of repeats separated by segments of exogenous DNA (of ~30 bp in length), called spacers. The repeat-spacer array is transcribed as a long precursor and processed within repeat sequences to generate small crRNAs that specify the target sequences (also known as protospacers) cleaved by Cas9 protein, the nuclease component of CRISPR system. CRISPR spacers are then used to recognize and silence exogenous genetic elements at the DNA level. Essential for cleavage is a three-nucleotide sequence motif (NGG) immediately downstream on the 3' end of the target region, known as the protospacer-adjacent motif (PAM). The PAM is present in the target DNA, but not the crRNA that targets it (see Figure 1).

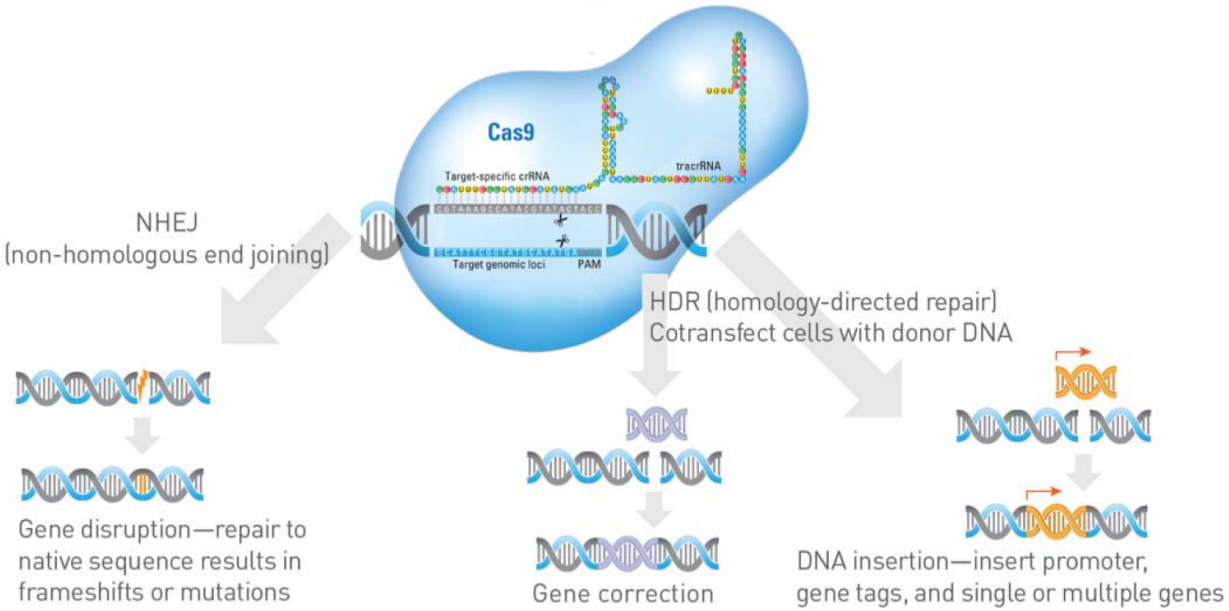

Figure 1 Schematic representation of CRISPR/Cas9-mediated target DNA cleavage

Overview of gRNA synthesis

The gRNA (guide RNA) is a fusion of the natural crRNA and tracrRNA components and is required for Cas9 endonuclease to specifically bind and cleave a target DNA sequence. The gRNA contains an 18–20 base variable sequence that can be changed to target any DNA sequence that is immediately upstream of PAM. The native Streptococcus pyogenes Cas9 bound to the gRNA will specifically bind the target genomic sequence and cut about three bases upstream of PAM, leaving a dsDNA break (see Figure 1).

Using a customer supplied forward and reverse overlapping oligonucleotides that contain the target DNA sequence (i.e., CRISPR sequence), the Precision gRNA Synthesis Kit can be used to generate a gRNA DNA template containing a T7 promoter by PCR. Subsequent in vitro transcription (IVT) of the gRNA template followed by spin column purification of the product yields >10 µg of gRNA at a concentration of >200 ng/µL. The purified gRNA can be immediately used for transfections or stored at –20°C.

Contents and storage

The Precision gRNA Synthesis Kit is composed of two modules:

- gRNA Prep Kit (Box 1, shipped on dry ice)
- gRNA Clean Up Kit (Box 2, shipped at ambient temperature)

Table 1 Components of the Precision gRNA Synthesis Kit (Cat. No. A29377)

Box	Component	Amount	Storage
1: gRNA Prep Kit	Phusion™ High-Fidelity PCR Master Mix	1000 µL	-20°C
	Tracr Fragment + T7 Primer Mix[1]	50 µL	
	TranscriptAid™ Enzyme Mix	100 µL	
	5X TranscriptAid™ Reaction Buffer	200 µL	
	DNase I, RNase-free (1 U/µL)	100 µL	
	Nuclease-free water	1.25 mL	
	NTP mix (25 mM each of ATP, GTP, CTP, UTP in Tris buffer)	200 µL	
	Control gRNA forward and reverse primers (10 µM mix)[2]	10 µL	
2: gRNA Clean Up Kit	Binding Buffer	2.5 mL	15–25°C
	Wash Buffer 1 (concentrated)	7.5 mL	
	Wash Buffer 2 (concentrated)	7.5 mL	
	Nuclease-free water	1.25 mL	
	GeneJET™ RNA Purification Micro Column & Collection Tubes	25 each	15–25°C For better long-term performance store at 2°C to 8°C.
	Elution Tubes, 1.5 mL	25 each	15–25°C

[1] Contains the universal PCR amplification primers (T7 forward primer and reverse primer) and the 80-nt constant region of the crRNA/tracrRNA.

[2] Control gRNA primers target the human HPRT locus.

Required materials not supplied

Unless otherwise indicated, all materials are available through **thermofisher.com**. "MLS" indicates that the material is available from **fisherscientific.com** or another major laboratory supplier.

Catalog numbers that appear as links open the web pages for those products.

Item	Source
Equipment and consumables	
Adjustable micropipettor	**MLS**
Microcentrifuge of ≥14,000 × *g* with rotor for appropriate centrifuge tubes	**MLS**
Target-specific forward and reverse oligonucleotides	**MLS**
Water, nuclease-free	**MLS**
Ethanol, 96–100% (molecular biology grade)	**MLS**
2% E-Gel™ EX Agarose Gel	G4010-02
2X RNA Gel Loading Dye	R0641
RNA Century™ Markers	AM7140
RNase-free pipette tips (sterile)	**MLS**
Microcentrifuge tubes (1.5 or 2.0mL)	**MLS**
Optional	
Qubit™ RNA BR Assay Kit	Q10210
NanoDrop™ spectrophotometer	**MLS**
Qubit™ Fluorometer	**thermofisher.com/qubit**

Workflow

Step	Action	Page
1	Design and order 34-nt forward and reverse target DNA oligonucleotides using the TrueDesign Genome Editor Software	page 9
2	PCR assemble the gRNA DNA template using the Phusion™ High-Fidelity PCR Master Mix	page 15
3	Generate the gRNA by in vitro transcription using the TranscriptAid™ Enzyme Mix	page 16
4	Remove the DNA template by DNase I degradation	page 17
5	Purify the in vitro transcribed gRNA using the GeneJET™ purification columns	page 18
6	Measure the purified gRNA concentration	page 20

2 Methods

Design gRNA DNA template

The first step in de novo CRISPR gRNA synthesis is the analysis of the sequence of interest to identify potential CRISPR targets. Once the CRISPR target sequence has been selected, the specific forward and reverse primers for the PCR assembly of the gRNA DNA template need to be designed. The forward and reverse primers are then be used to generate the gRNA by in vitro transcription (IVT).

This section presents an overview of the gRNA synthesis principles and provides general guidelines for designing the forward and reverse oligonucleotides that are required with the Tracr Fragment + T7 Primer Mix for the PCR assembly of the gRNA DNA template.

The design guidelines provided are:

- Choice of CRISPR target sequences. See “Recommendations for the CRISPR design tool” on page 9 and “Recommendations for choice of CRISPR target sequences” on page 10.
- gRNA DNA template design. See “Recommendations for the gRNA DNA template design” on page 11.
- Design of forward and reverse oligonucleotides for PCR assembly. See “Design of forward and reverse oligonucleotides for PCR assembly” on page 12.

Recommendations for the CRISPR design tool

For best results, use the TrueDesign Genome Editor Software available at (**thermofisher.com/truedesign**) to analyze the sequence of interest for potential CRISPR target sequences or search the Thermo Fisher™ database of >600,000 predesigned gRNA sequences using a gene name, symbol, or accession number, and to design and order primers for gRNA template assembly.

Based on input, the CRISPR design tool identifies the top six CRISPR target sequences with PAM sites, provides recommendations based on potential off-target effects for each CRISPR sequence, displays exon maps with gRNA binding sites, and allows one-click online ordering for custom primers used in gRNA template assembly (for countries with enabled online ordering).

Recommendations for choice of CRISPR target sequences

When performing CRISPR/Cas9-mediated DNA double-strand cleavage, the choice of the target sequence can significantly affect the degree of cleavage observed. The following guidelines below are recommended when choosing a target sequence.

Note: These are general recommendations only. Exceptions may occur.

- **Length**: Choose a target sequence 18–20 nucleotides in length that is adjacent to an NGG protospacer adjacent motif (PAM) on the 3' end of the target sequence. See Figure 2.

  IMPORTANT! Do not include the PAM sequence in the oligonucleotide primers.

- **Homology**: Make sure that the target sequence does not contain significant homology to other genomic sequences as this can increase off-target cleavage. gRNA-Cas9 nuclease complexes can potentially tolerate up to 1–4 mismatches.
- **Direction**: It is recommended to choose a target sequence encoding the sense or the antisense sequence of the target locus. This enables the generation of CRISPR RNA in two possible orientations, provided that it meets the PAM requirements on the 3' ends.
- **Cleavage**: Cas9 nuclease will generally make a dsDNA cut 3–4 bases upstream from the PAM site.

  Note: The cleavage efficiency of a CRISPR sequence at its target depends upon many factors that are not well understood. Therefore, it is recommend to choose at least three CRISPR sequences against a gene of interest and identify the CRISPR sequence with the best cleavage efficiency. For knocking out a gene, it is recommended to select targets within the first 3 exons.

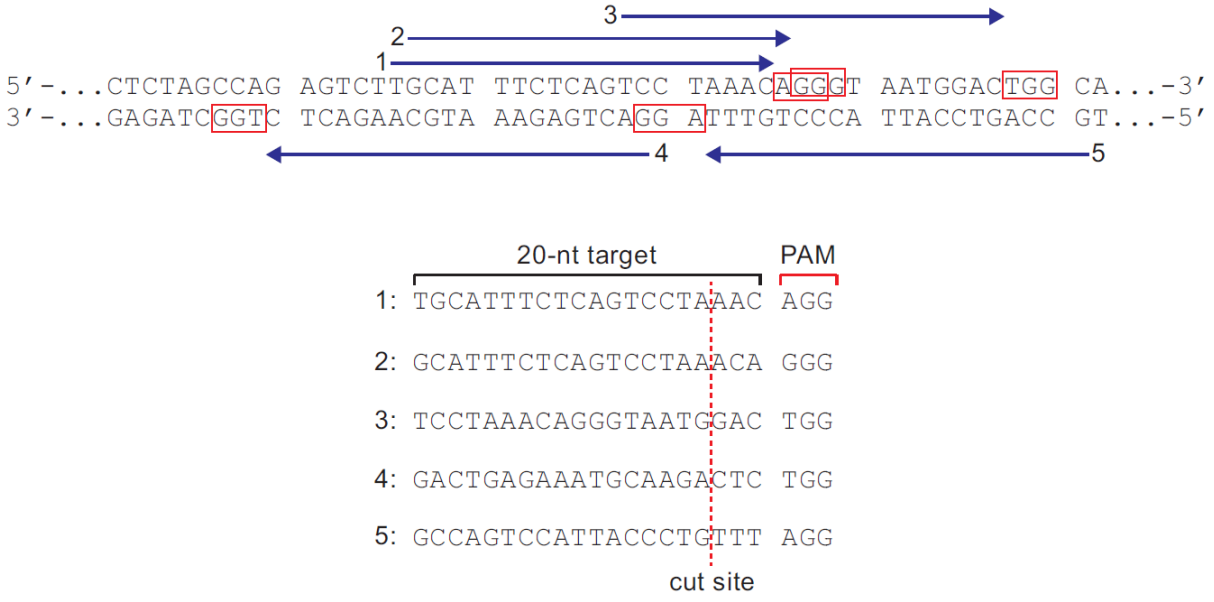

Figure 2 Identifying potential CRISPR targets PAMs are depicted as red boxes and potential targets as blue lines.

Recommendations for the gRNA DNA template design

Once the target sequence has been identified, the next step is to design the gRNA DNA template. The gRNA DNA template sequence is composed of the T7 promoter sequence, the sequence coding the target-specific gRNA, and the constant region of the crRNA/tracrRNA. See Figure 3.

```
5’-TAATACGACTCACTATAGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
   AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT-3’
```

Figure 3 gRNA DNA template sequence

- The T7 Promoter sequence is shown in blue and underlined.
- Transcription begins at and includes the bold G from the T7 promoter sequence.
- The target region represented by red Ns and is up to 20 bases in length (when designing the template, replace the Ns with the target sequence).

  Note: The use of only 18 bases (deleting the first 2 bases from the 5’end) can result in greater specificity.
- The 80-nt constant region of the crRNA/tracrRNA is shown in green.

Recommendations:

- To improve gRNA yield from the in vitro transcription (IVT) reaction, ensure there is at least one G at the start of the transcript. Two to three Gs can improve gRNA IVT yields, although usually one G is sufficient.

  Note: The T7 forward primer in the Tracr Fragment + T7 Primer Mix used for the gRNA template assembly always adds a 5’ G to the target sequence.

- Cutting efficiency decreases if the target region, including the added 5' Gs, is longer than 21 bases. Since transcription begins immediately after the TATA of the T7 promoter sequence, it is recommended to choose a target sequence that naturally includes one to two 5' Gs within the 20-base sequence. Alternatively, use the T7 promoter sequence to add a single G to the 5' end of the target sequence. See Figure 4.

Scenario 1: No existing 5' G in the 20-nt target sequence

5′-...TCTAGTCTTGCATTTCTCAGTCCTAAACAGGGTAATGGACT...-3′

gRNA DNA template: 5' G added from the T7 promoter sequence (20+1)

5′-TAATACGACTCACTATAGTGCATTTCTCAGTCCTAAACGTTTTAGAGCTAGAAATAGCA...-3′

Scenario 2: Existing 5' G in the 20-nt target sequence

5′-...TCTAGTCTTGCATTTCTCAGTCCTAAACAGGGTAATGGACT...-3′

gRNA DNA template: 5' G added from the T7 promoter sequence (20+1)

5′-TAATACGACTCACTATAGGCATTTCTCAGTCCTAAACAGTTTTAGAGCTAGAAATAGCA...-3′

Scenario 3: Use the G from the T7 promoter and 19 bases of the target

5′-...TCTAGTCTTGCATTTCTCAGTCCTAAACAGGGTAATGGACT...-3′

gRNA DNA template: 5' G added from the T7 promoter sequence (19+1)

5′-TAATACGACTCACTATAGCATTTCTCAGTCCTAAACAGTTTTAGAGCTAGAAATAGCA...-3′

Figure 4 Examples of gRNA DNA template sequence using the human HPRT gene

Design of forward and reverse oligonucleotides for PCR assembly

To generate the gRNA DNA template, design the forward and reverse oligonucleotides that will be PCR assembled with the Tracr Fragment + T7 Primer Mix included in the kit.

The Tracr Fragment + T7 Primer Mix contains the universal forward and reverse amplification primers and the 80-nt constant region of the crRNA/tracrRNA. See Figure 5.

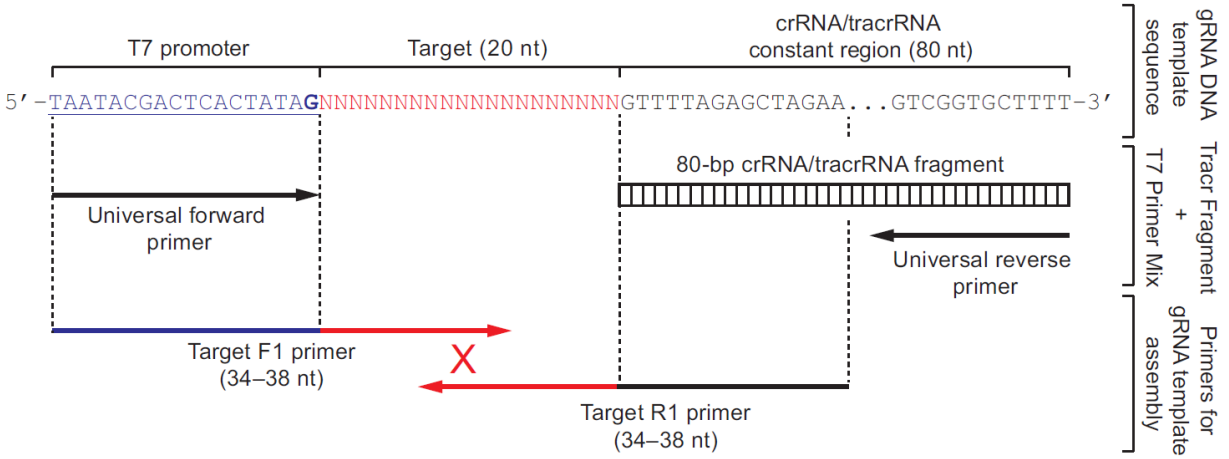

Figure 5 PCR assembly of gRNA DNA template using synthetic forward and reverse oligonucleotides with the Tracr Fragment + T7 Primer Mix

Two 34- to 38-bp oligonucleotides are needed to assemble the synthetic gRNA template:

- Target F1 forward primer harboring the T7 promoter sequence
- Target R1 reverse primer that overlaps with the Target F1 primer and the 5’ end of the crRNA/tracrRNA constant sequence. See Figure 6.

Note: Shorter oligonucleotide lengths (≤40 bases) are recommended for the target primers to reduce the chance of synthesis mistakes, which are more likely in long 11 oligonucleotides. By default, the TrueDesign Genome Editor Software returns forward and reverse target primer sequences that are 34-nt long.

Target F1: `TAATACGACTCACTATAG` + first 16–20 nt of the target sequence

Target R1: `TTCTAGCTCTAAAAC` + first 19–20 nt of the target sequence reverse complement

Figure 6 Sequences of the Target F1 forward and Target R1 reverse oligonucleotides required for synthetic gRNA template assembly

- If the target sequence already contains a 5’ G, this will result in an extra G being added from the T7 promoter primer. Alternatively, the first G of the target sequence can be removed, which will be added back by the T7 promoter primer.
- Using a target sequence of <20 bases can result in lower off-target cutting rates as improved specificity has been observed with target sequences of 17–19 bases (usually 18).
- The effect of the deletion is sequence dependent, so the exact target size that has the most efficient on-target cleavage and reduced off-target cutting will vary. Ideally, choose a target that will contain a 5’ G after the truncation; if such a sequence is not available, a 5’ G can be added later.

Example 1: Keeping the full 20-nt target sequence with a 5' G adds an extra G from the T7 forward primer to the final gRNA sequence (20+1 nt final target sequence)

HPRT gRNA target sequence: GCATTTCTCAGTCCTAAACA

Reverse complement: TGTTTAGGACTGAGAAATGC

HPRT Target F1: TAATACGACTCACTATA**G** + GCATTTCTCAGTCCTA

HPRT Target R1: TTCTAGCTCTAAAAC + TGTTTAGGACTGAGAAAT

gRNA sequence after IVT: **G**GCATTTCTCAGTCCTAAACAGTTTTAGAGCTAGA...

Example 2: 5' G not included in the 20-nt target sequence is restored from the T7 forward primer in the final gRNA sequence (19+1 nt final target sequence)

HPRT gRNA target sequence: ~~X~~CATTTCTCAGTCCTAAACA

Reverse complement: TGTTTAGGACTGAGAAATG~~X~~

HPRT Target F1: TAATACGACTCACTATA**G** + CATTTCTCAGTCCTA

HPRT Target R1: TTCTAGCTCTAAAAC + TGTTTAGGACTGAGAAAT

gRNA sequence after IVT: **G**CATTTCTCAGTCCTAAACAGTTTTAGAGCTAGA...

Example 3: 18-nt truncated target sequence results in a 19-nt final target sequence with a 5' G added from the T7 forward primer in the final gRNA sequence (19+1 nt final target sequence)

HPRT gRNA target sequence: ~~XX~~ATTTCTCAGTCCTAAACA

Reverse complement: TGTTTAGGACTGAGAAAT~~XX~~

HPRT Target F1: TAATACGACTCACTATA**G** + ATTTCTCAGTCCTA

HPRT Target R1: TTCTAGCTCTAAAAC + TGTTTAGGACTGAGAAAT

gRNA sequence after IVT: **G**ATTTCTCAGTCCTAAACAGTTTTAGAGCTAGA...

Figure 7 Designing Target F1 forward and Target R1 reverse oligonucleotides for high IVT yield and increased on-target cleavage

PCR assemble the gRNA DNA template

During the PCR assembly of the gRNA DNA template, the forward and reverse overlapping oligonucleotides that contain the target DNA sequence (i.e., CRISPR sequence) and the Tracr Fragment + T7 Primer Mix are annealed to their complements, and act both as a primer and a template in the PCR to generate the full length gRNA DNA template. See Figure 5.

Required materials

- 0.3 µM working solution of Target F1 forward and Target R1 reverse oligonucleotide mix
- Tracr Fragment + T7 Primer Mix (contains the universal PCR amplification primers and the 80-nt constant region of the crRNA/tracrRNA)
- Phusion™ High-Fidelity PCR Master Mix (2X)
- Nuclease-free water

Prepare 0.3 µM oligonucleotide mix working solution

Target oligonucleotides ordered using the TrueDesign Genome Editor Software are shipped lyophilized and must be resuspended before use.

1. Pellet the lyophilized oligonucleotides by brief centrifugation for 30 seconds at room temperature.
2. To prepare target oligonucleotide stock solution, resuspend each target oligonucleotide in 1X TE buffer to a concentration of 100 µM.
3. To prepare a 10 µM stock solution of target oligonucleotide mix, add 10 µL each of the 100 µM forward and reverse target oligonucleotide stock solution to 80 µL of nuclease-free water.
4. To prepare the 0.3 µM target oligonucleotide mix working solution, dilute 3 µL of the 10 µM target oligonucleotide mix stock solution in 97 µL of nuclease-free water.

Perform PCR assembly

- To ensure homogeneity and improve recovery, carefully mix and centrifuge all tubes before opening
- PCR set up can be performed at room temperature

1. Set up the PCR assembly reaction in a 25 µL volume, adding the reaction components in the order given. See Table 2.

Table 2 PCR assembly reaction

Component	Amount
Phusion™ High-Fidelity PCR Master Mix (2X)	12.5 µL
Tracr Fragment + T7 Primer Mix	1 µL
0.3 µM Target F1/R1 oligonucleotide mix	1 µL
Nuclease-free water	10.5 µL

2. Using the cycling parameters below, perform assembly PCR. See Table 3.

Note: Since the gRNA template DNA is very short (120 bp), a two-step PCR protocol is recommended. No separate extension step is needed during the 32 PCR cycles.

Table 3 Cycling parameters

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	10 seconds	1X
Denaturation	98°C	5 seconds	32X
Annealing	55°C	15 seconds	
Final extension	72°C	1 minute	1X
Hold	4°C	Hold[1]	1X

[1] Remove the product when ready to proceed to next step.

3. *(Optional)*: Confirm the template assembly by running 5 µL of the PCR product against a size marker on a 2% E-Gel™ EX Agarose Gel or an equivalent.

4. Proceed to "Generate the gRNA by in vitro transcription" on page 16.

Generate the gRNA by in vitro transcription

After the DNA template containing the T7 promoter and the gRNA sequence have been assembled, proceed to the in vitro transcription (IVT) reaction to generate gRNA using the TranscriptAid™ Enzyme Mix included in the kit.

Required materials

- gRNA DNA template. See "Perform PCR assembly" on page 15.
- TranscriptAid™ Enzyme Mix
- 5X TranscriptAid™ Reaction Buffer
- NTP mix (25 mM each of ATP, GTP, CTP, UTP in Tris buffer)
- DNase I, RNase-free (1 U/µL)
- Sterile, disposable plastic ware and RNase-free pipette tips
- 2% E-Gel™ EX Agarose Gel
- 2X RNA Gel Loading Dye
- RNA Century™ Markers
- (Optional) Qubit™ RNA BR Assay Kit

Procedural guidelines

- Maintain a separate area, dedicated pipettors, and reagents for RNA work.
- Wear gloves when handling RNA and reagents for work with RNA. Change gloves frequently.
- Use sterile RNase-free plastic tubes and pipette tips.
- Before use, thaw all frozen reaction components, mix, and centrifuge briefly to collect all drops.
- Keep the TranscriptAid™ Enzyme Mix and the nucleotides on ice.
- Keep the 5X TranscriptAid™ Reaction Buffer at room temperature.

Perform IVT reaction

If desired, perform a control IVT reaction in parallel using the Control gRNA forward and reverse primers included in the kit. The control gRNA primers contain two 5' Gs in the target sequence, resulting in high gRNA yields from the IVT reaction. The in vitro transcribed and purified control gRNA targets the human HPRT locus and shows very high genomic cleavage efficiencies.

1. Set up the following in vitro transcription reaction in a 20 µL volume.

IMPORTANT! Add the reaction components in the order given.

Table 4 In vitro transcription reaction

Component	Amount
NTP mix (25 mM each of ATP, GTP, CTP, UTP in Tris buffer)	8 µL
gRNA DNA template (see "Perform PCR assembly" on page 15)	6 µL
5X TranscriptAid™ Reaction Buffer	4 µL
TranscriptAid™ Enzyme Mix	2 µL

Note: If a higher gRNA yield is required, double the amounts used in the IVT reaction.

2. Mix the reaction components thoroughly. Centrifuge briefly to collect all drops then incubate for 2–3 hours at 37°C.

Note: For higher gRNA yields, extend the incubation up to 4 hours.

Remove the DNA template by DNase I digestion

To prevent the template DNA from interfering with downstream applications of the RNA transcript, remove it by DNase I digestion directly after the IVT reaction.
Add 1 µL of DNase I (at 1 U/µL; included in the kit) into the reaction mix immediately after the IVT reaction, then incubate for 15 minutes at 37°C.

Note: A white precipitate will form after the IVT reaction. The precipitate contains pyrophosphate and smaller amounts of RNA. It does not affect downstream steps (purification). Resuspending the precipitate and including it in the purification will increase the amount of RNA recovered.

Determine in vitro transcribed gRNA quality

1. Dilute 0.5 µL of the IVT product in 10 µL of DEPC-treated water.
2. Mix 10 µL of the diluted sample with 10 µL of 2X RNA Loading Dye Solution.
3. Heat the sample at 70°C for 10 minutes, then chill on ice prior to loading.
4. Run the sample on a 2% E-Gel™ EX Agarose Gel or an equivalent against an RNA Ladder that has a 100-base band (For example, RNA Century™ Markers).

Note: The expected gRNA transcript size is 100 bases. A discreet band at 100 bases indicates intact RNA.

Note: To check the quality of the gRNA sample, it is recommended to run the gRNA sample on a 10% Novex™ TBE-Urea Gel.

(Optional) Determine in vitro transcribed gRNA concentration

Determine the concentration of the gRNA transcript using the Qubit™ RNA BR Assay Kit. A NanoDrop™ spectrophotometer or an equivalent system can also be used, but there is a 2X variation in concentration estimation.

1. To determine the concentration of the gRNA transcript, dilute an aliquot of the gRNA transcript 1:100 in nuclease-free water.

Note: If not diluted, the salts in the in vitro transcription reaction can interfere with the reading.

2. Measure concentration using the Qubit™ RNA BR Assay Kit. See “Determine the purified gRNA concentration using the Qubit™ RNA BR Assay Kit” on page 20.

Purify the in vitro transcribed gRNA

After generating gRNA by IVT and removing the DNA template by DNAse I digestion, purify the gRNA using the gRNA Clean Up Kit (Box 2). The gRNA Clean Up Kit contains pre-assembled GeneJET™ RNA Purification Micro Columns and all the necessary buffers to effectively remove primers, dNTPs, unincorporated nucleotides, enzymes, and salts from PCR and IVT reaction mixtures.

Required materials

- IVT reaction mix containing the gRNA
- gRNA Clean Up Kit (Box 2 of the Precision gRNA Synthesis Kit)
- >96% Ethanol
- Nuclease-free water
- Microcentrifuge and 1.5- or 2-mL microcentrifuge tubes
- (Optional) Qubit™ RNA BR Assay Kit

Before first use of the kit

1. Add the indicated volume of ethanol (96–100%) to Wash Buffer I and Wash Buffer II before first use.

Component	Wash Buffer I	Wash Buffer II
Concentrated wash buffer	7.5 mL	7.5 mL
Ethanol (96–100%)	13 mL	30 mL

2. Mark the checkbox on the bottlecap to indicate that ethanol has been added to the bottle.

Procedural guidelines

- Examine the Binding Buffer for precipitates before each use. Re-dissolve any precipitate by warming the solution to 37°C and cooling to 25°C.
- Wear gloves when handling the Binding Buffer as this solution contains irritants.
- Perform all purification steps at room temperature (15–25°C).

Purify in vitro transcribed gRNA

1. Adjust the volume of the IVT reaction to 200 µL with nuclease-free water.
2. Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.
3. Add 300 µL of ethanol (>96%), then mix by pipetting.
4. Transfer the mixture to the GeneJET™ RNA Purification Micro Column (pre-assembled with a collection tube).
5. Centrifuge at 14,000 × *g* for 30–60 seconds. Discard the flow-through, then place the column back into the collection tube.
6. Add 700 µL Wash Buffer 1 supplemented with ethanol (see “Before first use of the kit” on page 19) to the purification column, then centrifuge at 14,000 × *g* for 30–60 seconds.
7. Discard the flow-through, then place the column back into the collection tube.
8. Add 700 µL Wash Buffer 2 supplemented with ethanol (see “Before first use of the kit” on page 19) to the purification column, then centrifuge at 14,000 × *g* for 30–60 seconds.
9. Repeat step 8. Discard the flow-through, then place the column back into the collection tube.
10. Centrifuge the empty purification column at 14,000 × *g* for an additional 60 seconds to completely remove any residual Wash Buffer.

    Note: This step is essential to avoid residual ethanol in the purified RNA solution. The presence of ethanol in the RNA sample may inhibit downstream enzymatic reactions.

11. Transfer the purification column to a clean 1.5-mL Collection Tube.

12. Add 10 µL of nuclease-free water to the center of the purification column filter, then centrifuge at 14,000 × *g* for 60 seconds to elute the RNA.

Note:

- Depending on the desired concentration of the eluted RNA, use 6 µL to 20 µL of nuclease-free water for the elution step. Using an elution volume of <10 µL slightly decreases the RNA yield.
- When purifying larger amounts of RNA (>5 µg), double the elution volume or perform two elution cycles.

Use the eluted gRNA immediately or store at –20°C until use. For prolonged storage (>1 month), store the RNA at –80°C.

Determine the purified gRNA concentration using the Qubit™ RNA BR Assay Kit

Determine the concentration of the gRNA transcript using the Qubit™ RNA BR Assay Kit. A NanoDrop™ spectrophotometer or an equivalent system can also be used, but there is a 2X variation in concentration estimation.

1. Set up 2 assay tubes for the standards and 1 tube for each user sample.
2. Dilute an aliquot of the gRNA transcript 1:100 in nuclease-free water before proceeding with the assay.
3. Prepare 200 µL of Qubit™ Working Solution for each standard and sample by diluting the Qubit™ RNA BR Reagent 1:200 in Qubit™ RNA BR Buffer.
4. Prepare the assay tubes (use 0.5-mL PCR tubes) according to the following table.

Component	Standards	Samples
Working solution. See step 3.	190 µL	199 µL
Standard (from Qubit™ Assay Kit)	10 µL	–
gRNA sample (diluted 1:100)	–	1 µL

5. Vortex standards and samples for 2–3 seconds, then incubate at room temperature for 2 minutes.
6. Select RNA Broad Range Assay on a Qubit™ Fluorometer to calibrate with standards and read the samples.

Note: The typical yield of gRNA is 10–40 µg.

A Ordering information

Precision gRNA Synthesis Kit and related products

The following products supplied in the Precision gRNA Synthesis Kit are available separately from Thermo Fisher™. Ordering information is provided below. For more information, refer to our website (**www.thermofisher.com**) or contact Technical Support.

Product	Amount	Cat. No.
Precision gRNA Synthesis Kit	1 kit	A29377
Phusion™ High-Fidelity PCR Master Mix (2X)	100 reactions	F531S
TranscriptAid™ T7 High Yield Transcription Kit	50 reactions	K0441
GeneJET™ RNA Cleanup and Concentration Micro Kit	50 preps	K0841

Accessory products

The following accessory products are suitable for use with the Precision gRNA Synthesis Kit and are available separately from Thermo Fisher™. Ordering information is provided below. For more information, refer to our website (**www.thermofisher.com**) or contact Technical Support.

Product	Amount	Cat. No.
Novex™ TBD-Urea Gels, 10%	1 box	EC68752BOX
DNase/RNase-free Microfuge Tubes	500 tubes	AM12400
RNase*Zap*™ RNase Decontamination Solution	250 mL	AM9780
2% E-Gel™ EX Agarose Gel	10 gels	G4010-02
2X RNA Gel Loading Dye	1 mL	R0641
RNA Century™ Markers	50 µg	AM7140
Qubit™ RNA BR Assay Kit	100 assays	Q10210

Products for CRISPR/Cas9-mediated genome editing

Thermo Fisher™ offers a variety of products for CRISPR/Cas9-mediated genome editing. Ordering information is provided below. For more information, refer to our website (**www.thermofisher.com**) or contact Technical Support.

Product	Amount	Cat. No.
TrueCut™ Cas9 Protein v2 (1 µg/µL)	10 µg	A36496
TrueCut™ Cas9 Protein v2 (1 µg/µL)	25 µg	A36497
GeneArt™ CRISPR Nuclease mRNA	15 µg	A29378
GeneArt™ Genomic Cleavage Detection Kit	20 reactions	A24372
MEGAshortscript™ T7 Transcription Kit	25 reactions	AM1354
MEGAclear™ Transcription Clean-Up Kit	20 reactions	AM1908
Lipofectamine™ CRISPRMAX™ Transfection Reagent	0.75 mL	CMAX00008
Lipofectamine™ RNAiMAX™ Transfection Reagent	0.75 mL	13778075
Lipofectamine™ MessengerMAX™ Transfection Reagent	0.3 mL	LMRNA003

B Quick reference for gRNA synthesis

The following gRNA synthesis and purification protocols are provided for experienced users of the Precision gRNA Synthesis Kit. First time users should follow the detailed protocols provided in the user guide.

PCR assemble the gRNA DNA template

1. To prepare the 0.3 µM target oligonucleotide mix working solution, dilute 10 µM target oligonucleotide mix stock solution in nuclease-free water.

2. Set up the PCR assembly reaction:

Table 5 PCR assembly reaction

Component	Amount
Phusion™ High-Fidelity PCR Master Mix (2X)	12.5 µL
Tracr Fragment + T7 Primer Mix	1 µL
0.3 µM Target F1/R1 oligonucleotide mix	1 µL
Nuclease-free water	10.5 µL

3. Perform assembly PCR using the cycling parameters below.

Note: Since the gRNA template DNA is very short (120 bp), a two-step PCR protocol is recommended. No separate extension step is needed during the 32 PCR cycles.

Table 6 Cycling parameters

Cycle step	Temperature	Time	Cycles
Initial denaturation	98°C	10 seconds	1X
Denaturation	98°C	5 seconds	32X
Annealing	55°C	15 seconds	
Final extension	72°C	1 minute	1X
Hold	4°C	Hold[1]	1X

[1] Remove the product when ready to proceed to next step.

4. *(Optional)*: Confirm the template assembly by running 5 µL of the PCR product against a size marker on a 2% E-Gel™ EX Agarose Gel or an equivalent.

Perform in vitro transcription

1. Set up the following in vitro transcription reaction, adding the reaction components in the order given.

   Table 7 In vitro transcription reaction

Component	Amount
NTP mix (25 mM each of ATP, GTP, CTP, UTP in Tris buffer)	8 µL
gRNA DNA template (see "Perform PCR assembly" on page 15)	6 µL
5X TranscriptAid™ Reaction Buffer	4 µL
TranscriptAid™ Enzyme Mix	2 µL

2. Incubate at 37°C for 2–3 hours.
3. Add 1 µL of DNase I into the reaction mix after the transcription reaction, then incubate at 37°C for 15 minutes.

Purify in vitro transcribed gRNA

1. Adjust the volume of the IVT reaction to 200 µL with nuclease-free water.
2. Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.
3. Add 300 µL of ethanol (>96%), then mix by pipetting.
4. Transfer the mixture to the GeneJET™ RNA Purification Micro Column, then centrifuge at 14,000 × *g* for 30–60 seconds. Discard the flow-through.
5. Add 700 µL Wash Buffer 1 supplemented with ethanol (see "Before first use of the kit" on page 19), then centrifuge at 14,000 × *g* for 30–60 seconds. Discard the flow-through.
6. Add 700 µL Wash Buffer 2 supplemented with ethanol (see "Before first use of the kit" on page 19), then centrifuge at 14,000 × *g* for 30–60 seconds. Discard the flow-through and repeat.
7. Centrifuge the empty purification column at 14,000 × *g* for an additional 60 seconds to completely remove any residual Wash Buffer. Transfer the purification column to a clean 1.5-mL collection tube.
8. Add 10 µL of nuclease-free water to the center of the purification column filter, then centrifuge at 14,000 × *g* for 60 seconds to elute the gRNA.

C

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, visit **thermofisher.com/support**.

Documentation and support

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  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and its affiliates warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have questions, contact Life Technologies at **www.thermofisher.com/support**.