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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

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Revision C.0

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

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Revision	Date	Description
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. Updated Safety and Documentation and support appendices. Removed GeneArt™ from product name. Updated products in Ordering information appendix. Updated GeneArt™ CRISPR Search and Design Tool name to Invitrogen™ TrueDesign Genome Editor. Removed GeneArt™ CRISPR Search and Design Tool appendix. Removed references.
B.0	22 June 2016	Updated product name
A.0	15 September 2015	New user guide

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

Table of contents

gRNA synthesis procedure for experienced users

Introduction

The following gRNA synthesis and purification protocols are provided for experienced users of the Precision gRNA Synthesis Kit. If you are performing the gRNA synthesis procedure for the first time, follow the detailed protocols provided in the user guide.

PCR assemble the gRNA DNA template

1. Prepare a 0.3 µM target oligonucleotide mix working solution by diluting the 10 µM target oligonucleotide mix stock solution in nuclease-free water.
2. Set up the PCR assembly reaction:

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.

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*	1X

Perform *in vitro* transcription

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

NTP mix (25 mM each of ATP, GTP, CTP, UTP)	8 µL
gRNA DNA template (from PCR assembly, page 14)	6 µL
5X TranscriptAid™ Reaction Buffer	4 µL
TranscriptAid™ Enzyme Mix	2 µL

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

Purify *in vitro* transcribed gRNA

7. Adjust the volume of the IVT reaction to 200 µL with nuclease-free water.
8. Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.
9. Add 300 µL of ethanol (>96%) and mix by pipetting.
10. Transfer the mixture to the GeneJET™ RNA Purification Micro Column and centrifuge for 30–60 seconds at 14,000 × *g*. Discard the flow-through.
11. Add 700 µL Wash Buffer 1(diluted with 13 mL of >96% ethanol) and centrifuge for 30–60 seconds at 14,000 × *g*. Discard the flow-through.
12. Add 700 µL Wash Buffer 2 (diluted with 30 mL of >96% ethanol) centrifuge for 30–60 seconds at 14,000 × *g*. Discard the flow-through and repeat.
13. Centrifuge the empty purification column for an additional 60 seconds at 14,000 × *g* to completely remove any residual Wash Buffer and transfer the purification column to a clean 1.5-mL collection tube.

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

Product information

Product description

Precision gRNA Synthesis Kit

The Precision gRNA Synthesis Kit provides a fast workflow for generating full length gRNA for use with *Streptococcus pyogenes* Cas9 (either protein or mRNA) in CRISPR/Cas9-mediated genome editing.

Kit contents and storage

Kit contents

The Precision gRNA Synthesis Kit (Cat. No. A29377) is composed of two modules: gRNA Prep Kit (Box 1, shipped on dry ice) and gRNA Clean Up Kit (Box 2, shipped at ambient temperature).

	Component	Amount	Storage
Box 1: gRNA Prep Kit	Phusion™ High-Fidelity PCR Master Mix (2X)	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 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	
Box 2: gRNA Clean Up Kit	Binding Buffer	2.5 mL	Room temperature
	Wash Buffer 1 (concentrated)[3]	7.5 mL	
	Wash Buffer 2 (concentrated)[4]	7.5 mL	
	Nuclease-free water	1 mL	
	GeneJET™ RNA Purification Micro Column & Collection Tubes	25 each	
	Elution Tubes, 1.5 mL	25 each	

[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.

[3] Add 13 mL of >96% EtOH before use (63% final EtOH).

[4] Add 30 mL of >96% EtOH before use (80% final EtOH).

Materials required but included

The following materials are not included with the Precision gRNA Synthesis Kit, but are necessary to perform gRNA synthesis.

- Target-specific forward and reverse oligonucleotides
- Nuclease-free water
- *Optional*: Qubit™ RNA BR Assay Kit (Cat. No. Q10210)

Description of the system

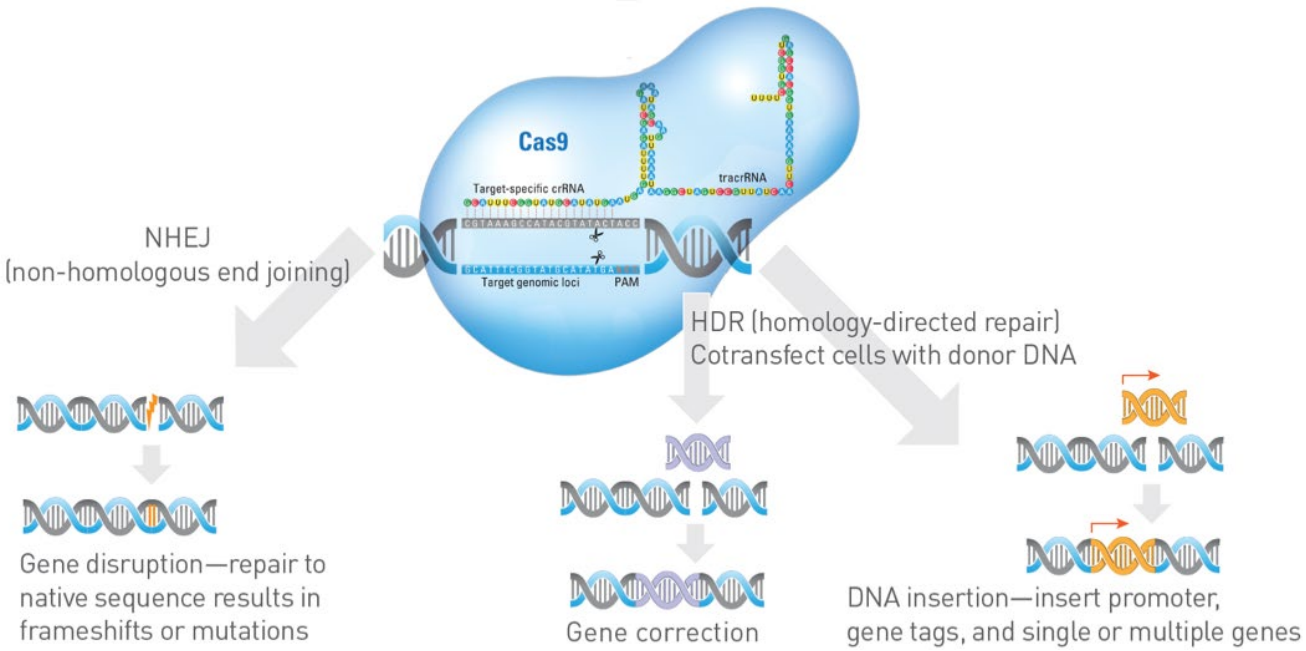
Overview of CRISPR/Cas9-mediated DNA cleavage

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 (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 (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 (Figure 1, page 5).

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.

Workflow

Step	Action	Page
1	Design and order 34-nt forward and reverse target DNA oligonucleotides using the Invitrogen™ TrueDesign™ Genome Editor (**thermofisher.com/truedesign**)	7
2	PCR assemble the gRNA DNA template using the Phusion™ High-Fidelity PCR Master Mix	13
3	Generate the gRNA by *in vitro* transcription using the TranscriptAid™ Enzyme Mix	15
4	Remove the DNA template by DNase I degradation	16
5	Purify the *in vitro* transcribed gRNA using the GeneJET™ purification columns	17
6	Measure the purified gRNA concentration	18

Methods

Guidelines for gRNA design and assembly

Introduction

The first step in *de novo* CRISPR gRNA synthesis is the analysis of your sequence of interest to identify potential CRISPR targets. Once you have selected your CRISPR target sequence, you need to design specific forward and reverse primers for the PCR assembly of the gRNA DNA template, which will 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 (included in the kit) for the PCR assembly of the gRNA DNA template.

The design guidelines provided are:

- Choice of CRISPR target sequences (page 8)
- gRNA DNA template design (page 9)
- Design of forward and reverse oligonucleotides for PCR assembly (page 10)

Invitrogen™ TrueDesign™ Genome Editor

For best results, use the Invitrogen™ TrueDesign™ Genome Editor available at (**thermofisher.com/truedesign**) to analyze your sequence of interest for potential CRISPR target sequences or search our 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 your 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).

Choice of CRISPR target sequences

When performing CRISPR/Cas9-mediated DNA double-strand cleavage, your choice of the target sequence can significantly affect the degree of cleavage observed. We recommend following the guidelines below when choosing your target sequence. Note that these are general recommendations only; exceptions may occur.

- **Length:** Choose a target sequence 18–20 nucleotides in length that is adjacent to an NGG proto-spacer adjacent motif (PAM) on the 3′ end of the target sequence.

  Note: In the example below (Figure 2, page 8), PAMs are depicted as red boxes and potential targets as blue lines.

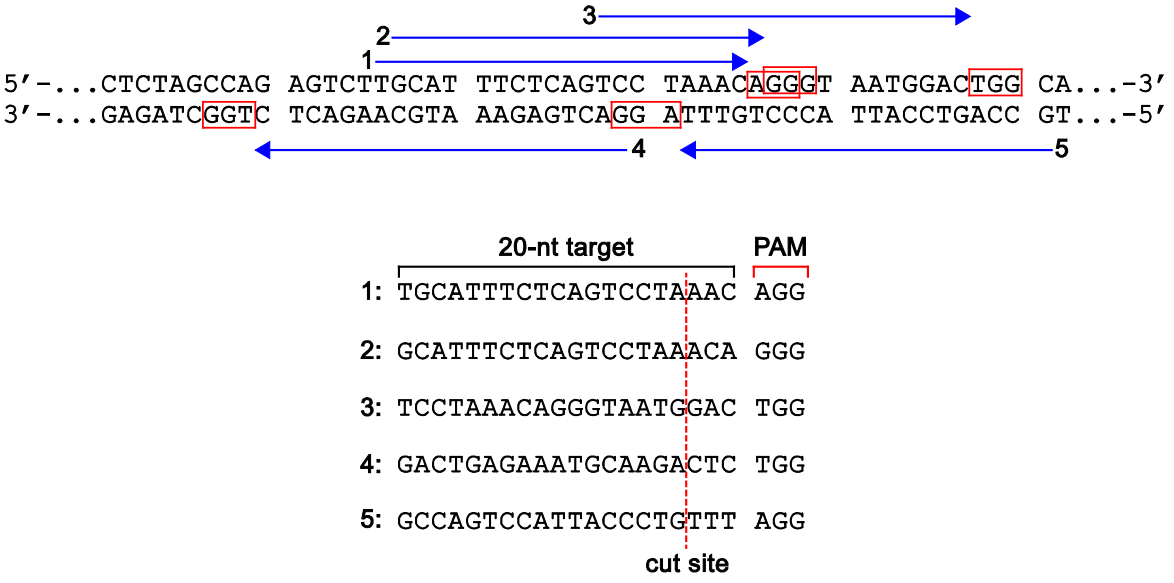
- **Homology:** Make sure that the target sequence does **not** contain significant homology to other genomic sequences as this can increase off-target cleavage. Recently published work has shown that gRNA-Cas9 nuclease complexes can potentially tolerate up to 1–4 mismatches. Refer to published articles for more insights into choosing target sequence

- **Direction:** You may choose a target sequence encoding the **sense** or the **antisense** sequence of the target locus. Thus, you can generate 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, we recommend that you 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, we recommend targets within the first 3 exons.

Figure 2. Identifying potential CRISPR targets



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

gRNA DNA template design

Once you identify your target sequence, 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 (Figure 3, below).

- 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 your template, replace the Ns with your target sequence).

  Note: The use of only 18 bases (deleting the first 2 bases from the 5′end) can result in greater specificity (Fu et. al. 2014).
- The 80-nt constant region of the crRNA/tracrRNA is shown in green.

Figure 3. gRNA DNA template sequence

5′–TAATACGACTCACTATAGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAAT
AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT–3′

We recommend having at least one G at the start of the transcript to improve gRNA yield from the *in vitro* transcription (IVT) reaction. Although we have seen improved gRNA IVT yields with two to three Gs, 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.

We have also observed decreased cutting efficiency if the target region with the added 5′ Gs is longer than 21 bases. Therefore, since transcription starts immediately after the TATA of the T7 promoter sequence, we recommend that you choose a target sequence that naturally includes one to two 5′ Gs within the 20 base sequence or use the T7 promoter sequence to add a single G to the 5′ end of the target sequence (as shown in Figure 4).

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

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)

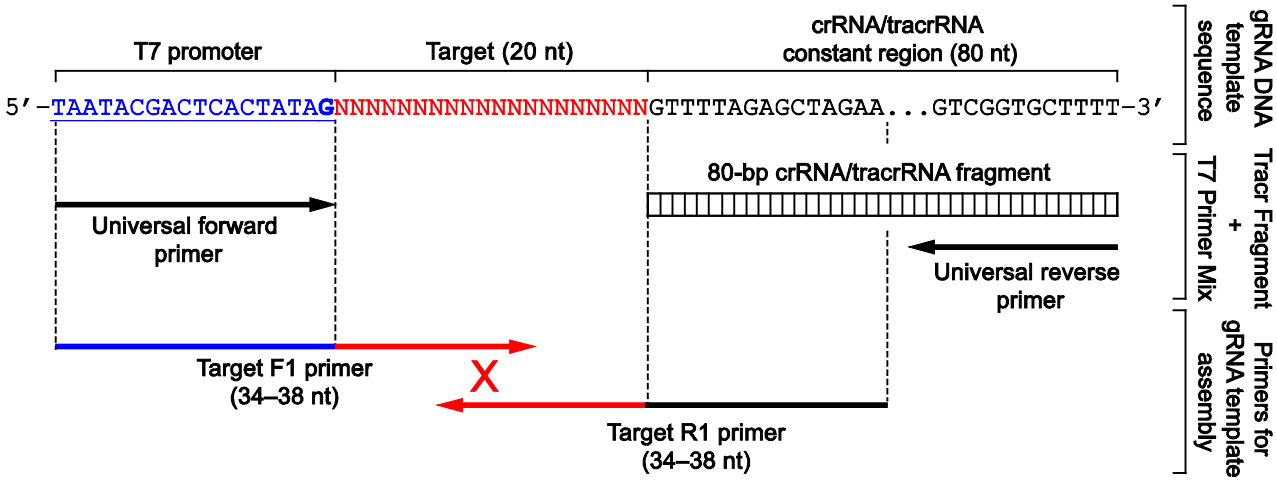
5′-TAATACGACTCACTATAGCATTTCTCAGTCCTAAACAGTTTTAGAGCTAGAAATAGCA...-3′

Design of forward and reverse oligonucleotides for PCR assembly

Once you have identified the final target sequences, you need to design the forward and reverse oligonucleotides that will be PCR assembled with the Tracr Fragment + T7 Primer Mix included in the kit to generate your gRNA DNA template.

The Tracr Fragment + T7 Primer Mix contains the universal forward and reverse amplification primers and the 80-nt constant region of the crRNA/tracrRNA (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: a Target F1 forward primer harboring the T7 promoter sequence and a Target R1 reverse primer that overlaps with the Target F1 primer and the 5′ end of the crRNA/tracrRNA constant sequence (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

oligonucleotides. By default, the Invitrogen™ TrueDesign™ Genome Editor returns forward and reverse target primer sequences that are 34-nt long.

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

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

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

- If the target sequence already contains a 5′ G, you can choose to keep it, which will result in an extra G being added from the T7 promoter primer.

  Alternatively, you can remove the first G of the target sequence, 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.

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

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...

PCR assemble the gRNA DNA template

Overview

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, page 10).

Materials required

- 0.3 µM working solution of Target F1 forward and Target R1 reverse oligonucleotide mix (see below)
- 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 Invitrogen™ TrueDesign™ Genome Editor are shipped lyophilized and must be resuspended before use.

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

Perform PCR assembly

Carefully mix and centrifuge all tubes before opening to ensure homogeneity and improve recovery. You can perform the PCR set up at room temperature.

1. Set up the following PCR assembly reaction in a 25-µL volume, adding the reaction components in the order given.

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. Perform assembly PCR using the cycling parameters below.

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

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*	1X

   * Remove the product when ready to proceed to next step.

3. *Optional checkpoint*: At this point you can 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 *in vitro* transcription to generate the gRNA, page 15.

Generate the gRNA by *in vitro* transcription

Overview

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

Materials required

- gRNA DNA template (from PCR assembly, page 14)
- 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 (Cat. No. G4010-02)
- 2X RNA Gel Loading Dye (Cat. No. R0641)
- RNA Century™ Markers (Cat. No. AM7140)
- *Optional*: Qubit™ RNA BR Assay Kit (Cat. No. Q10210)

Guidelines for IVT

- 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

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

   IMPORTANT! Add the reaction components in the order given.

NTP mix (25 mM each of ATP, GTP, CTP, UTP in Tris buffer)	8 µL
gRNA DNA template (from PCR assembly, page 14)	6 µL
5X TranscriptAid™ Reaction Buffer	4 µL
TranscriptAid™ Enzyme Mix	2 µL

   Note: You can double the amounts used in the IVT reaction, if higher gRNA yield is required.

2. Mix the reaction components thoroughly, centrifuge briefly to collect all drops, and incubate at 37°C for 2 hours.

   Note: You can extend the incubation up to 4 hours for higher gRNA yields.

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:

1. Add 1 µL of DNase I (at 1 U/µL; included in the kit) into the reaction mix immediately after the IVT reaction and incubate at 37°C for 15 minutes.

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 your purification will increase the amount of RNA recovered.

Control reactions

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.

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 and 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 (e.g., RNA Century™ Markers).

   Note: You can also check the quality of the gRNA sample by running it on a 10% Novex™ TBE-Urea Gel.
5. The expected gRNA transcript size is 100 bases. A discreet band at 100 bases indicates intact RNA.

Optional: Determine *in vitro* transcribed gRNA concentration

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

To determine the concentration of the gRNA transcript, dilute an aliquot of the gRNA transcript 1:100 in nuclease-free water before proceeding with the Qubit™ RNA BR Assay and follow the procedure described on page 18.

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

Purify the *in vitro* transcribed gRNA

Overview

After you have generated your gRNA by IVT and removed the DNA template by DNAse I digestion, purify the gRNA using the gRNA Clean Up Kit (Box 2 of the Precision gRNA Synthesis Kit). 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.

Materials required

- IVT reaction mix containing the gRNA
- gRNA Clean Up Kit (Box 2 of the Precision gRNA Synthesis Kit)
- >96% EtOH
- Nuclease-free water (user supplied)
- Microcentrifuge and 1.5- or 2-mL microcentrifuge tubes.
- *Optional*: Qubit™ RNA BR Assay Kit (Cat. No. Q10210)

Important notes

- Prior to the initial use of the gRNA Clean Up Kit, dilute the Wash Buffer 1 with 13 mL of >96% ethanol (63% final ethanol) and Wash Buffer 2 with 30 mL of >96% EtOH before use (80% final ethanol).

  After the ethanol has been added, mark the check box on the bottle to indicate the completed step.
- 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.

  Note: 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 (supplied by the user).
2. Add 100 µL of Binding Buffer. Mix thoroughly by pipetting.
3. Add 300 µL of ethanol (>96%) and mix by pipetting.
4. Transfer the mixture to the GeneJET™ RNA Purification Micro Column (pre-assembled with a collection tube) and centrifuge for 30–60 seconds at 14,000 × *g* (10,000–14,000 rpm, depending on the rotor type).
5. Discard the flow-through and place the column back into the collection tube.
6. Add 700 µL Wash Buffer 1(diluted with 13 mL of >96% ethanol) to the purification column and centrifuge for 30–60 seconds at 14,000 × *g*.
7. Discard the flow-through and place the column back into the collection tube.
8. Add 700 µL Wash Buffer 2 (diluted with 30 mL of >96% ethanol) to the purification column and centrifuge for 30–60 seconds at 14,000 × *g*.
9. Discard the flow-through and place the column back into the collection tube.
10. Repeat Step 8, discard the flow-through, and place the column back into the collection tube.

11. Centrifuge the empty purification column for an additional 60 seconds at 14,000 × *g* 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.

12. Transfer the purification column to a clean 1.5-mL Collection Tube (included).
13. Add 10 µL of nuclease-free water to the center of the purification column filter, and centrifuge for 60 seconds at 14,000 × *g* to elute the RNA.

    Note: Depending on the desired concentration of the eluted RNA, you can use from 6 µL to 20 µL of nuclease-free water for the elution step. Note that using an elution volume of <10 µL slightly decreases the RNA yield.

    Note: When purifying larger amounts of RNA (>5 µg), double the elution volume or perform two elution cycles.

14. 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

Determine the concentration of the purified gRNA using the Qubit™ RNA BR Assay Kit. NanoDrop™ spectrophotometer or an equivalent system can also be used, but we have seen up to a 2X variation in concentration estimation.

To determine the concentration of the purified gRNA using the Qubit™ RNA BR Assay Kit:

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 Qubit™ RNA BR 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 (from 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 and incubate at room temperature for 2 minutes.
6. Select RNA Broad Range Assay on the Qubit™ 2.0 Fluorometer to calibrate with standards and read the samples. The typical yield of gRNA is 10–40 µg.

Appendix 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 Scientific. Ordering information is provided below. For more information, refer to our website (**www.thermofisher.com**) or contact Technical Support (see page 23).

Product	Amount	Catalog 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 suitable for use with the Precision gRNA Synthesis Kit are available separately from Thermo Fisher Scientific. Ordering information is provided below. For more information, refer to our website (**www.thermofisher.com**) or contact Technical Support (see page 23).

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

Products for CRISPR/Cas9-mediated genome editing

Thermo Fisher Scientific 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 (see page 23).

Product	Amount	Catalog 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™ Cas9 Transfection Kit	0.75 mL	CMAX00008
Lipofectamine™ RNAiMAX™ Transfection Reagent	0.75 mL	13778-075
Lipofectamine™ MessengerMax™ Reagent	0.3 mL	LMRNA003

Appendix B: Safety

General 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, etc.). To obtain SDSs, see visit **thermofisher.com/support**.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, Biosafety in Microbiological and Biomedical Laboratories (BMBL), 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020 www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associatedmonographs) www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Appendix C: Documentation and support

Customer and technical support

Visit **www.thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
- Order and web support
- Product documentation, including:
  - 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/or its affiliate(s)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 any questions, please contact Life Technologies at **www.thermofisher.com/support**.