Takara Bio USA, Inc.

Guide-it™ sgRNA In Vitro Transcription and Screening Systems User Manual

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(100824)

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I. Introduction

A. Summary

CRISPR/Cas9 is a breakthrough genome editing technology that permits site-specific cleavage of DNA targets in the genomes of various organisms and mammalian cells. Disruptive mutations are created at those cleavage sites by error-prone repair or by using homologous recombination to change or insert sequences. The power of this technology derives from its simplicity, since all it requires is a Cas9 nuclease enzyme combined with a single guide RNA (sgRNA) that determines its target specificity. Each sgRNA is designed by the user to contain a short sequence homologous to a target sequence of choice, with which it directs the Cas9 nuclease to introduce a double-stranded break at that sequence (Figure 1).

This user manual describes how to use the following kits:

- The **Guide-it sgRNA *In Vitro* Transcription Kit** (Cat. No. 632635) is used to produce high yields of sgRNAs following *in vitro* transcription (IVT) reactions. This kit includes the **Guide-it IVT RNA Clean-Up Kit** (Cat. No. 632638) to purify sgRNA for direct use in transfection, electroporation, or *in vitro* cleavage assays.
- The **Guide-it IVT RNA Clean-Up Kit** (Cat. No. 632638) is a phenol-free kit used to purify sgRNA following *in vitro* transcription (IVT) reactions. The purified sgRNA is ready for use in transfection, electroporation, or *in vitro* cleavage assays.
- The **Guide-it sgRNA Screening Kit** (Cat. No. 632639) enables the user to test the efficacy of different sgRNAs *in vitro* prior to using them in studies involving Cas9-mediated gene editing. With this kit, a template containing an sgRNA target site is created by PCR, then combined with an sgRNA that you wish to test and Cas9 nuclease. The efficiency with which Cas9 nuclease cleaves the template can be measured using agarose gel electrophoresis.
- The **Guide-it Complete sgRNA Screening System** (Cat. No. 632636) is a combination of the **Guide-it sgRNA *In Vitro* Transcription Kit** (Cat. No. 632635) and **Guide-it sgRNA Screening Kit** (Cat. No. 632639) described above and is used to produce and test the efficacy of sgRNAs. The kit contains all of the components needed to *in vitro* transcribe sgRNAs, purify the sgRNAs, and test their efficacy against a PCR target fragment using recombinant Cas9 nuclease.

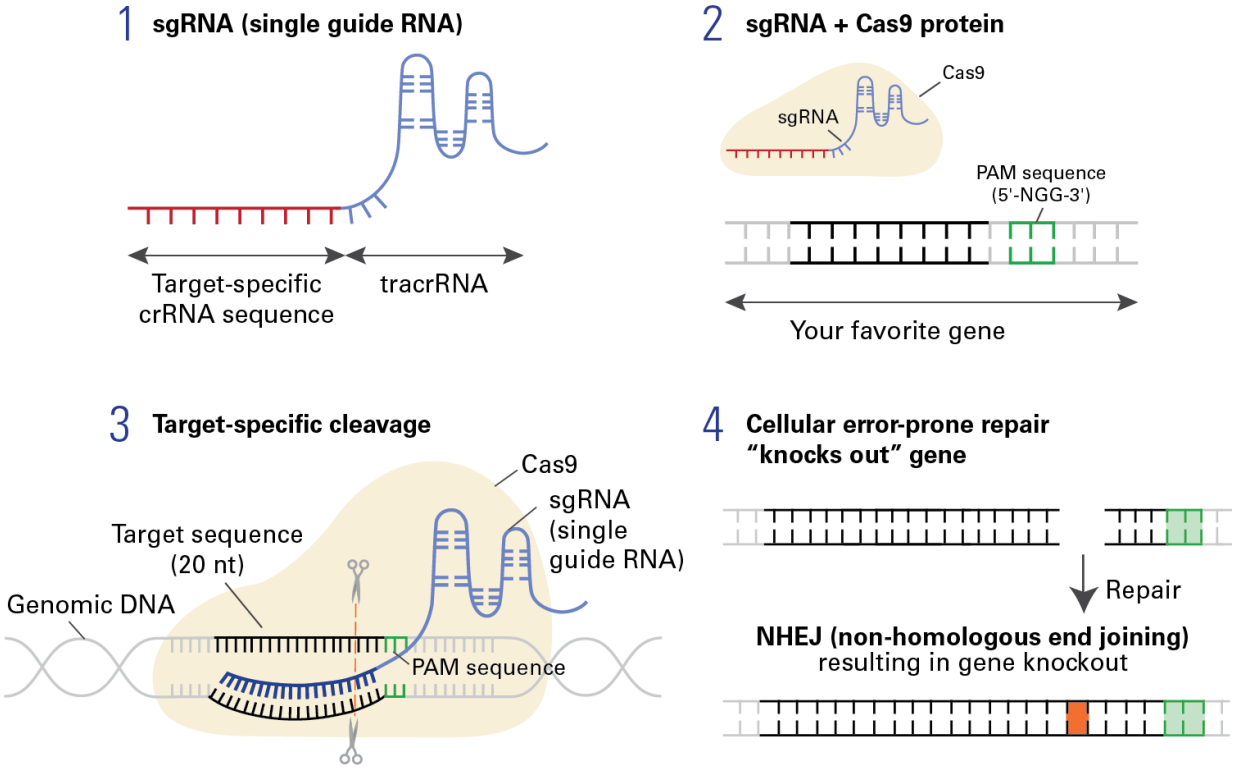


Figure 1. Using Guide-it technology to perform genome editing. An sgRNA consists of a single RNA strand with a crRNA sequence that binds to a specific DNA target, and a tracrRNA sequence that binds to Cas9 protein. When an sgRNA engineered to contain a 20-nucleotide (nt) target sequence of interest binds to a recombinant form of Cas9 protein that has double-stranded DNA endonuclease activity, the resulting complex will produce target-specific double-stranded cleavage. Cellular repair, which is error-prone, will take place at the cleavage site, and may result in a mutation that can knock out a gene.

B. Protocol Overview

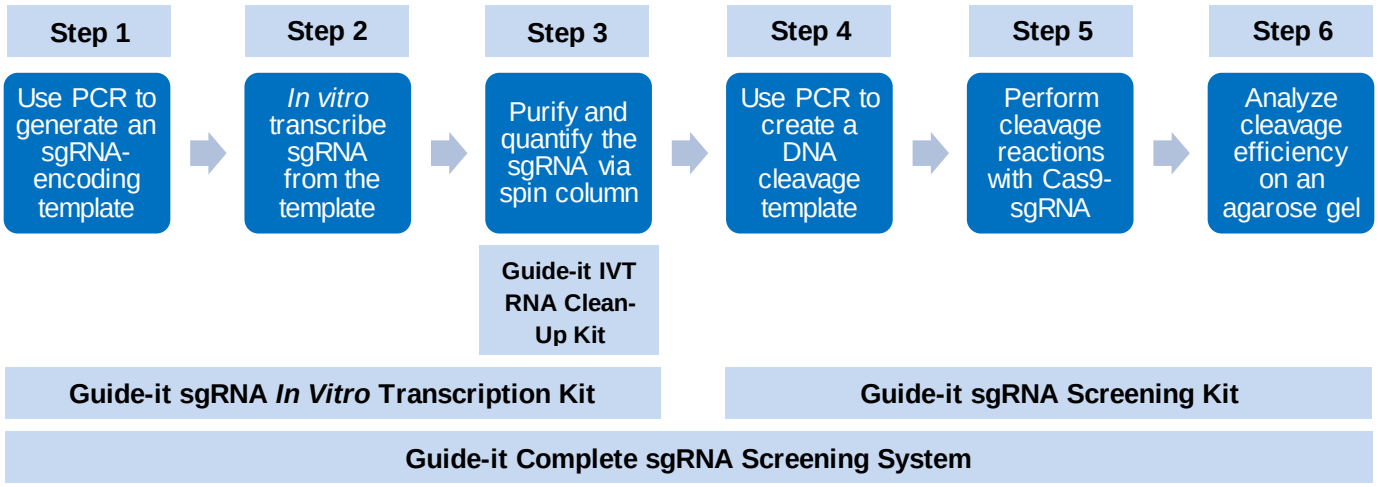


Figure 2. Guide-it Complete sgRNA Screening System protocol.

The Guide-it Complete sgRNA Screening System can be used to synthesize and test the efficacy of sgRNAs as follows (Figure 2):

1. Generate a DNA template that contains your sgRNA-encoding sequence under the control of a T7 promoter by performing a PCR reaction with the included Guide-it Scaffold Template and a primer you design (Section V.A).
2. *In vitro* transcribe this template with the included Guide-it T7 Polymerase Mix to create an sgRNA containing your target sequence (Section V.B).
3. Purify your sgRNA after digestion with Recombinant DNase I (RNase-Free) using the Guide-it IVT RNA Clean-Up Kit; and measure its concentration using a NanoDrop 2000 spectrophotometer or equivalent (Section VI).
4. Create a cleavage template for screening your purified sgRNA by amplifying a 600- to 800-bp fragment of genomic DNA that contains your target sequence in an asymmetric position that will produce two cleavage fragments of unequal size (Sections VII.A–C).
5. Perform a cleavage reaction on this template using your purified sgRNA in combination with the included Guide-it Recombinant Cas9 Nuclease (Section VII.D).
6. Analyze the efficiency of your cleavage reactions on an agarose gel to determine if your target sequence was successfully incorporated (Section VII.D).

II. List of Components

- Store Guide-it IVT RNA Clean-Up Kit at room temperature. Store all other components at –20°C.
- Avoid repeated freeze/thaw cycles.

Guide-it sgRNA In Vitro Transcription Kit (Cat. No. 632635; 50 rxns)

Guide-it sgRNA *In Vitro* Transcription Components v2 (Cat. No. 632637; 50 rxns; Not sold separately)

50 µl	Guide-it Scaffold Template (1 ng/µl)
350 µl	Guide-it *In Vitro* Transcription Buffer
1 ml	RNase Free Water
150 µl	Guide-it T7 Polymerase Mix (33 U/µl)
625 µl	PrimeSTAR® Max Premix (2X)
100 µl	Recombinant DNase I (RNase-Free) (5 U/µl)

Guide-it IVT RNA Clean-Up Kit (Cat. No. 632638; 50 rxns)

13 ml	RNase Free Water
3 ml	IVT Binding Buffer
50 columns	IVT RNA Clean-Up Spin Columns
2 x 6 ml	IVT Wash Buffer
50 tubes	Collection Tubes (2 ml)

Guide-it IVT RNA Clean-Up Kit (Cat. No. 632638; 50 rxns)

13 ml	RNase Free Water
3 ml	IVT Binding Buffer
50 columns	IVT RNA Clean-Up Spin Columns
2 x 6 ml	IVT Wash Buffer
50 tubes	Collection Tubes (2 ml)

Guide-it sgRNA Screening Kit (Cat. No. 632639; 50 rxns)

25 µl	Guide-it Recombinant Cas9 Nuclease (500 ng/µl)
50 µl	15X Cas9 Reaction Buffer
50 µl	15X BSA
15 µl	Control sgRNA (50 ng/µl)
150 µl	Control Fragment (20 ng/µl)
50 µl	Terra™ Direct PCR Polymerase Mix (1.25 U/µl)
1.25 ml	2X Terra Direct PCR Buffer (with Mg^{2+}, dNTP)
5 ml	RNase Free Water
5 ml	Extraction Buffer 1
0.5 ml	Extraction Buffer 2

Guide-it Complete sgRNA Screening System (Cat. No. 632636; 50 rxns)

Guide-it sgRNA *In Vitro* Transcription Components v2 (Cat. No. 632637; 50 rxns; Not sold separately)

50 µl	Guide-it Scaffold Template (1 ng/µl)
350 µl	Guide-it *In Vitro* Transcription Buffer
1 ml	RNase Free Water
150 µl	Guide-it T7 Polymerase Mix (33 U/µl)
625 µl	PrimeSTAR® Max Premix (2X)
100 µl	Recombinant DNase I (RNase-Free) (5 U/µl)

Guide-it IVT RNA Clean-Up Kit (Cat. No. 632638; 50 rxns)

13 ml	RNase Free Water
3 ml	IVT Binding Buffer
50 columns	IVT RNA Clean-Up Spin Columns
2 x 6 ml	IVT Wash Buffer
50 tubes	Collection Tubes (2 ml)

Guide-it sgRNA Screening Kit (Cat. No. 632639; 50 rxns)

25 µl	Guide-it Recombinant Cas9 Nuclease (500 ng/µl)
50 µl	15X Cas9 Reaction Buffer
50 µl	15X BSA
15 µl	Control sgRNA (50 ng/µl)
150 µl	Control Fragment (20 ng/µl)
50 µl	Terra Direct PCR Polymerase Mix (1.25 U/µl)
1.25 ml	2X Terra Direct PCR Buffer (with Mg^{2+}, dNTP)
5 ml	RNase Free Water
5 ml	Extraction Buffer 1
0.5 ml	Extraction Buffer 2

III. Additional Materials Required

The following materials are required but not supplied:

- 96–100% ethanol
- Isopropanol
- 1.5-ml microcentrifuge tubes
- 200-µl PCR tubes
- NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Cat. No. ND-2000) or equivalent

IV. *In Vitro* Transcription of an sgRNA Containing the Desired Target Sequence

Create an sgRNA containing your target sequence as follows:

1. Using the included Guide-it Scaffold Template and a primer you design (Section IV.A), PCR amplify a DNA template that contains your sgRNA encoding sequence under the control of a T7 promoter (Section V.A).
2. Perform an *in vitro* transcription reaction with the PCR product from Step 1 to generate your sgRNA (Section V.B), then purify and quantify the newly synthesized sgRNA (Section VI.A).

A. Guidelines for Designing PCR Primers

Use the following guidelines to design a forward primer to be used in a PCR reaction with the included Guide-it Scaffold Template to create a DNA template for *in vitro* transcription of your sgRNA. This primer should contain the T7 promoter sequence, followed by your sgRNA target sequence, and the Guide-it Scaffold Template-specific sequence (Figure 3).

Choosing the correct DNA target sequence

Choose the DNA target sequence that will correspond to your actual sgRNA target sequence as shown in Figure 3, Panel A, according to the following guidelines:

a. The DNA target sequence you choose must end with the proto-spacer adjacent motif (PAM) sequence, NGG, on its 3' end. Only DNA sequences that are 20 nucleotides upstream of a PAM sequence can be used for CRISPR/Cas9.

b. Any target sequence can be used if the sequence is followed by the PAM sequence, NGG. However, to minimize off-target cleavage events, the entire target sequence (including the PAM) should have at least three base mismatches with any other non-targeted genomic sequence. Off-target events should be especially low if the mismatches are in, or adjacent to, the PAM. The majority of online tools for sgRNA design will predict off-target sequences for a given sgRNA target sequence. To learn more, visit http://www.clontech.com/sgRNA-design-tools.

Designing a 56- to 58-nt forward PCR primer

The forward (sense) primer must contain the following four sequence elements, as shown in Figure 3, Panel B.

a. A T7 promoter sequence plus four extra bases (21 total nt) at the 5' end of the primer.

b. A transcription initiation site (0–2 guanine (G) residues): The number of Gs added is dependent on the 5' end of the target sequence. The T7 promoter requires at least two Gs for efficient transcription.

NOTE: If your specific target sequence (see item c, below) already contains two Gs, there is no need to add extra Gs for transcription initiation. Extra Gs could reduce cleavage efficiency.

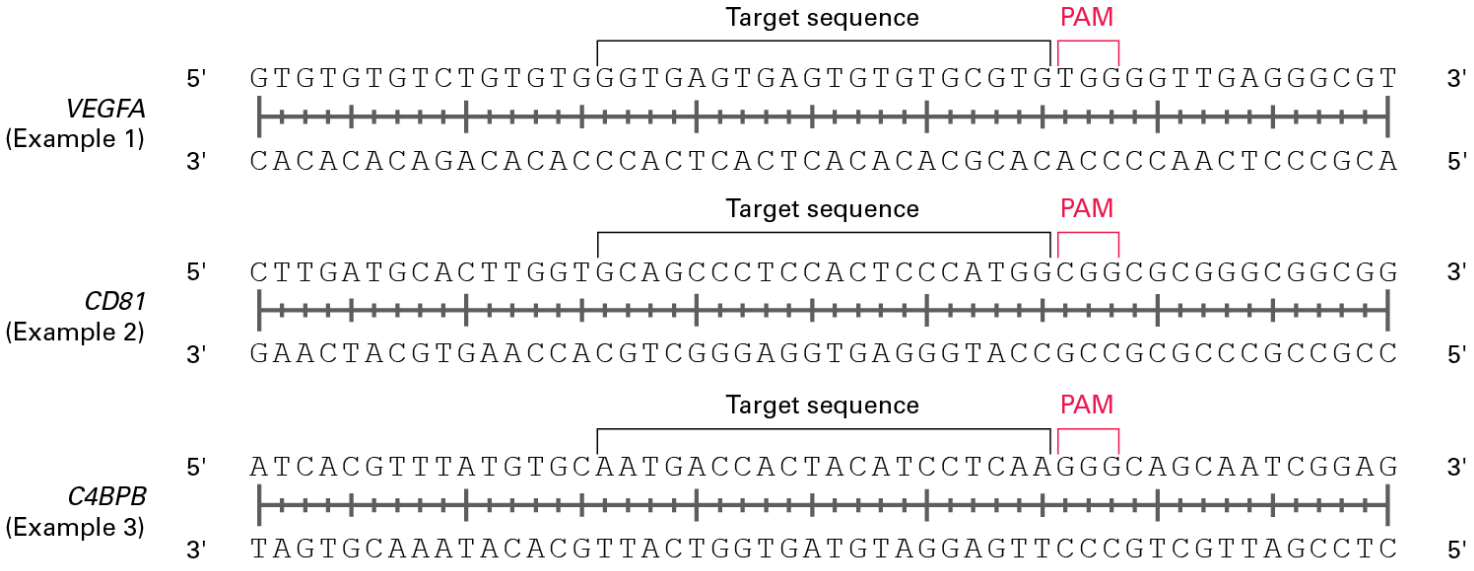
c. Your specific sgRNA target sequence (20 nt).

d. The Guide-it Scaffold Template-annealing sequence (15 nt at the 3'end of the primer)

NOTES:

- A reverse (antisense) primer comes premixed with the Guide-it Scaffold Template.
- The forward primer should be subjected to salt-free purification following synthesis and diluted to a concentration of 10 µM in PCR-grade water.

A Choosing a DNA target sequence



B Designing a 56-to 58-nt forward primer to create the DNA template for your sgRNA

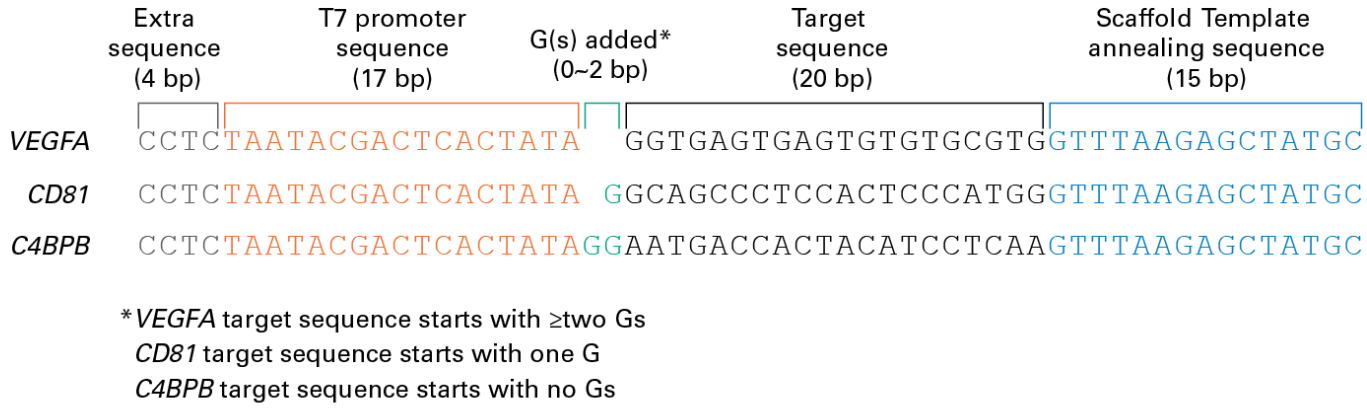


Figure 3. Designing a forward PCR primer to generate a DNA template for an sgRNA containing your target sequence. **Panel A.** Choose the DNA target sequence that will correspond to your actual sgRNA target sequence. **Panel B.** Design the forward primer to create the *in vitro* transcription template you will use to generate your sgRNA. Do not include the PAM sequence between your 20 bp target sequence and the Scaffold Template annealing sequence. (**NOTE:** The T7 promoter sequence (with four extra bases) and the Scaffold Template-specific sequence do **not** change.)

V. Protocol: *In Vitro* Transcription of sgRNA

For use with the Guide-it sgRNA *In Vitro* Transcription Kit.

A. PCR Amplification of sgRNA Template

1. Combine the following components in a 200-µl PCR tube. Briefly vortex and spin down to collect the reagents at the bottom of the tube.

Reagent	Amount (µl)
PrimeSTAR Max Premix (2X)	12.5
Guide-it Scaffold Template	1
Your forward primer (10 µM)	0.5
RNase Free Water	11
Total	25

2. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

33 cycles:
98°C 10 sec
68°C 10 sec
4°C forever

3. Run and analyze 5 µl of the PCR product on a 2% agarose gel with a 100-bp DNA ladder. You should see a single band at ~130 bp (see Figure 4).

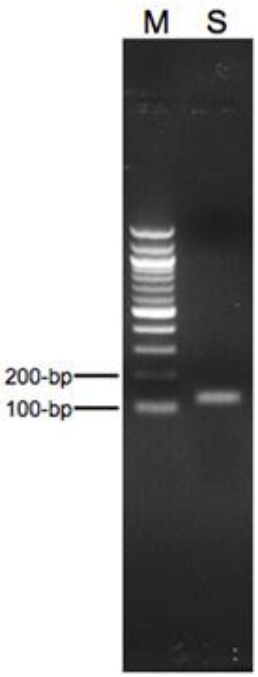


Figure 4. Gel electrophoresis of the PCR product. M = 100-bp DNA ladder. S = 5 µl of sample.

B. *In Vitro* Transcription (IVT) Reaction

The PCR product amplified in Section V.A Step 2 is directly used as template for the IVT reaction without purification.

1. Combine the following components in a 200-µl PCR tube. Briefly vortex and spin down to collect the reagents at the bottom of the tube.

Reagent	Amount (µl)
sgRNA PCR template (Step A2)	5
Guide-it *In Vitro* Transcription Buffer	7
Guide-it T7 Polymerase Mix	3
RNase Free Water	5
Total	20

NOTE: If you require a higher amount of sgRNA, you can scale up the total reaction size (e.g., to 50 µl) without changing the quality of the sgRNA.

2. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

37°C	4 hr
4°C	forever

NOTE: We recommend a 4-hr incubation, but a shorter incubation time is acceptable if you do not need to maximize your sgRNA yield (see Figure 5). We have observed a clear, sharp band via Agilent Bioanalyzer following 8 hr of incubation, indicating no drop in sgRNA quality.

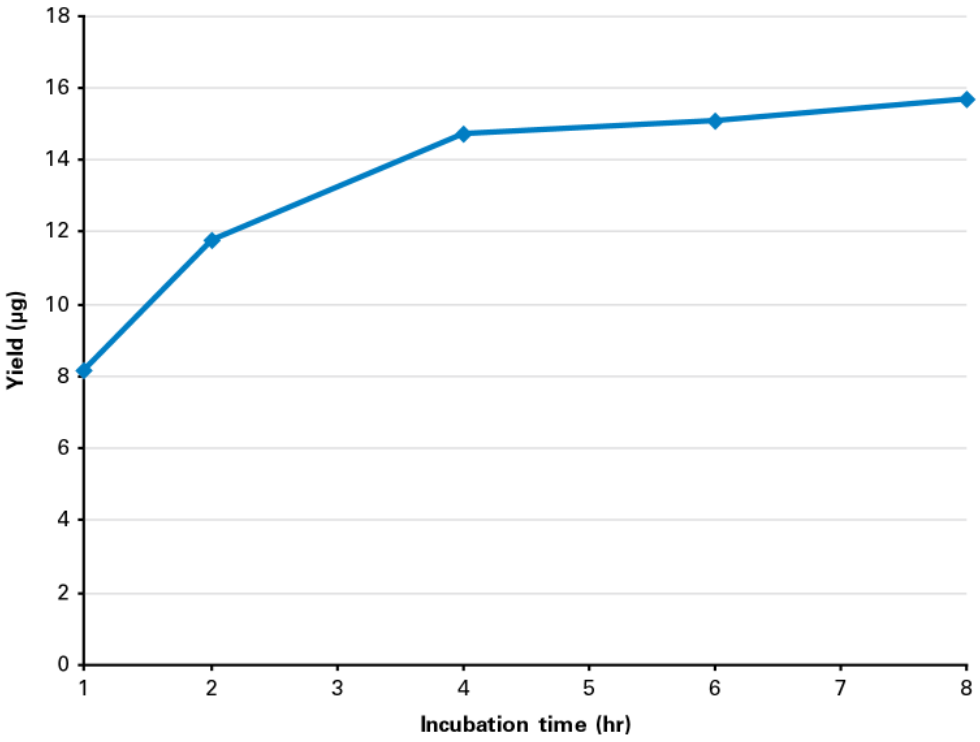


Figure 5. sgRNA yield over time with the Guide-it sgRNA *In Vitro* Transcription Kit. 20 µl of sgRNA IVT reactions were incubated for different times at 37°C. The reactions were purified using the Guide-it IVT RNA Clean-Up Kit and quantified using a NanoDrop spectrophotometer.

3. Following incubation, add 2 µl of Recombinant DNase I (RNase-Free) to the 20-µl IVT reaction. Briefly vortex and spin down to collect the reagents at the bottom of the tube.
4. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

37°C	15 min
4°C	forever

VI. Protocol: Purification of Transcribed sgRNA

For use with the Guide-it IVT RNA Clean-Up Kit.

NOTE: Before purifying your sgRNA, prepare the IVT Wash Buffer by adding 24 ml of 96–100% ethanol.

1. Add 78 µl of RNase Free Water to the reaction mixture (Section V, Step B4) for a total volume of 100 µl. Transfer all 100 µl to a 1.5-ml microcentrifuge tube.
2. Add 30 µl of IVT Binding Buffer and vortex for 5 sec.
3. Add 130 µl of isopropanol and vortex for 5 sec.
4. Place an IVT RNA Clean-Up Spin Column in a Collection Tube and load the sample from Step 3 onto the column. Centrifuge at 11,000*g* for 30 sec at room temperature.
5. Discard the flowthrough and place the column back in the Collection Tube.
6. Add 600 µl of IVT Wash Buffer and centrifuge at 11,000*g* for 30 sec at room temperature.
7. Discard the flowthrough and place the column back in the Collection tube.
8. Add 250 µl of IVT Wash Buffer and centrifuge at 11,000*g* for 2 min at room temperature.
9. Place the IVT RNA Clean-Up Spin Column in a new 1.5-ml microcentrifuge tube.
10. Add 20 µl of RNase Free Water directly onto the silica membrane of the Spin Column and incubate for 1 min at room temperature.

NOTE: With an elution volume of 20 µl, a concentration of >0.5 µg/µl is expected. This is appropriate for applications such as transfection, electroporation, and in vitro cleavage assay. If you need a higher concentration of sgRNA, the elution volume can be reduced down to 5 µl (see Table I, below).

Table I. Examples of sgRNA concentration and yield using different elution volumes. Different elution volumes were used for purification of sgRNA using the Guide-it IVT RNA Clean-Up Kit. A smaller elution volume can be used without a significant loss of total yield of sgRNA if the higher sgRNA concentration is required for your experiment.

Elution volume	5 µl	10 µl	20 µl
Concentration	2.2 µg/µl	1.3 µg/µl	0.7 µg/µl
Yield	11.2 µg	12.8 µg	13.2 µg

11. Centrifuge at 11,000*g* for 1 min at room temperature.
12. Use 1 µl to measure the OD using a NanoDrop Spectrophotometer (or equivalent). A yield of 10–20 µg of sgRNA is expected.

VII. Protocol: Screening sgRNAs for Effective Cleavage of their Targets

For use with the Guide-it sgRNA Screening Kit.

NOTE: The screening kit includes sufficient reagents for 50 preps of genomic DNA in a 48-well format. Choose a format appropriate for your cells and use the appropriate method below (Section A or B) for DNA extraction. Cells that are 50–90% confluent are preferred.

A. Extraction of Genomic DNA (Individual Sample Method)

In this method, cells are harvested from wells prior to crude DNA extraction.

1. Harvest your cells from their individual wells and add an appropriate volume of Extraction Buffer 1 to the cell pellet, depending on the plate type used (see Table II, below). Vortex briefly.
2. Incubate at 95°C for 10 min.
3. Add an appropriate volume of Extraction Buffer 2 (see Table II, below). Vortex briefly.
4. Dilute the lysate (Step 3) 1:9 with RNase Free Water (e.g., 5 µl of lysate + 45 µl of RNase Free Water).

   NOTE: Depending on the amplification efficiency of the target, a range of dilutions (from undiluted to 1:50) may be required to achieve clear amplification results.

5. The diluted lysate contains genomic DNA and will be used directly for PCR (Section VIIC).

B. Extraction of Genomic DNA (Plate Method)

In this method, crude DNA extracts are prepared directly from cells while in the plate wells.

1. Remove the medium from the wells of the plate. Add an appropriate volume of Extraction Buffer 1 (see Table II, below).
2. Incubate at 70°C for 10 min.
3. Vortex for 5 min.
4. Add an appropriate volume of Extraction Buffer 2 (see Table II, below) and vortex for 30 sec.
5. Move the lysate (Step 3) to a fresh 1.5-ml microcentrifuge tube.
6. Dilute the lysate 1:9 with RNase Free Water (e.g., 5 µl of lysate + 45 µl of RNase Free Water).

   NOTE: Depending on the amplification efficiency of the target, a range of dilutions (from undiluted to 1:50) may be required to achieve clear amplification results.

7. The diluted lysate contains genomic DNA and will be used directly for PCR (Section VIIC).

Table II. Reference volumes for genomic DNA extraction.

Plate type (wells)	96	48	24	12
Extraction Buffer 1	36 µl	90 µl	180 µl	360 µl
Extraction Buffer 2	4 µl	10 µl	20 µl	40 µl

C. PCR Amplification of Target DNA

1. Design primers to amplify the region targeted by CRISPR/Cas9. The optimal amplicon size is 600–800 bp, with the sgRNA target sequence located asymmetrically within the amplicon; each cleavage fragment should be at least 250 bp, and there should be a >100 bp size difference between the fragments after Cas9 cleavage (Figure 6). These guidelines ensure efficient amplification and good assay resolution.

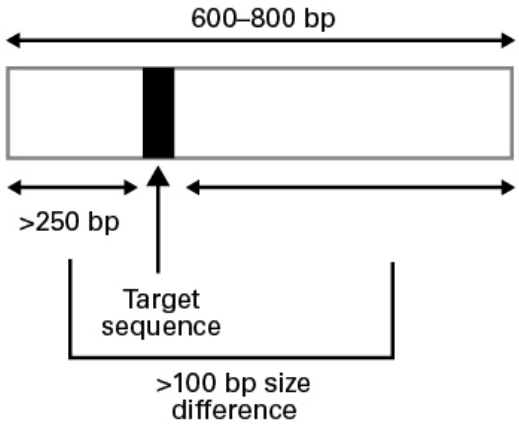


Figure 6. PCR amplicon design.

2. Combine the following components in a 200-µl PCR tube. Briefly vortex and spin down to collect the reagents at the bottom of the tube.

Reagent	Amount (µl)
2X Terra Direct PCR Buffer (with Mg^{2+}, dNTP)	25
Forward primer (10 µM)	1.5
Reverse primer (10 µM)	1.5
Terra Direct PCR Polymerase Mix (1.25 U/µl)	1
RNase Free Water	19
Prepared lysate (Section VIIA, Step 5 or Section VIIB Step 7)	2
Total	50

3. Place reactions in a preheated thermal cycler with a heated lid and run the following program:

	98°C	2 min
30–40 cycles:		
	98°C	10 sec
	60°C	15 sec
	68°C	1 min/kb
	4°C	forever

NOTE: Adjustments to the cycling conditions may be necessary, depending on your target fragment.

4. Use 5 µl of the PCR product for agarose gel electrophoresis; run the sample on a 1.5–2% agarose gel.

NOTE: Make sure that your PCR yields a strong, single band; 100–250 ng of PCR product (in a volume of ≤5 µl) will be required for the subsequent Cas9 cleavage assay. Using this amount ensures that the bands remain easily visible after the cleavage assay without overwhelming the Cas9/sgRNA.

D. Cas9 Cleavage Assay

1. Combine the following components in a 200-µl PCR tube. If you have multiple samples, make a master mix.

Reagent	Amount (µl)
Target-specific sgRNA (50 ng/µl) or Control sgRNA (50 ng/µl)	1
Guide-it Recombinant Cas9 Nuclease (500 ng/µl)	0.5
Total	1.5

2. Mix well, very gently, by pipetting. Incubate using a thermal cycler with the following conditions:

37°C	5 min
4°C	forever

3. Combine the following components in a 200-µl PCR tube in the order listed. If you have multiple samples, make a master mix.

NOTE: The volume of PCR sample used should not exceed 5 µl; carryover of PCR reaction buffer might inhibit Cas9 activity. The optimal range of DNA input is 100–250 ng.

Reagent	Amount (µl)
PCR reaction solution (100–250 ng) or Control Fragment	Up to 5
15X Cas9 Reaction Buffer	1
15X BSA	1
RNase Free Water	6.5
Cas9/sgRNA mix (from Step 2 above)	1.5
Total	15

4. Mix well, very gently, by pipetting. Incubate using a thermal cycler with the following conditions:

37°C	1 hr
80°C	5 min
4°C	forever

5. Add an appropriate volume of loading buffer and run the entire sample on a 1.5–2% agarose gel along with an appropriate DNA marker.

NOTE: The sgRNA may also be slightly visible after agarose gel electrophoresis, usually appearing near the 130-bp position in a 1.5–2% agarose gel. If this interferes with assay interpretation, the reaction samples can be treated with RNase (e.g., 5 µg RNase for 30 min at 37°C) prior to loading the gel.

6. Analyze the results. Refer to Figure 7 for assay interpretation.

NOTE: The control fragment included in this kit has a size of 614 bp; it is cleaved by the control sgRNA/Cas9 complex to form two fragments that are 350 bp and 264 bp, respectively.

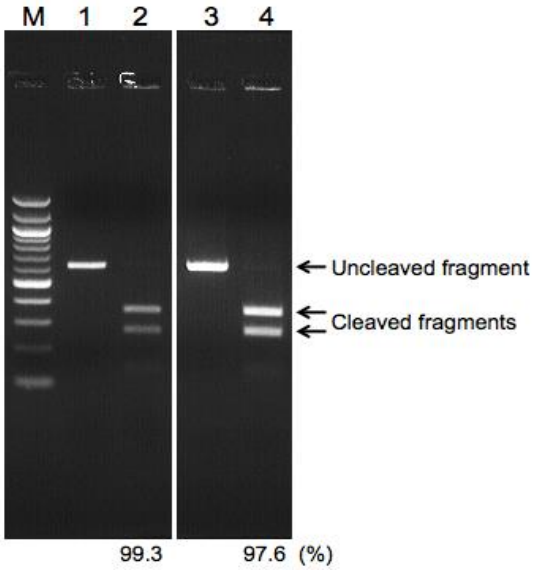


Figure 7. Analysis of cleavage products. An sgRNA sequence was synthesized and tested against its target in human chromosome 1. A 614-bp target fragment was amplified, and an sgRNA designed to cleave the amplified sequence was *in vitro* transcribed (using the Guide-it Complete sgRNA Screening System). The target fragment, the sgRNA, and recombinant Cas9 enzyme (also included in kit) were combined in an *in vitro* cleavage reaction according to the protocol. Densitometer analysis of the agarose gel indicated that this sgRNA directed Cas9-mediated cleavage of the target with 97.6% efficiency (Lane 4). Lane 1 shows the untreated Control Fragment, Lane 2 shows the treated Control Fragment, Lane 3 shows the untreated target fragment, and Lane 4 shows the treated target fragment.

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