IG® PE pegRNA Synthesis Kit

Manual

Catalog #	3404	3406
Volume	20 µl RXN	100 µl RXN
Package Size	10 reactions	

Important!

-20°C Storage Required

* Immediately inspect packages
* Freeze upon receipt

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Intact Genomics, Inc.

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Description:

Intact Genomics is your resource for *in vitro* gene editing using CRISPR Cas systems. Prime-editing is gaining popularity as a precision gene-editing technique. Prime-editing (PE) enzymes are a fusion of the Cas9 nickase mutant (H840A) with a modified Murine Moloney Leukemia Virus-Reverse Transcriptase (MMLV-RT) (1,2). One of the resulting optimized prime-editing fusions is known as PEmax enzyme (3). Prime-editing enzymes enable precise gene-editing without the need for double-stranded DNA breaks (DSBs) or donor DNA templates. PE enzymes require a unique long gRNA known as a "pegRNA" shown schematically in Fig.1.

We understand your need to generate sufficient quantities of pegRNA for *in vitro* gene editing projects. The IG® PE pegRNA synthesis kit is simple to use, and is scalable to help you reach necessary yields of pegRNA. The kit can be purchased with an optional IG® RNA Cleanup Kit (Cat.#4003 or #4005) for purification of pegRNA.

The process of pegRNA synthesis by *in vitro* transcription (IVT) is intricate but allows for successful creation of longer pegRNAs (e.g. 100 nt or more) that cannot be easily chemically synthesized and purified at a low cost. pegRNA synthesis is often complicated, and the maximum yield of pegRNA depends on the edit region. Only Intact Genomics provides an IVT pegRNA synthesis kit for PE enzymes. Our kit provides at least 7ug of pegRNA per 20 µL reaction, enough for *in vitro* DNA sequence editing in most cases. This kit also provides a protocol for an optional 100 µL pegRNA synthesis volume to achieve more yield. Higher yields from the IG® PE pegRNA synthesis kit will enhance your chances of experimental success.

Intact Genomics is with you every step of the way to complete your projects. We offer expert advice, fast ordering and delivery, and product customization. For successful pegRNA synthesis, a scientist on your team must identify a DNA sequence to edit and order unique primers targeting that sequence. (See oligo design instructions in this manual.) From there, a simple workflow details the *in vitro* pegRNA synthesis steps, and you may progress to prime-editing using PE enzyme, optionally, IG® PEmax enzyme (Cat.#3473 or #3476).

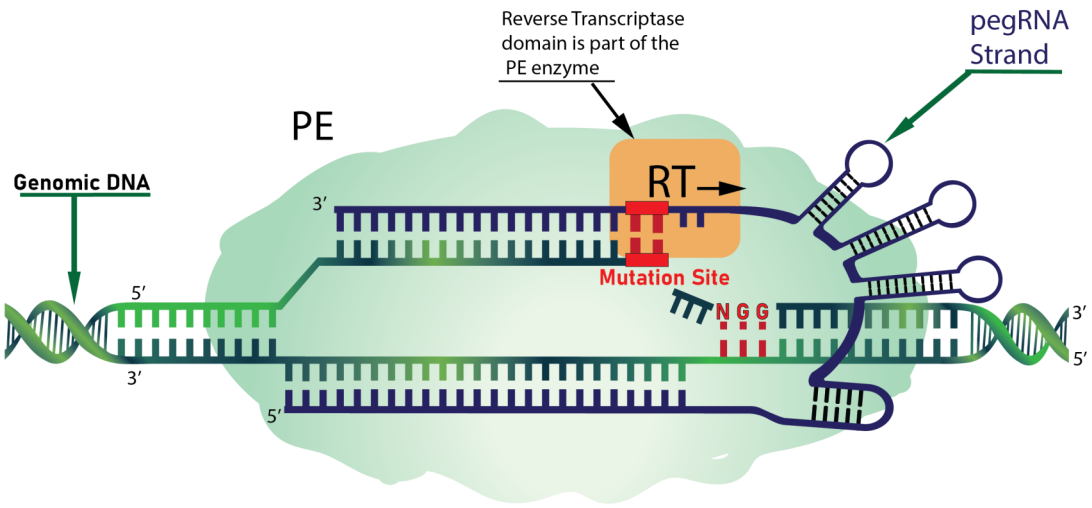

Figure 1. Schematic diagram of prime-editing two bases in genomic DNA (in red) by a PE enzyme and pegRNA.

Benefits:

The IG® PE pegRNA Synthesis Kit is a perfect choice for a variety of pegRNA synthesis needs. Below is a sampling of some of the key benefits.

- IG® PE pegRNA Synthesis Kit includes everything but your target oligos (all buffers, enzymes, scaffolds).
- User simply needs to provide the oligos for your target: a pair of sgRNA oligo and pegRTpbs oligo generated using the template design in this manual.
- Rapid Workflow (Less than 1 hour)
- Scalable Yield (100 µl reaction)
- Customer support – our team is available to aid with your success

Product Components and Storage:

IG® PE pegRNA Synthesis Kit contains the items below. Store all components at -20°C.

- IG® PE pegRNA Enzyme Mix (10X)
- IG® PE pegRNA Reaction Mix. (4X)
- igDNase I (RNase-free, 2 Units/µL)
- IG® PE pegRNA Control Oligo (1 µM)
- Dithiothreitol (DTT, 0.2 M)
- NTPs mix (25 mM)

Items needed but not provided in this kit:

- Nuclease-Free Water
- Custom nucleotide oligos **(see instructions below to design your oligos)**
- Nuclease-free pipet tips and microcentrifuge tubes

IG® PE pegRNA Synthesis Protocol:

Step 1: Design your custom pair of sgRNA oligo and pegRTpbs oligo.

These steps will help to design the sequence of the target DNA oligos needed to use the kit. These oligos are not included and should be ordered and manufactured separately to use with this kit.

1. Use a target site selection webtool to find a pair of sgRNA and pegRTpbs oligos following the 5' PAM 'NGG' in your target DNA. (pegFinder: http://pegfinder.sidichenlab.org/ for example). We use a pUC19 sequence BELOW as an example to change "CC" into "AA":
   GTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTG(CC/AA)
   TTCCTGTTTTTGCTCACCCAGAAACGCT GGTGAAAGTAAAAGATGCTGA
2. Webtools provide recommendations for DNA templates needed for pegRNA design. For example:

   sgRNA (This is the genomic DNA target sequence) : CTGGGTGAGCAAAAACAGGA
   RT template (12 nt) (RT, Reverse Transcriptase): ATTTTGAATTCC
   PBS (14 nt) (PBS, Primer Binding Site): TGTTTTTGCTCACC
   Sense 3' extension (RT+PBS): ATTTTGAATTCCTGTTTTTGCTCACC
   An example of a full-length cDNA template that is assembled and transcribed into pegRNA by combining custom oligos with the IGÒ PE pegRNA Synthesis Kit is shown below:
   CTGGGTGAGCAAAAACAGGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTG
   AAAAAGTGGCACCGAGTCGGTGCATTTTGAATTCCTGTTTTTGCTCACC

Here is a break down of the design steps:

A. A target-specific DNA sequence for sgRNA/PE enzyme is selected.

Example: 5' CTGGGTGAGCAAAAACAGGAAGG 3'

Remove these three nucleotides: the PAM sequence (NGG, which are NOT highlighted in red) is required for PE enzyme recognition of the target site and is NOT part of the sgRNA sequence as BELOW:

5' CTGGGTGAGCAAAAACAGGA3'

B. Add "G" to the 3' end of a T7 promoter sequence highlighted in blue to ensure transcription, because at least one G is necessary for efficient T7 RNA polymerase binding. Then, attach it to the above DNA sequence of the specific sgRNA below:

5' TTCTAATACGACTCACTATA**G**CTGGGTGAGCAAAAACAGGA3'

C. Append the 14 nucleotide-overlap DNA sequence shown in black. The Blue and Black sequences are always the same. Your customized oligo will have a different Red sequence. A DNA oligo with a total length of 55 bp is to be ordered. Example shown BELOW: PEpUC19pegR11

5' TTCTAATACGACTCACTATA**G**CTGGGTGAGCAAAAACAGGAGTTTTAGAGCTAGA 3'

D. The IG® PE pegRNA synthesis kit includes the oligo shown below. This sequence matches the *S.pyogenes* Cas9 scaffold DNA sequence: PE-scaffold-HP

5'GCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAA AC 3'

E. Prefix the 14 nucleotide-overlap DNA sequence shown in black (always the same) with pegRT template and PBS sequences (Sense 3' extension). Your custom oligos will have different purple and blue DNA sequences. A DNA oligo of a total length of 40 bp is to be ordered. Our example is shown below for PEpUC19pegRTpbs:

5'CACCGAGTCGGTGCATTTTGAATTCCTGTTTTTGCTCACC 3'

When setting up the IG® sgRNA synthesis reaction, the overlapped DNA oligos are oriented as shown below:

5'TTCTAATACGACTCACTATAGCTGGGTGAGCAAAAACAGGAGTTTTAGAGCTAGA……
……
3'……
CAAAATCTCGATCTTTATCGTTCAATTTTATTCCGA

TCAGGCAATAGTTGAACTTTTTCACCGTGGCTCAGCCACG……3'

……CACCGAGTCGGTGCATTTTGAATTCCTGTTTTTGCTCACC 5'

Following completion of the incubation, the double-stranded DNA reaction product from DNA polymerase activity is shown below:

5'TTCTAATACGACTCACTATAGCTGGGTGAGCAAAAACAGGAGTTTTAGAGCTAGATCAGGCAATAGTTGAACTTTTT

3'AAGATTATGCTGAGTGATATCGACCCACTCGTTTTTGTCCTCAAAATCTCGATCTAGTCCGTTATCAACTTGAAAAA

CACCGTGGCTCAGCCACGTAAAACTTAAGGACAAAAACGAGTGG3'

GTGGCACCGAGTCGGTGCATTTTGAATTCCTGTTTTTGCTCACC5'

The final example pUC19 PE pegRNA sequence is:

5'GCUGGGTGAGCAAAAACAGGAGUUUUAGAGCUAGAUCAGGCAAUAGUUGAACUUUUUCACCGUGGCUCAGCCA CGUAAAACUUAAGGACAAAAACGAGUGG 3'

Step 2: Utilize the IG® PE pegRNA Synthesis Kit

You've done the hard part, now let IG make this next part easy. Simply choose whether to follow the SMALL SCALE or the PREPARATIVE SCALE table in the protocol.

1. Prewarm an incubator or water bath for microcentrifuge tubes to 37°C.
2. Thaw each component of the IG® PE pegRNA Synthesis Kit and keep the unmixed kit reagents on ice.
3. Prepare a mixture of your custom oligos, 1 µM EACH in solution (e.g. PEpUC19pegR11, PEpUC19pegRTpbs).
4. Choose to follow one of the two tables below. In a DNase/RNase-free 0.2 mL PCR tube or 1.5 mL microcentrifuge tube for Preparative scale, mix the following IG® PE pegRNA Synthesis Kit components IN ORDER on ice. (Choose EITHER the "Small Scale" OR the "Preparative Scale").

Small Scale

Reagent	Amount
Nuclease-free Water	3 µL
4X IG PE pegRNA reaction mix	5 µL
Your custom oligo mixture (1 µM EACH)	5 µL
DTT (0.2M)	1 µL
NTPs mix (25 mM)	4 µL
IG PE pegRNA enzyme mix	2 µL
Total	**20 µL**

Preparative Scale

Reagent	Amount
Nuclease-free Water	15 µL
4X IG PE pegRNA reaction mix	25 µL
Your custom oligo mixture (1 µM EACH)	25 µL
DTT (0.2 M)	5 µL
NTPs mix (25 mM)	20 µL
IG PE pegRNA enzyme mix	10 µL
Total	**100 µL**

5. Mix thoroughly by tapping the tube 10 times, (do not vortex) and then centrifuge the reaction droplets (for less than 5 seconds) to the bottom of the tube in a microcentrifuge. This mixture is the pegRNA synthesis reaction.
6. Transfer the pegRNA mixture to the prewarmed 37°C incubator for 30 minutes. The reaction is usually complete in about 25 minutes, and there are no negative effects if left for under an hour at 37°C.
7. Transfer the reaction to ice.
8. Add igDNAse-I (RNase-Free) and Nuclease-free Water as described in the table below that matches

Small Scale

Reagent	Amount
Tube with your pegRNA mixture	Already in tube (20 µL)
Nuclease-free Water	30 µL
igDNase-I (RNase-free)	2 µL
Total	**50 µL**

Preparative Scale

Reagent	Amount
Tube with your pegRNA mixture	Already in tube (100 µL)
Nuclease-Free Water	150 µL
igDNase-I (RNase-free)	10 µL
Total	**260 µL**

9. Mix thoroughly by tapping the tube 10 times, and then centrifuge (less than 5 seconds) all sample droplets to the bottom of the tube in a microcentrifuge.
10. Transfer the pegRNA mixture to the prewarmed 37°C incubator for 15 minutes.
11. For *in vitro* downstream CRISPR applications, we recommend purifying the pegRNA using the IG® RNA purification kit (follow the kit protocol) or analyzing your pegRNA by gel electrophoresis using RNasefree tips, tubes and buffers. We have demonstrated specific, RNA-guided nicking activity of target DNA using the example pegRNA synthesized with this kit (Fig.2) and PEmax *in vitro* RT activities (not shown).

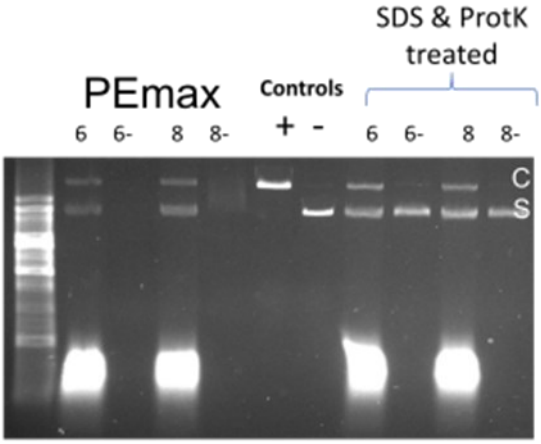

Figure 3. pegRNA activates nicking by PE enzyme. The PE nicking assay shows circular (C) and supercoiled (S) dsDNA. The positive (+) control uses Nb.SssSI, a nicking restriction enzyme from NEB, which nicks DNA so that it runs mostly as circular DNA (lane +). The negative (–) control is dsDNA with NO enzyme added. The DNA is mostly supercoiled; the circular DNA is too faint to see (lane –). PEmax is unable to nick without pegRNA (lanes 6– and 8-). However, in the presence of pegRNA, PEmax nicks dsDNA generating more circular dsDNA (lanes 6 and 8). For better visibility of resulting nicked DNA, SDS and Proteinase K were added to the reaction sample to remove proteins so that more DNA could enter the gel.

References:

1. Anzalone AV, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature. 2019;576:149–157.

2. Lee J, Lim K, Kim A, et. al. Prime editing with genuine Cas9 nickases minimizes unwanted indels. Nat Commun. 2023 Mar 30;14(1):1786.

3. Chen PJ, Hussmann JA, Yan J, et. al. Enhanced prime editing systems by manipulating cellular determinants of editing outcomes. Cell. 2021 Oct 28;184(22):5635-5652.e29.

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