Klenow Fill-In Kit

Instruction Manual

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Revision C.0

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Klenow Fill-In Kit

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MATERIALS PROVIDED

Materials provided[a]	Quantity
Klenow polymerase	125 U (5 U/µl)
10× fill-in buffer[c,d]	1.0 ml
pUC19/*Bam*H I-digested control DNA	20 µg (1 µg/µl)
dNTPs[c] (separate 10 mM stocks)	150 µl each

[a] The Klenow Fill-In Kit contains enough reagents to perform 25 partial fill-in reactions, 25 end-labeling reactions or 12 complete fill-in reactions. The Klenow Fill-In Kit does not contain radiolabeled nucleotides.

[b] Do not store the Klenow polymerase or dNTPs in a frost-free freezer.

[c] We recommend aliquoting the 10× fill-in buffer and 10 mM dNTPs into smaller volumes following initial thawing. Avoid multiple freeze–thaw cycles for the highest incorporation.

[d] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

Klenow Polymerase: **–20°C**
10× Fill-In Buffer: **–20°C**
Control DNA: **–20°C**
10 mM dNTPs (pH 7.5):**–20°C for up to 3 months, –80°C for long-term storage**

ADDITIONAL MATERIALS REQUIRED

30–50 µCi of [α-^{32}P]dNTP or ^{35}S-dNTP (End-labeling protocol)

Revision C.0

INTRODUCTION

The Agilent Klenow Fill-In Kit provides the components needed to perform partial fill-in reactions to prepare genomic DNA for insertion into the Lambda FIX II vector. Partial fill-in of vector arms containing 5´ overhangs increases the accuracy of representation of genomic libraries constructed using this technique.[1] Partial fill-in of genomic inserts prevents self-ligation, significantly reducing the number of recombinant molecules containing multiple inserts, leading to an accurate representation of adjacent genomic sequences.

The Klenow Fill-In Kit can also be used to perform a complete fill-in of a 5´ overhang to generate blunt ends, which can be directly ligated. Alternatively, linkers or adaptors can be ligated to the blunt-ended fragments, resulting in the introduction of a new restriction site. In addition, the kit can be used to radioactively end label a 5´ overhang with [α-^{32}P]dNTP.

CRITICAL PRECAUTIONARY NOTES

- The sample DNA should be extracted with phenol–chloroform and ethanol-precipitated before performing a fill-in reaction.
- Add the Klenow polymerase to the reaction mixture last—after the DNA, fill-in buffer and dNTPs—and do not let the reaction mixture sit on ice for an extended period of time.

PROTOCOLS

Standard Complete Fill-In Reaction Conditions

1. Add the following components to two sterile 1.5-ml tubes:

Control Reaction	Sample Reaction
1–1.5 µg control DNA	0–1.5 µg DNA
2.5 µl 10× fill-in buffer	2.5 µl 10× fill-in buffer
1 µl dATP (10 mM)	1.0 µl each desired dNTP[‖] (10 mM)
1 µl dCTP (10 mM)	2 µl Klenow polymerase (5 U/µl)
1 µl dGTP (10 mM)	dH_2O to a total volume of 25 µl
1 µl dTTP (10 mM)	
2 µl Klenow polymerase (5 U/µl)	
dH_2O to a total volume of 25 µl	

   Mix the components of both tubes by gently pipetting the mixtures up and down with a pipet tip.

2. Incubate the control and sample reactions at room temperature (22°C) for 15 minutes.

3. In order to minimize DNA loss during the extractions, add 475 µl of 1× STE buffer (see *Preparation of Media and Reagents*) to each reaction to adjust the total reaction volumes to 500 µl.

4. Add an equal volume (500 µl) of phenol–chloroform to each reaction. Vortex and microcentrifuge both reactions for 2 minutes at room temperature at maximum speed. Transfer the upper aqueous layer of each reaction to a fresh tube. Repeat this step if there is an interface.

5. Add an equal volume (500 µl) of chloroform to each reaction in order to extract any residual phenol. Vortex, then microcentrifuge both reactions for 2 minutes at room temperature at maximum speed. Transfer the upper aqueous layer of each reaction to a fresh tube.

6. Add 2 volumes of 100% (v/v) ethanol (1 ml) to each reaction and mix the reactions well. Precipitate the DNA for 30 minutes at –20°C.

7. Microcentrifuge the reactions at high speed for 10 minutes at 4°C. Discard the supernatants and wash the pellets with ice-cold 70% (v/v) ethanol. Lyophilize the pellets until dry.

8. Resuspend the DNA of the sample reaction in a desired volume of TE buffer (see *Preparation of Media and Reagents*) and resuspend the DNA of the control reaction in 3.5 µl of TE buffer.

[‖] Only the dNTPs complementary to the overhang need to be added. For example, for the *Eco*R I 5′ overhang of TTAA, only dATP and dTTP are required for the fill-in reaction.

Analysis of Complete Fill-in Control Reactions

9. Add the following components to 1 µg (1 µl) of control DNA that has been completely filled in:

    0.5 µl of 10× fill-in buffer
    0.5 µl of 10 mM rATP (pH 7.5)
    0.5 µl of T4 DNA ligase (4 Weiss U/µl)
    2.5 µl of dH2O for a final volume of 5 µl

10. In addition, to a separate sterile 1.5-ml tube, add the following components:

    1.0 µl of pUC19/*Bam*H I-digested control DNA (1 µg)
    0.5 µl of 10× fill-in buffer
    0.5 µl of 10 mM rATP (pH 7.5)
    0.5 µl of T4 DNA ligase (4 Weiss U/µl)
    2.5 µl of dH_2O for a final volume of 5 µl

11. Mix the reaction components by gently pipetting the reactions up and down with a pipet tip.

12. Incubate the tubes overnight at 4°C.

13. Analyze the control reactions that have been ligated overnight on a 1.0% (w/v) agarose gel. In addition, load 1 µg of pUC19/*Bam*H I-digested control DNA.

Expected Results

The completely filled-in and ligated control reaction (from step 9, above) should form a ligation ladder.

The pUC19/*Bam*H I-digested control DNA that has been ligated, but not filled in (from step 10, above), should ligate and form a smear in the upper part of the gel.

Standard Partial Fill-In Reaction Conditions

The example below for the sample reaction is for performing a partial fill-in of an insert to be ligated into the Lambda FIX II vector or an *Xho* I cut and partially filled-in Lambda ZAP II vector. Assume 50 µg of insert digested with either *Mbo* I, *Sau* 3A or *Bam*H I.

1. Add the following components to two sterile 1.5-ml tubes:

Control Reaction	Sample Reaction
1–1.5 µg control DNA	50 µg digested insert DNA
2.5 µl 10× fill-in buffer	30 µl 10× fill-in buffer
1 µl dATP (10 mM)	5 µl dATP (10 mM)
1 µl dGTP (10 mM)	5 µl dGTP (10 mM)
1 µl Klenow polymerase (5 U/µl)	3 µl Klenow polymerase (5 U/µl)
dH_2O to a total volume of 25 µl	dH_2O to a total volume of 300 µl

   Mix the components of both tubes by gently pipetting the reactions up and down with a pipet tip.

2. Incubate the control and sample reactions at room temperature (22°C) for 15 minutes.

3. Add 150 µl of 1× STE buffer to the sample reaction reaction to adjust the total volume to 450 µl. Add 475 µl of 1× STE buffer to the control

4. Add 50 µl of 10× STE buffer to the sample reaction only.

5. Add an equal volume (500 µl) of phenol–chloroform to each reaction. Vortex, then microcentrifuge both reactions for 2 minutes at room temperature at maximum speed. Transfer the upper aqueous layer of each reaction to a fresh tube. Repeat this step if there is an interface.

6. Add an equal volume (500 µl) of chloroform to each reaction in order to extract any residual phenol from the DNA. Vortex and microcentrifuge both reactions for 2 minutes at room temperature at maximum speed. Transfer the upper aqueous layer of each reaction to a fresh tube.

7. Add two volumes of 100% (v/v) ethanol (1 ml) to each reaction and mix well. Precipitate the DNA for 30 minutes at –20°C.

8. Microcentrifuge both reactions at high speed for 10 minutes at 4°C. Discard the supernatants and wash the pellets with ice-cold 70% (v/v) ethanol. Lyophilize the pellets until dry.

9. Resuspend the DNA of the sample reaction in 25 µl of TE buffer and resuspend the DNA of the control reaction in 3.5 µl of TE buffer.

Analysis of Partial Fill-in Control Reactions

10. Add the following components to 1 µg (1 µl) of control DNA that has been partially filled in:

    0.5 µl of 10× fill-in buffer
    0.5 µl of 10 mM rATP (pH 7.5)
    0.5 µl of T4 DNA ligase (4 Weiss U/µl)
    2.5 µl of dH_2O for a final volume of 5 µl

11. In addition, add the following components to a separate sterile 1.5-ml tube:

    1.0 µl of pUC19/*Bam*H I-digested control DNA (1 µg)
    0.5 µl of 10× fill-in buffer
    0.5 µl of 10 mM rATP (pH 7.5)
    0.5 µl of T4 DNA ligase (4 Weiss U/µl)
    2.5 µl of dH_2O for a final volume of 5 µl

12. Mix the reaction components by gently pipetting the reactions up and down with a pipet tip.

13. Incubate the tubes overnight at 4°C.

14. Analyze the control reactions that have been ligated overnight on a 1.0% (w/v) agarose gel. In addition, load 1 µg of pUC19/*Bam*H I-digested control DNA.

Expected Results

The partially filled-in and ligated control reaction (from step 10, above) should not show any ligation at all and will migrate in the same manner as the pUC19/*Bam*H I-digested control DNA.

The pUC19/*Bam*H I-digested control DNA that has been ligated, but not filled in (from step 11, above), should ligate and form a smear in the upper part of the gel.

Standard End-Labeling Reaction Conditions

1. Add the following components to a sterile 1.5-ml tube:

   0–1 µg of DNA
   5 µl of 10× fill-in buffer
   1 µl of each of the required dNTPs (10 mM), **except** the radiolabeled dNTP[‖]
   30–50 µCi of [α-^{32}P]dNTP or ^{35}S-labeled dNTP
   1 µl of Klenow polymerase (5 U/µl)
   dH_2O to a total volume of 50 µl

   Note *Radiolabeled dNTPs should not be stored at room temperature for extended periods of time. For optimal incorporation, use fresh radiolabeled dNTPs.*

   Mix the components by gently pipetting the reaction up and down with a pipet tip.

2. Incubate the reaction at room temperature (22°C) for 15 minutes.

3. Incubate the reaction at 70°C for 5–10 minutes to inactivate the enzyme.

4. Purify the unincorporated nucleotides from the labeled probe using the NucTrap probe purification columns. Alternatively, the sample can be purified with a G-50 spin column. The radioactive probes should be stored at–20°C.

   Note *After purification, sample purity can easily be checked by thin-layer chromatography (TLC).*

[‖] Only the dNTPs complementary to the overhang need to be added. For example, for the *Eco*R I 5´ overhang of TTAA, only dATP and dTTP are required for the fill-in reaction. For end labeling, one of these dNTPs must be radiolabeled. The best results are obtained when an inner base is filled-in with the labeled nucleotide. When end-labeling DNA that has been cut with *Eco*R I, radiolabeled dATP and cold dTTP are the nucleotides of choice.

PREPARATION OF MEDIA AND REAGENTS

10× Fill-In Buffer	10× STE Buffer
60 mM Tris-HCl (pH 7.5) 60 mM NaCl 60 mM $MgCl_2$ 0.5% gelatin 10 mM dithiothreitol (DTT)	1 M NaCl 200 mM Tris-HCl (pH 7.5) 100 mM EDTA
TE Buffer 10 mM Tris-HCl (pH 7.5) 1 mM EDTA	

REFERENCE

1. Frischauf, A. M., Lehrach, H., Poustka, A. and Murray, N. (1983) *J Mol Biol* 170(4):827-42.

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.