QuickClone™ PCR Cloning Kit

Cat. No: PGK121

CONTENTS

COMPONENTS OF THE KIT

Component	**PGK121** 30 reactions
pTZ57R/T Vector, 55 ng/µL	90 µL
5X Ligation Buffer	300 µL
T4 DNA Ligase, 5 u*/µL	30 µL
Control PCR Fragment, 42 ng/µL 953 bp long	20 µL
Control DNA 1, 0.1 µg/µL circular supercoiled plasmid vector pTZ57R DNA without insert, 2886 bp	30 µL
Control DNA 2, 0.1 µg/µL circular supercoiled plasmid vector pTZ57R DNA with inserted control PCR fragment, 3839 bp	30 µL
Water, nuclease-free	1.25 mL

STORAGE

All components of the kit should be stored at -20 °C.

DESCRIPTION

The Purgene's QuickClone™ PCR Cloning Kit is a TA system for direct one-step cloning of PCR products with 3'-dA overhangs (1). The high quality TA cloning vector pTZ57R/T is ready to use for efficient ligation with PCR products providing high cloning yields and low background

CLONING PRINCIPLE

The QuickClone PCR Cloning Kit takes advantage of the terminal transferase activity of *Taq* DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert. Recombinant clones are selected based on blue/white screening.

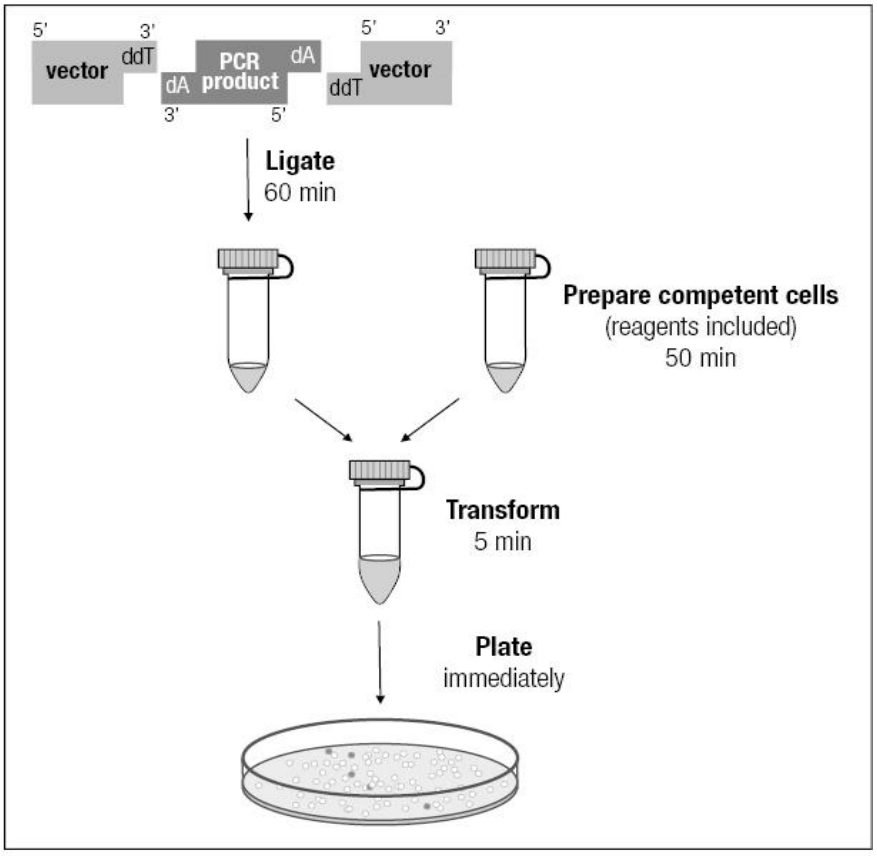

Fig. 1. PCR product cloning with QuickClone PCR Cloning Kit.

IMPORTANT NOTES

- Include final extension step in the PCR cycling protocol to ensure efficient 3'-dA tailing of the PCR product. The final extension step prolonged to 20-30 minutes generally yields 3-4 fold higher numbers of recombinant clones.
- Thoroughly mix every vial before use.
- The QuickClone PCR Cloning Kit is compatible with all PCR buffers.
- Gel-analyze the PCR product for specificity and yield before cloning.
- Specific PCR products of <1 kb appearing as one discrete band on the gel can be used for ligation directly from PCR reaction mixture without any purification.
- Do not use more than 4 µL of unpurified PCR product in the ligation reaction. Excess salts from the PCR reaction mixture may reduce the efficiency of the cloning procedure.
- Gel purification of the PCR product is recommended to increase the number of recombinants containing full length inserts in following cases:
  – PCR product is longer than 1 kb;
  – PCR product is contaminated with non-specific PCR products;
  – PCR product is contaminated with primer-dimers;
  – PCR template contains β-lactamase (ampicillin resistance) gene, which may result in background colonies on LB-ampicillin agar plates.
- For efficient cloning of gel-purified DNA fragments, it is important to avoid DNA damage by ethidium bromide and UV light. Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit DNA exposure to UV to a few seconds. Keep the gel on a glass plate or on a plastic plate during UV illumination. Alternatively, use dyes, like crystal violet, to visualize DNA in ambient light.
- The optimal insert/vector ratio is 3:1. Refer to Table 1 to calculate the amount of PCR product, required for efficient ligation with 0.165 µg (3 µL, 0.172 pmol ends) of the pTZ57R/T vector or use dedicated software for calculations.

Table 1. Recommended amount of PCR product for the ligation reaction.

Length of PCR product (bp)	Optimal PCR product quantity for ligation reaction, (0.52 pmol ends)
100	17 ng
300	51 ng
500	86 ng
1000	172 ng
2000	343 ng
3000	515 ng
4000	686 ng
5000	858 ng

- To enable blue/white screening, choose only strains having lacZΔM15 mutation, for example, XL1-Blue, ER1727, JM109 or other.

CONSIDERATIONS FOR CLONING LONG PCR PRODUCTS

Short DNA fragments (<1 kb) are cl*oned wi*th a much higher efficiency compared to long ones. Therefore, long PCR products must be purified to remove an smaller fragments from the solution.

- Optimize PCR conditions to increase specificity and yield of full length PCR product.
- Gel-purify PCR products to minimize presence of primer dimmers or non-specific short PCR products in the ligation reaction, even if these are not visible on the gel.
- Protect long PCR products from mechanical sharing and damage by nucleases:
  – store the PCR product at -20 °C if it is not used immediately;
  – use clean labware, razor blade and electrophoresis tank. Prepare fresh electrophoresis running buffer for gel purification procedure.
- Avoid DNA damage by UV light
- Before ligation, verify the quantity and quality of the purified PCR product on a gel. The optimal insert/vector ratio is 3:1. Refer to Table 1 to calculate the amount of PCR product (0.52 pmol ends), required for efficient ligation with 0.165 µg (3 µL, 0.172 pmol ends) of the pTZ57R/T vector or use dedicated software for calculations.
- Success in cloning of long PCR products may also depend on the DNA sequence of the insert. PCR products may contain toxic sequences not tolerated by *E. coli*, therefore multicopy vectors like pTZ57R/T may not be suitable for cloning these PCR products.

CLONING PROTOCOL

Please read all IMPORTANT NOTES on page 4 before starting.

Ligation

1. Set up the ligation reaction:

Component	Volume
pTZ57R/T Vector, (0.17 pmol ends)	3 µL
5X Ligation Buffer	6 µL
PCR product (0.52 pmol ends)	variable*
Nuclease-free Water	to 29 µL
T4 DNA Ligase	1 µL
Total volume	**30 µL**

* Do not use more than 4 µL of unpurified PCR product in the ligation reaction. Excess salts from the PCR reaction mixture may reduce the efficiency of the cloning procedure.

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the ligation mixture at room temperature (22 °C) for 1 hour. If maximal number of transformants is required, incubate overnight at 4 °C.

   Note. During the ligation prepare competent *E. coli* cells using the provided set of solutions for preparation of competent cells –Bacterial Transformation Kit. To enable blue/white screening, choose only strains having lacZ∆M15 mutation, for example, XL1-Blue, ER1727, JM109 or other. Refer to transformation protocols on next page.

3. Use 2.5 µL of the ligation mixture directly for bacterial transformation.

   Note. Keep the ligation mixture at -20 °C if transformation is postponed. Thaw on ice and mix carefully before transformation.

Transformation

All common *E. coli* laboratory strains can be used. To enable blue/white screening, choose only strains having lacZ∆M15 mutation, for example, XL1-Blue, ER1727, JM109 or other. Typical transformation efficiencies are more than 10_7 transformants per µg of supercoiled plasmid DNA.

CONTROL EXPERIMENT

The Control PCR Fragment is a 953 bp purified amplicon generated with *Taq* DNA polymerase, which adds extra nucleotides to the 3'-ends of PCR products.

1. Set up the ligation reaction:

Component	Volume
pTZ57R/T Cloning vector, (0.17 pmol ends)	3 µL
5X Ligation Buffer	6 µL
Control PCR Fragment (0.52 pmol ends)	4 µL
Nuclease-free Water	16 µL
T4 DNA Ligase	1 µL
Total volume	**30 µL**

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the ligation mixture at room temperature (22°C) for 1 hour. Use 2.5 µL of the ligation mixture directly for bacterial transformation. Keep the ligation mixture at -20 °C if transformation is postponed. Thaw on ice and mix carefully before transformation.

   Note. To enable blue/white screening, choose only strains having lacZΔM15 mutation, for example, XL1-Blue, ER1727, JM109 or other.

3. Transform 50 µL of competent *E. coli* cells with 2.5ul of the ligation mixture. Plate the cells on LB amplicillin XGal/IPTG plates
4. Transformation with Control DNA 1 (vector without insert) will yield all blue colonies and control transformation with Control DNA 2 (vector with insert) will yield all white colonies.

MAP AND FEATURES OF pTZ57R/T CLONING VECTOR

The pTZ57R/T cloning vector is linearized and ddT tailed for direct use in cloning of PCR products, generated with *Taq, Tth, Tfl* or other DNA polymerases or polymerase mixtures, which add extra adenines to the ends of PCR products.
The map and the MCS region of the vector are represented below:

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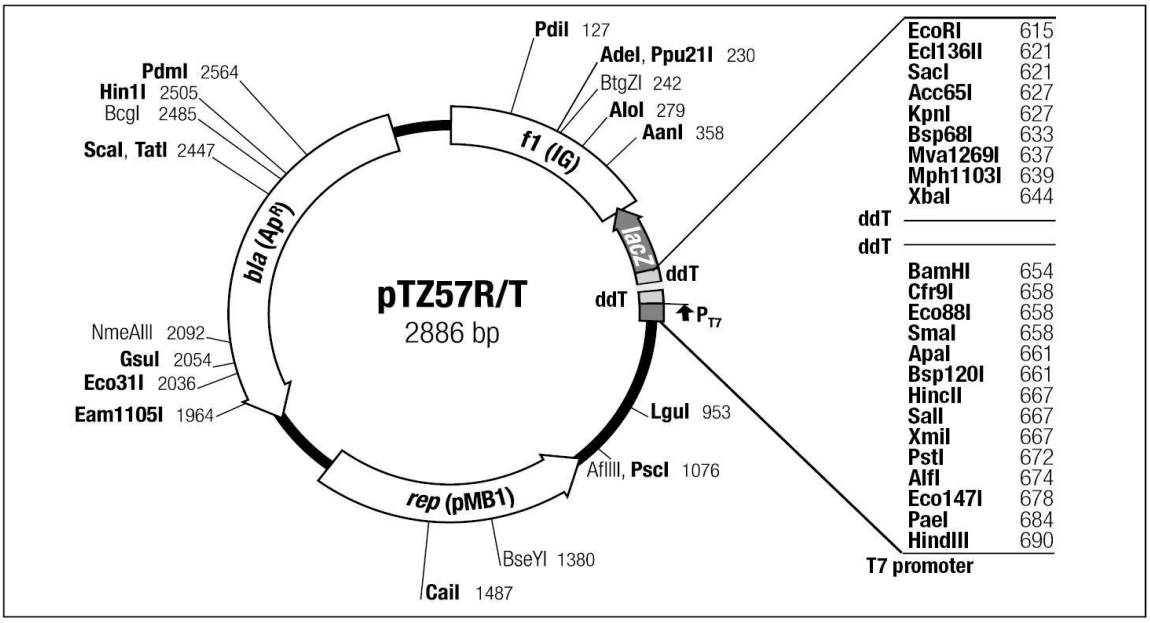

Fig. 2. Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated.

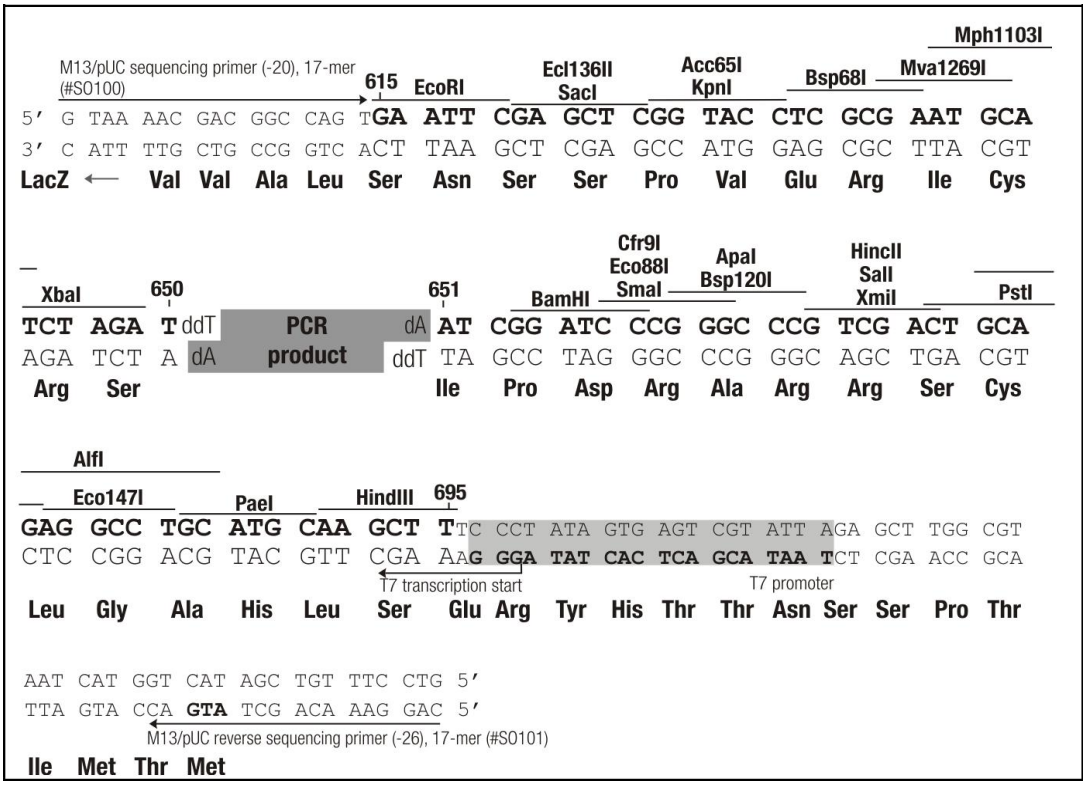

Fig. 3. DNA sequence of MCS region.

Genetic elements of pTZ57R/T cloning vector

Element	Function	Position (bp)
rep (pMB1)	A replicon (rep) from the pMBI plasmid is responsible for the replication.	1122-1736
Replication start	Initiation of the replication.	1136 (±1)
bla (Ap_R)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells.	1896-2756
LacZ α-peptide	Blue/white screening of recombinant clones	449-739
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	615-695
Cloning site	3'-ddT tailed DNA ends for ligation with insert	650-651
Phage f1 origin	Synthesis of a single-stranded DNA	2-457
T7 promoter	*In vitro* transcription of insert DNA with T7 RNA polymerase	697-716
M13/pUC forward sequencing primer (-20)	Sequencing of insert, colony PCR	599-614
M13/pUC reverse sequencing primer (-26)	Sequencing of insert, colony PCR	735-751
T7 promoter sequencing primer	Sequencing of insert, colony PCR	697-716

Restriction enzymes, that do not cut pTZ57R/T:

AarI, AatII, AbsI, AjiI, AjuI, BaeI, BbvCI, BclI, BcuI, BglII, BoxI, BpiI, BplI, Bpu10I, Bpu1102I, BseJI, BseRI, BsgI, BshTI, Bsp119I, Bsp1407I, BspOI, BspTI, Bst1107I, BstAPI, BstXI, Bsu15I, BtgI, BveI, Cfr42I, CpoI, CspCI, Eco32I, Eco47III, Eco52I, Eco72I, Eco81I, Eco91I, Eco105I, Eco130I, EcoO109I, EheI, Esp3I, FalI, FaqI, FseI, FspAI, Kpn2I, KspAI, MauBI, MLsI, MLuI, MreI, MssI, MunI, NcoI, NdeI, NheI, NotI, OliI, PacI, PasI, PauI, Pfl23II, PfoI, Psp5II, PspXI, PsrI, PsyI, SanDI, SdaI, SexAI, SfaI, SfiI, SgfI, SgrAI, SgrDI, SgsI, SmiI, SrfI, TstI, Van91I, XagI, XcmI, XhoI, XmaJI.

Restriction enzymes, that cut pTZ57R/T once:

AanI	358	Bsp120I*	661	Hin1I	2505	Ppu21I	230
Acc65I*	627	BtgZI	242	HincII*	667	PscI	1076
AdeI	230	CaiI	1487	HindIII*	690	PstI*	672
AflIII	1076	Cfr9I*	658	KpnI*	627	SacI*	621
AlfI*	674	Eam1105I	1964	LguI	953	SalI*	667
AloI	279	Ecl136II*	621	Mph1103I*	639	ScaI	2447
ApaI*	661	Eco31I	2036	Mva1269I*	637	SmaI*	658
BamHI*	654	Eco88I*	658	NmeAIII	2092	TatI	2447
BcgI	2485	Eco147I*	678	PaeI*	684	XbaI*	644
BseYI	1380	EcoRI*	615	PdiI	127	XmiI*	667
Bsp68I*	633	GsuI	2054	PdmI	2564	* – MCS	

Problem	Cause and Solution
Few or no transformants	**Low transformation efficiency of competent cells.** Use only high transformation efficiency cells. Perform a control transformation with 0.1 ng of Control DNA 1 (supercoiled pTZ57R DNA). Transformation efficiency should exceed 10_6 cfu/µg DNA. **Proofreading DNA polymerase was used for PCR.** If *Pfu* DNA polymerase, or other proofreading DNA polymerase, was used in PCR, the PCR product is blunt-ended and is not compatible with TA cloning method. Use *Taq* DNA polymerase to generate PCR product for cloning. **T4 DNA Ligase was inhibited by salts present in the PCR buffer.** If using non-purified PCR product, do not add more than 4 µL of the PCR mixture to the ligation reaction to avoid inhibition of T4 DNA ligase by salts. **PCR product was damaged by UV light during excision from the agarose gel.** For efficient cloning of gel-purified DNA fragments, it is important to avoid DNA damage by ethidium bromide and UV light. Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit DNA exposure to UV to a few seconds. Keep the gel on a glass plate or on a plastic plate during UV illumination. Alternatively, use dyes, like crystal violet, to visualize DNA in ambient light. (2,3). **Insert:vector ratio is suboptimal.** The optimal insert/vector ratio is 3:1. Refer to Table 1 on page 4 to calculate the amount of PCR product, required for efficient ligation with 0.165 µg (3 µL, 0.172 pmol ends) of the pTZ57R/T vector or use dedicated software for calculations.
Background colonies without plasmid	**Insufficient amount of antibiotic in agar medium.** Use 100 µg/mL of ampicillin in LB-ampicillin agar plates. Allow the LB medium to cool to 55 °C before addition of the ampicillin
Background colonies that contain plasmids with incorrect inserts	**PCR products are contaminated with a template which encodes ampicillin resistance.** Gel-purify the PCR product if the PCR template encodes a β-lactamase to avoid background colonies on LB-ampicillin agar. **Non-specific PCR products or primer dimers were cloned.** Gel-analyze the PCR product prior to ligation. If non-specific PCR products or primer-dimers were generated during the PCR reaction, gel-purify the target PCR product. Otherwise, optimize the PCR conditions to increase specificity.

Problem	Cause and Solution
Background colonies that contain plasmids with incorrect inserts	**Large PCR product (>1 kb) was cloned without purification.** Short DNA fragments (<1 kb) are cloned with a much higher efficiency compared to long ones. Therefore, long PCR products must be purified to remove any smaller fragments from the solution. **Nuclease contamination.** To guaranty DNA integrity, preserve long PCR product from both mechanical sharing and damage by nucleases: • Use only components provided with the kit. Nuclease contamination can impair the integrity of the cloning vector, thus disabling blue/white selection of recombinant clones. • Store PCR product at -20 °C if it is not used immediately. Use clean labware and razor blade, prepare fresh electrophoresis running buffer for gel purification procedure.
False negatives in colony PCR	**False-negatives in colony PCR.** Due to considerable amount of recircularized vector plated on the surface of the plate, colony PCR may give some false-negative results. Prior to clone analysis propagate short strikes of individual colonies on ampicillin plates. Then use small amount of each for colony PCR.
Transformation efficiency is too low	**Competent cells prepared from non-fresh bacterial culture.** Seed overnight culture from a freshly streaked bacterial culture plate. Refresh bacterial strains weekly. For seeding of overnight *E. coli* DH5α culture, use only one day old culture plates.
Sequence errors in the cloned insert	**PCR product was damaged by UV light during excision from agarose gel.** Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When a short-wavelength (254-312 nm) light-box is used, limit DNA exposure to UV to a few seconds. Keep the gel on a glass or on plastic plate during UV illumination. Alternatively, use dyes visible in ambient light to visualize DNA in standard agarose gels (2, 3). **Errors in PCR primers.** If the cloned PCR product contains sequence errors or is missing 5' bases and the same error persists in more than one clone, re-order the PCR primers from a reliable supplier and repeat the procedure starting from the PCR step.

RECIPES

Ampicillin stock solution (50 mg/mL)

Dissolve 2.5 g of ampicillin sodium salt in 50 mL of deionized water. Filter-sterilize and store in aliquots at -20 °C.

X-Gal stock solution (20 mg/mL)

Dissolve 200 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (#R0404) in 10 mL N,N-dimethylformamide. Store at -20 °C in the dark. Alternatively, use X-Gal Solution, ready-to-use (#R0941). Use 40 µL per plate.

IPTG stock solution (100 mM)

Dissolve 1.2 g IPTG (isopropyl- β -D-thiogalactopyranoside) (#R0392) in 50 mL deionized water. Filter-sterilize, aliquote and store at 4 °C. Alternatively, use IPTG Solution, ready-to-use (#R1171). Use 40 µL per plate.

LB-ampicillin X-Gal/IPTG Plates

- Prepare LB-agar medium (1 liter), weigh out:

  Bacto™ Tryptone 10 g,
  Bacto Yeast extract 5 g,
  NaCl 5 g.

  Dissolve in 800 mL of water, adjust pH to 7.0 with NaOH and adjust the volume with water to 1000 mL. Add 15 g of agar and autoclave.

- Before pouring the plates, allow the medium to cool to 55 °C. Then, add 1 mL of ampicillin stock solution (50 mg/mL) to a final concentration of 50 µg/mL. Mix gently and pour the plates. Allow the LB-ampicillin agar medium to solidify. Dry plates opened at room temperature under UV light for 30 min.
- Add 40 µL of X-Gal stock solution (20 mg/mL) or X-Gal Solution, ready-to-use (#R0941) and 40 µL of IPTG 100 mM or IPTG Solution, ready-to-use (#R1171), spread evenly with a sterile spatula.

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