SureVector Cloning Kits

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G7518A

Protocol

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Notices

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In this Guide...

This document describes how to use the SureVector cloning kits to create custom vectors.

1 Before You Begin

This chapter provides important information on getting started with SureVector cloning.

2 Protocol

This chapter provides guidelines and instructions on how to perform the SureVector cloning protocol.

3 Troubleshooting

This chapter contains suggestions for troubleshooting your SureVector cloning.

4 Reference Information

This chapter provides recipe information for preparation of the bacterial growth media.

1
Before You Begin

This chapter provides important information on getting started with SureVector cloning.

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Overview of SureVector cloning

Agilent's SureVector cloning protocol creates custom cloning vectors using a unique enzyme mix that allows a gene-of-interest to be cloned into a fully customizable vector backbone. Along with the SureVector Enzyme Mix, the SureVector core cloning kits (described on page 9) contain various DNA modules that each serve a specific functional purpose in the resulting vector. The modules include selectable markers, origins of replication, expansion elements, and transcriptional promoters with a HIS6 tag that is expressed as an N-terminal fusion with the gene-of-interest.

You choose which modules to include, then combine them in a single tube for assembly into a custom vector. You then combine the assembly reaction with XL1-Blue Supercompetent cells to transform the vector into *E. coli* (see Figure 1 on page 7).

For assistance in designing your custom vector using the SureVector cloning kits, visit Agilent's online SureVector design site at: www.genomics.agilent.com/surevector/vectorfragment.jsp.

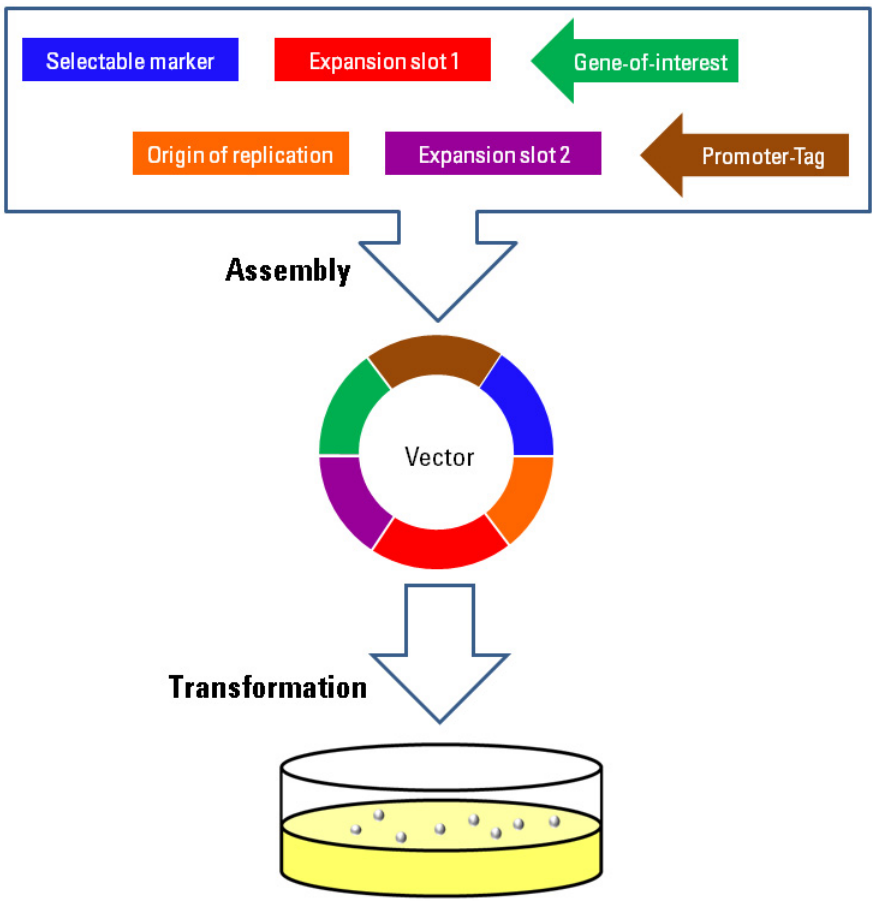

Figure 1 SureVector cloning overview

Required reagents and equipment

Table 1 contains the list of reagents and equipment that are required for the protocol.

Table 1 Required Equipment and Reagents

Equipment or reagent
Agilent SureVector Core Kit (p/n G7514A) or SureVector *E. coli* Selection Kit (p/n G7518A), see "SureVector kit contents" on page 9 for information
Agilent XL1-Blue Supercompetent Cells (p/n 200236) or other suitable competent cells, if using the SureVector *E. coli* Selection Kit
Prepared gene-of-interest DNA fragment, see "Gene-of-interest insert" on page 13 for information
Agilent SureCycler 8800 thermal cycler, or other programmable thermal cycler
0.2-mL thin-wall PCR tubes, or other tubes suitable for your thermal cycler
Water baths set to 37°C and 42°C
NZY medium, see "Preparation of media" on page 23 for recipe
LB-agar plates with IPTG, X-gal, and the appropriate antibiotic, see "Preparation of media" on page 23 for recipe
LB-agar plates with ampicillin (for pUC18 control transformations), see "Preparation of media" on page 23 for recipe
14-mL BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059)
DNase-free dH_20

SureVector kit contents

Agilent offers two SureVector cloning kits:

- **SureVector Core Kit, part number G7514A**
  - Provides sufficient reagents for 15 cloning reactions
  - Includes functional modules for *E. coli*, mammalian cells, and yeast
  - Includes the Agilent XL1-Blue Supercompetent Cells Kit for transformation of assembled vectors
- **SureVector *E. coli* Selection Kit, part number G7518A**
  - Provides sufficient reagents for 5 cloning reactions
  - Includes functional modules for *E. coli*
  - Does not include competent cells; Agilent XL1-Blue Supercompetent Cells Kit can be purchased separately (p/n 200236)

Both kits provide the SureVector Enzyme Mix, 10× SureVector Buffer, dNTP Mix, *Dpn* I restriction enzyme, and 5× SureSolution.

Table 2 lists the quantities and descriptions for the materials provided in each kit.

Table 2 SureVector cloning kits – Quantities and descriptions of kit components

Materials provided	SureVector Core Kit	SureVector *E. coli* Selection Kit	Description
Bacterial Selectable Markers – *select 1 per reaction*			
SureVector Amp^R Selectable Marker	30 µL	10 µL	Ampicillin selection in *E. coli*
SureVector Kan^R Selectable Marker	30 µL	10 µL	Kanamycin selection in *E. coli*
SureVector Chl^R Selectable Marker	30 µL	10 µL	Chloramphenicol selection in *E. coli*
Bacterial Origins of Replication – *select 1 per reaction*			
SureVector pUC Origin	30 µL	10 µL	*E. coli* origin of replication (100–200 copies/cell)
SureVector p15a Origin	30 µL	—	*E. coli* origin of replication (10–12 copies/cell)
SureVector pBR322 Origin	30 µL	—	*E. coli* origin of replication (10–20 copies/cell)

Table 2 SureVector cloning kits – Quantities and descriptions of kit components

Materials provided	SureVector Core Kit	SureVector *E. coli* Selection Kit	Description
XP1 Expansion Site Modules – *select 1 per reaction*			
SureVector XP1 Linker	30 µL	10 µL	Linker for expansion site 1
SureVector yARS	30 µL	—	Yeast autonomous replication sequence in *S. cerevisiae*
XP2 Expansion Site Modules – *select 1 per reaction*			
SureVector XP2 Linker	30 µL	—	Linker for expansion site 2
SureVector Neo^R Mammalian Selectable Marker	30 µL	—	Neomycin selection in mammalian cells
SureVector LEU2 Yeast Selectable Marker	30 µL	—	Leucine auxotroph selection in *S. cerevisiae*
SureVector LacI Repressor	30 µL	10 µL	Expression of *lacI* in *E. coli*
Promoters – *select 1 per reaction*			
SureVector T7-HIS6 *E. coli* Promoter	30 µL	10 µL	Bacteriophage T7 promoter fused to HIS6 tag
SureVector CMV-HIS6 Mammalian Promoter	30 µL	—	Mammalian CMV promoter fused to HIS6 tag
SureVector GAL1-HIS6 Yeast Promoter	30 µL	—	*S. cerevisiae* GAL1 promoter fused to HIS6 tag
Control Gene Insert – *use as a control insert*			
SureVector LacZ Control (N-term)	30 µL	10 µL	Expression of *lacZ* in *E. coli*
Assembly Reagents			
SureVector Enzyme Mix	15 µL	15 µL	Assembly reagent
10× SureVector Buffer	30 µL	30 µL	Assembly reagent
dNTP Mix	30 µL	30 µL	Assembly reagent
5× SureSolution	100 µL	100 µL	Assembly reagent
Dpn I	15 µL	15 µL	Assembly reagent

Table 2 SureVector cloning kits – Quantities and descriptions of kit components

Materials provided	SureVector Core Kit	SureVector *E. coli* Selection Kit	Description
Transformation Reagents – XL-1 Blue Supercompetent Cells Kit			
XL1-Blue Supercompetent Cells	5 × 200 µL	—	Transformation reagent
pUC18 Control Plasmid (0.1 ng/µL in TE buffer)	10 µL	—	Transformation reagent
β-Mercaptoethanol	25 µL	—	Transformation reagent

Kit storage

SureVector Core Kit

Upon receipt of the kit, immediately place the XL1-Blue Supercompetent Cells at the bottom of a –80°C freezer directly from the dry ice shipping container. Do not store the cells in liquid nitrogen.

Store all other components of the SureVector Core Kit at –20°C.

SureVector *E. coli* Selection Kit

Store all components at –20°C.

2 Protocol

This chapter provides guidelines and instructions on how to perform the SureVector cloning protocol.

Preprotocol considerations

Control reactions

Agilent recommends assembling positive and negative control vectors.

The positive control assembly reaction contains the SureVector LacZ Control in place of the gene-of-interest DNA insert. The SureVector LacZ Control is ready for use in the assembly protocol with 5' and 3' ends that are compatible with the adjacent SureVector modules. This control can help you identify potential problems with your gene-of-interest fragment. On the transformation plate, XL1-Blue colonies that include the SureVector LacZ Control in the assembled vector are blue in color due to the presence of IPTG and X-gal in the plates.

The negative control assembly reaction contains water in place of the gene-of-interest DNA insert.

Gene-of-interest insert

Your gene-of-interest DNA insert needs to have 5' and 3' ends that overlap the 5' and 3' ends of the adjacent SureVector modules. The easiest way to accomplish this is to PCR-amplify your gene-of-interest insert with PCR primers that include the appropriate overlap sequences. This method is illustrated in Figure 2, and the overlap sequences are listed in Table 3. The overlaps are 30 nucleotides and the portion of the primer that compliments the gene-of-interest sequence needs to be 12–20 nucleotides. Thus, the resulting primers are 42–50 nucleotides long.

In the upstream primer, in order for the gene-of-interest to be in the same reading frame as the HIS6 tag, the first three nucleotides that complement the gene-of-interest need to encode the first amino acid codon for the gene-of-interest. In the downstream primer, the region that complements the gene-of-interest needs to include a stop codon.

Figure 2 PCR method for adding overlap sequences to the 5′ and 3′ PCR primers

Table 3 Overlap sequences for gene-of-interest PCR primers

Overlap position	DNA Sequence
Upstream of the gene-of-interest	5′ GGTGGCGGAGGTTCTGGAGGCGGTGGAAGT 3′
Downstream of the gene-of-interest	5′ CTCGAGGAGATATTGTACACTAAACCAAATG 3′

NOTE If desired, you can encode a protease cleavage site in the upstream PCR primer to induce cleavage between the HIS6 tag and the translated gene-of-interest.

The SureVector cloning protocol works well with gene-of-interest inserts up to 3 kb. For inserts >3 kb, assembly efficiency may not be optimal, and you may need to screen a greater number of colonies on the transformation plate to identify one that contains the correct plasmid.

Your gene-of-interest DNA insert needs to be purified and stored in dH_20 at a concentration of 0.05 pmol/µL. Agilent recommends Herculase II Fusion DNA Polymerase (p/n 600675) for PCR amplification of the gene-of-interest insert and the StrataPrep DNA Gel Extraction Kit (p/n 400766) for purification of the insert.

NOTE Purification of the PCR-amplified gene-of-interest insert is necessary to reduce carry over of the parental DNA into the assembly reactions.

For quality purposes, run a sample of your insert on an agarose gel to make sure that only one band is present and that the band is the expected size.

Protocol

Prepare the SureVector reagents

dNTP Mix

The SureVector cloning kits include dNTP Mix.

- After the initial thawing of the dNTP Mix, aliquot the mixture into single-use volumes and store the aliquots at –20°C to avoid multiple freeze-thaw cycles.
- Use the dNTP Mix that is provided with the kit. Do not use other sources of dNTPs.

SureSolution

The SureVector cloning kits include a 5× stock of SureSolution. After the initial thawing of the 5× SureSolution, dilute the 5× stock to 1×.

1. Transfer 100 µL of the 5× SureSolution to a DNase-free 1.5-mL tube.
2. Add 400 µL of DNase-free dH_20 directly to the tube to dilute the SureSolution to a 1× concentration. Mix well by vortexing.
3. Aliquot the 1× SureSolution into single-use volumes and store the aliquots at –20°C to avoid multiple freeze-thaw cycles.

   Use the 1× SureSolution in the assembly protocol.

Assemble the vectors

1 Thaw the gene-of-interest DNA inserts and the needed SureVector reagents on ice.

NOTE Each assembly reaction needs to include one SureVector module from each of the functional groups (bacterial selectable markers, bacterial origins of replication, XP1 expansion site modules, XP2 expansion site modules, and promoters). See Table 2 on page 9 for a list of modules in each functional group.

2 Program the thermal cycler with the program in Table 4, then pre-warm the thermal block to 95°C.

Table 4 Thermal cycling program for assembly

Segment	Cycles	Temperature	Duration
1	1	95°C	1 minute
2	8	95°C	20 seconds
		60°C	20 seconds
		68°C	1 minute
3	1	68°C	1 minute
4	1	4°C	2 minutes

3 Using the volumes listed in Table 5, prepare the assembly reactions in tubes that are suitable for your thermal cycler (e.g. 0.2-mL thin-wall tubes). Mix each reaction gently by pipetting up and down or tapping the tube.

Table 5 Reagent volumes for assembly reactions

Reagent	Gene-of-interest reaction	Negative control reaction	Positive control reaction
10× SureVector Buffer	2 µL	2 µL	2 µL
DNase-free dH_20	2 µL	4 µL	2 µL
Bacterial selectable marker	2 µL	2 µL	2 µL
Bacterial origin of replication	2 µL	2 µL	2 µL
XP1 expansion site module	2 µL	2 µL	2 µL
XP2 expansion site module	2 µL	2 µL	2 µL
Promoter	2 µL	2 µL	2 µL
Gene-of-interest insert (0.05 pmol/µL stock)	2 µL	—	—
SureVector LacZ Control (N-term)	—	—	2 µL
dNTP Mix	1 µL	1 µL	1 µL
SureSolution (diluted to 1×)	2 µL	2 µL	2 µL
SureVector Enzyme Mix	1 µL	1 µL	1 µL

4 At the conclusion of the thermal cycling program, transfer the reactions to ice.

5 Add 1 µL of *Dpn* I to each reaction then transfer to a 37°C heat block or thermal cycler pre-heated to 37°C. Incubate the reactions at 37°C for 5 minutes then transfer to ice.

   Use the *Dpn* I enzyme that is provided with the kit. Do not use other sources of *Dpn* I.

At this point, you can proceed directly to "Perform the transformations", below, or store the assembly reactions at –20°C until needed.

Perform the transformations

NOTE The transformation protocol provided here is appropriate for use with Agilent XL-1 Blue Supercompetent Cells (provided with the SureVector Core Kit and sold separately as p/n 200236). If you are using another competent cell line, follow the manufacturer's instructions for transformation.

1. Label the appropriate number of 14-mL polypropylene round-bottom tubes, then chill the tubes on ice. You will need one tube for each assembly reaction (including positive and negative controls) *plus* one additional tube for the pUC18 transformation control reaction.
2. Preheat the NZY medium to 42°C.
3. Thaw the XL1-Blue Supercompetent Cells on ice. Once thawed, gently mix the cells, then aliquot 50 µL to each pre-chilled polypropylene tube, keeping the tubes on ice. (Each provided tube of XL1-Blue Supercompetent Cells contains 200 µL.)
4. Add 0.8 µL of β-Mercaptoethanol to each aliquot of cells. Swirl the tubes gently to mix.

   Use the β-Mercaptoethanol provided with the kit.
5. Incubate the cell samples on ice for 10 minutes, swirling gently every 2 minutes.
6. Add 1 µL of the appropriate assembled vector to each cell sample. For the cell sample for the pUC18 transformation control, add 1 µL of the pUC18 Control Plasmid provided in the kit. Swirl the tubes gently to mix.
7. Incubate the cell samples on ice for 30 minutes.
8. Heat-pulse the cell samples in a 42°C water bath for 45 seconds. The 45-second duration is critical.
9. Incubate the cell samples on ice for 2 minutes.
10. Add 450 µL of pre-warmed NZY medium to each cell sample. Incubate the samples at 37°C for 1 hour with shaking at 225–250 rpm.

11 Spread samples of each transformation onto the appropriate LB-agar plates.

- For transformations with an assembled vector (including positive and negative controls), spread the following volumes onto LB-agar plates that contain IPTG, X-gal, and the appropriate antibiotic.
  - 10 µL
  - 50 µL
  - 100 µL
- For the pUC18 transformation, spread 2.5 µL onto an LB-agar plate containing ampicillin.

12 Incubate the plates at 37°C overnight (18–20 hours).

You can now analyze the transformants or store the plates at 4°C until needed.

Analyze transformants

1 Count or estimate the number colonies on each transformation plate. The expected number of colonies for each plate is listed in Table 6. Note that the number of colonies varies based on the volume of transformed cells that you spread on the plate.

Table 6 Expected number of colonies per plate for each vector

Vector		Expected # of colonies
Assembled vector	Gene-of-interest	100–300 (white)
	Positive control	100–300 (blue) <5–15 (white)
	Negative control	<10 (white)
pUC18 control vector		~100 (white)

The presence of very few colonies on the negative control plates compared to the gene-of-interest and positive control plates is indicative of successful vector assembly.

2 Verify the composition of your assembled gene-of-interest vector using restriction analysis, sequencing, or another method of DNA analysis.

3 Troubleshooting

This chapter contains suggestions for troubleshooting your SureVector cloning.

Troubleshooting suggestions

Table 7 Troubleshooting suggestions based on potential observations

Observation	Possible Cause	Suggestion
Fewer than expected colonies on the gene-of-interest and positive control transformation plates	Inefficient vector assembly	Verify the concentration of your gene-of-interest insert. Make sure you are using 0.1 pmol in the assembly reaction.
	Inefficient transformation	Make sure you are using 14-mL BD Falcon polypropylene round-bottom tubes (BD Biosciences Catalog #352059) for the transformation protocol, since other tubes may be degraded by β-mercaptoethanol. In addition, the duration of the heat-pulse step has been optimized using these tubes.
		Make sure that the cells are heat-pulsed at 42°C for 45–50 seconds. Efficiency decreases sharply when cells are heat-pulsed for <45 seconds or for >60 seconds. Do not exceed 42°C.
		Agilent recommends XL1-Blue Supercompetent Cells for use in the SureVector transformation protocol. If you are experiencing inefficient transformation with another competent cell line, try the protocol with the Agilent XL1-Blue Supercompetent Cell Kit (p/n 200236).
Expected number of blue colonies on the positive control plate (with the SureVector LacZ control insert) but fewer than expected colonies on the gene-of-interest transformation plate	The gene-of-interest insert does not have compatible 5′ and 3′ ends	Review the information in "Gene-of-interest insert" on page 13.
	The gene-of-interest insert contains contaminants that interfere with assembly.	Run the insert on an agarose gel then use the Agilent StrataPrep DNA Gel Extraction Kit (p/n 400766) to purify the insert.
Excessive number of colonies on the negative control plate	The transformation reactions are contaminated.	Start with fresh reagents and sterilized labware.
No blue colonies on the positive control plate (with the SureVector LacZ control insert)	Beta-galactosidase is not being expressed or is not functioning.	Make sure that X-gal and IPTG were included in the plates. See "Preparation of media" on page 23.

4
Reference Information

This chapter provides recipe information for preparation of the bacterial growth media.

Preparation of media

Use the recipes below to prepare the *E. coli* growth media needed for the transformation protocol.

NZY Broth (per liter)

In a clean flask combine:

- 10 g of NZ amine (casein hydrolysate)
- 5 g of yeast extract
- 5 g of NaCl
- 2 g of $MgSO_4 \cdot 7H_20$

Add deionized H_20 to a final volume of 1 liter then autoclave.

LB-agar plates with ampicillin (per liter)

In a clean flask combine:

- 10 g of NaCl
- 10 g of tryptone
- 5 g of yeast extract
- 20 g of agar

Add deionized H_20 to a final volume of 1 liter. Adjust pH to 7.0 with 5 N NaOH then autoclave.

When cooled to 55°C, add ampicillin to a final concentration of 100 µg/mL.

Pour into 100 × 15 mm petri dishes.

LB-agar plates with IPTG, X-gal, and antibiotic (per liter)

In a clean flask combine:

- 10 g of NaCl
- 10 g of tryptone
- 5 g of yeast extract
- 20 g of agar

Add deionized H_20 to a final volume of 1 liter. Adjust pH to 7.0 with 5 N NaOH then autoclave.

When cooled to 55°C, add:

- IPTG (for a final concentration of 0.1 mM)
- X-gal (for a final concentration of 60 µg/mL)
- Appropriate antibiotic (see table below for concentrations)

Antibiotic	Final concentration
Ampicillin	100 µg/mL
Kanamycin	50 µg/mL
Chloramphenicol	34 µg/mL

Pour into 100 × 15 mm petri dishes.

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In this book

This document describes how to use the Agilent SureVector cloning kits to create custom cloning vectors that can be used in a variety of downstream applications.

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