Vitality hrGFP Subcellular Localization Vectors phrGFP-Nuc, phrGFP-Mito, phrGFP-Golgi, and phrGFP-Perox

Instruction Manual

Catalog #240042 (phrGFP-Mito)
#240043 (phrGFP-Nuc)
#240044 (phrGFP-Golgi)
#240063 (phrGFP-Perox)

Revision D

Research Use Only. Not for Use in Diagnostic Procedures.

240042-12

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VITALITY HRGFP SUBCELLULAR LOCALIZATION VECTORS

phrGFP-Nuc, phrGFP-Mito, phrGFP-Golgi, and phrGFP-Perox

CONTENTS

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MATERIALS PROVIDED

Material provided	Concentration	Quantity
phrGFP-Mito vector (Catalog #240042)	1.0 µg/µl	20 µg
phrGFP-Nuc vector (Catalog #240043)	1.0 µg/µl	20 µg
phrGFP-Golgi vector (Catalog #240044)	1.0 µg/µl	20 µg
phrGFP-Perox vector (Catalog #240063)	1.0 µg/µl	20 µg

STORAGE CONDITIONS

All Components: –20°C

OPTIONAL ADDITIONAL MATERIALS

Cre recombinase [Catalog #600270]
pExchange module EC-Hyg [Catalog #211181]
pExchange module EC-Puro [Catalog #211182]
pExchange module EC-Neo [Catalog #211183]
XL1-Blue supercompetent cells [Catalog #200236]
GeneJammer transfection reagent [Catalog #204130]

Revision D

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INTRODUCTION

The green fluorescent protein (GFP) has become an extremely versatile tool for tracking and quantifying biological entities in the fields of biochemistry, molecular and cell biology, as well as high throughput screening and gene discovery.[1, 2] GFPs have been identified in a wide range of coelenterates, and while recently the number of cloned GFPs has expanded, to date the best characterized proteins are those from the jellyfish *Aequorea victoria*. *Aequorea* GFP forms weak homodimers at moderate to low concentrations, and is often cytotoxic when expressed at low levels. Due to this latter characteristic, researchers have often been frustrated in their attempts to produce stable GFP-expressing cells lines using the *Aequorea* protein.[3]

We have isolated a cDNA clone for GFP from a novel marine organism, and have fully humanized the gene using codons preferred in highly expressed human genes. The fluorescence spectrum for the cloned GFP protein is essentially identical to the published spectrum for the purified native protein, with the major excitation peak at 500 nm and the emission peak at 506 nm. We have expressed the protein in a wide range of human, rodent, and simian cell lines, and observed levels of fluorescence comparable to that for the red-shifted, humanized variant of *Aequorea* GFP (EGFP) in all cell-types tested. In viability experiments, we find that high level expression of functional fluorescent protein in retrovirus-transduced cells is substantially more consistent and less toxic over time and passage number for the humanized recombinant GFP (hrGFP) than for EGFP. Thus the stable GFP-expressing cell lines are produced much more efficiently using Agilent's hrGFP compared with EGFP.

The Vitality hrGFP subcellular localization vectors allow real-time monitoring of the fate of peroxisomes, mitochondria, golgi and nuclei for both transient and stable applications.

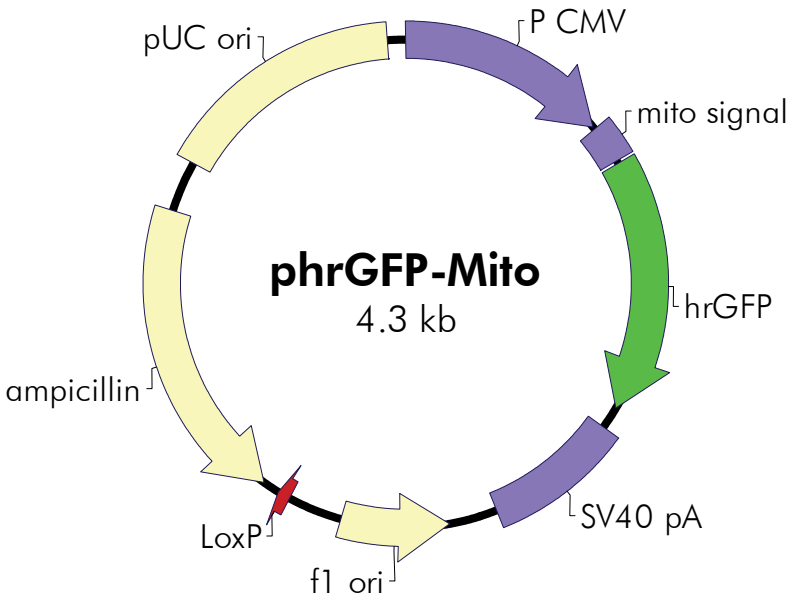
Description of the Vectors

The hrGFP-based subcellular localization vectors are derived from the vector pExchange-1, and thus take advantage of *Cre*-mediated site-specific recombination to allow quick and efficient directional insertion of prefabricated modules. With these vectors, a wide variety of drug-resistant markers may be readily substituted into the core hrGFP vector bearing the gene of interest.

Figures 1-4 show circular maps and locations of important features for the subcellular localization vectors.

The presequence of subunit VIII of cytochrome c oxidase[4] is fused at the N-terminus of hrGFP for localization to mitochondria in the vector phrGFP-Mito. The vector phrGFP-Nuc contains the SV40 nuclear localization peptide,[5] fused at the C-terminus of hrGFP. The vector phrGFP-Golgi contains the transmembrane domain of sialyltransferase[6] fused at the N-terminus for targeting to the golgi, and phrGFP-Perox contains a peroxisome signal sequence fused at the C-terminus of hrGFP for targeting to peroxisomes.[7] Images of hrGFP localization in HeLa cells using the Vitality subcellular localization vectors can be found in reference 8, or visit *www.genomics.agilent.com* and navigate to the Vitality vectors product page.

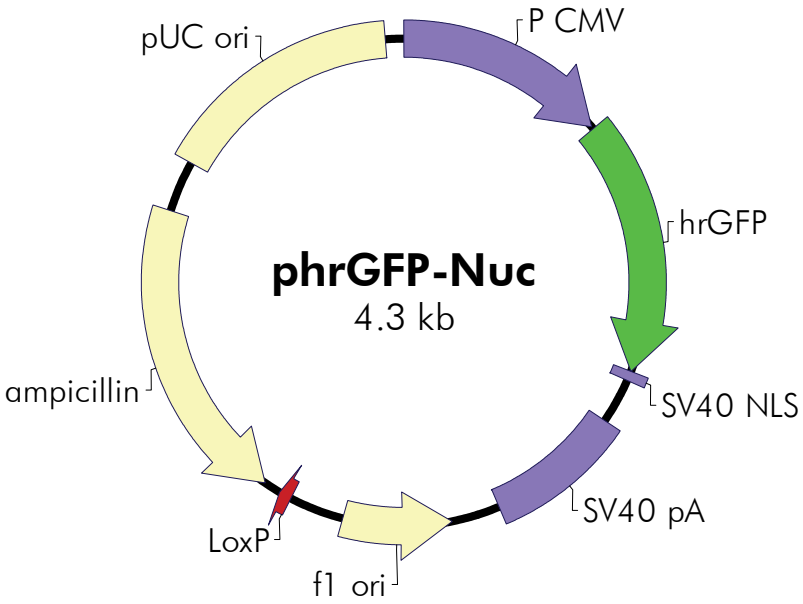
The phrGFP-Mito Vector



Feature	Nucleotide Position
CMV promoter	1–597
mitochondrial localization signal	606–713
hrGFP ORF	723–1439
T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′]	1471–1492
SV40 polyA signal	1504–1887
f1 origin of ss-DNA replication	2025–2331
LoxP sequence	2489–2523
ampicillin resistance (bla) ORF	2572–3429
pUC origin of replication	3576–4243

FIGURE 1 Features of the phrGFP-Mito subcellular localization vector.

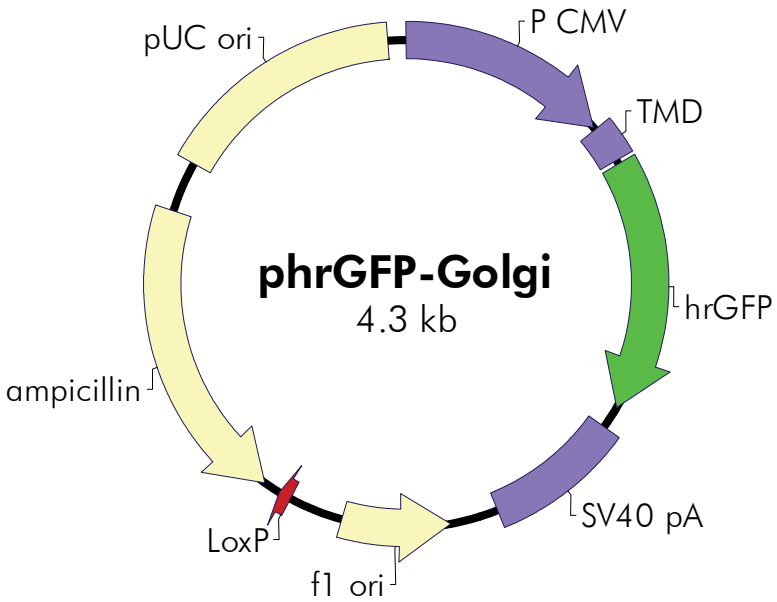
The phrGFP-Nuc Vector



Feature	Nucleotide Position
CMV promoter	1–597
hrGFP ORF	606–1322
SV40 nuclear localization signal (NLS)	1323–1349
T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′]	1447-1468
SV40 polyA signal	1479–1863
f1 origin of ss-DNA replication	2001–2307
LoxP sequence	2465–2499
ampicillin resistance (bla) ORF	2548–3405
pUC origin of replication	3552–4219

FIGURE 2 Features of the phrGFP-Nuc subcellular localization vector.

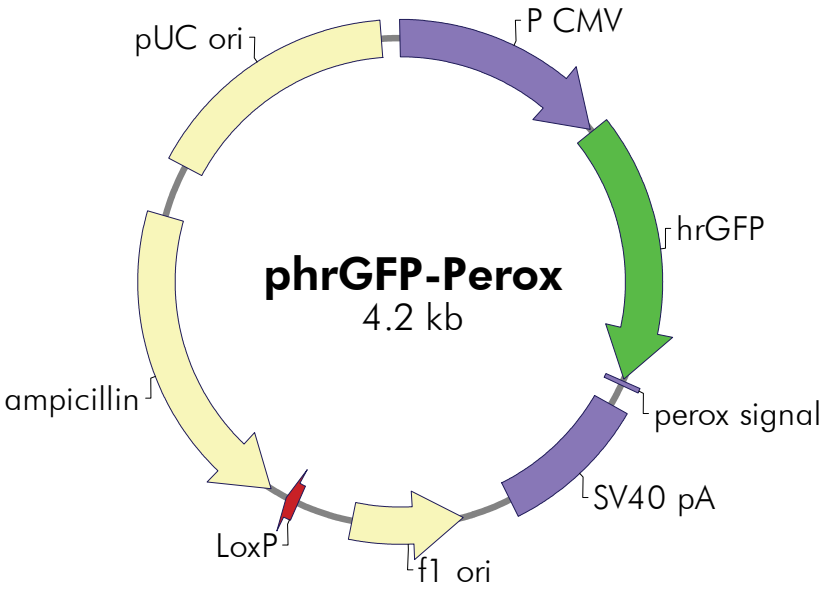
The phrGFP-Golgi Vector



Feature	Nucleotide Position
CMV promoter	1–597
sialyltransferase transmembrane domain (TMD)	606–710
hrGFP ORF	723–1436
T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′]	1468-1489
SV40 polyA signal	1501–1884
f1 origin of ss-DNA replication	2022–2328
LoxP sequence	2486–2520
ampicillin resistance (*bla*) ORF	2569–3426
pUC origin of replication	3573–4240

FIGURE 3 Features of the phrGFP-Golgi subcellular localization vector.

The phrGFP-Perox Vector



Feature	Nucleotide Position
CMV promoter	1–597
hrGFP ORF	606–1322
peroxosomal localization signal	1329–1340
T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′]	1369-1390
SV40 polyA signal	1402–1785
f1 origin of ss-DNA replication	1923–2229
LoxP sequence	2387–2421
ampicillin resistance (*bla*) ORF	2470–3327
pUC origin of replication	3474–4141

FIGURE 4 Features of the phrGFP-Perox subcellular localization vector.

OVERVIEW OF *CRE*-MEDIATED SITE-SPECIFIC RECOMBINATION

The Agilent Vitality hrGFP subcellular localization vectors utilize *Cre*-mediated site-specific recombination to allow the quick and efficient directional insertion of prefabricated modules. The site-specific recombination catalyzed by *Cre* recombinase is dependent on the presence and orientation of two *LoxP* sites. Each *LoxP* site has two 13-bp inverted repeats, which serve as recognition sites for the recombinase, on opposite sides of an 8-bp nonpalindromic region. The relative orientations of these *LoxP* sites determine the effects of the recombination event. Two sites oriented as tandem repeats results in the excision of the intervening sequence, while two sites in opposite orientations lead to an inversion of the DNA sequence between the two sites. In addition to these intramolecular reactions, site-specific recombination can be intermolecular, leading to the formation of a co-integrate, which is a new molecule formed by the insertion of one DNA molecule into another. A schematic diagram of the recombinant molecule formed by inserting a pExchange module into the hrGFP-based vector is shown in Figure 5. The same type of recombination reaction is possible using any of the three pExchange modules (see Figure 6) and any of the Vitality vectors.

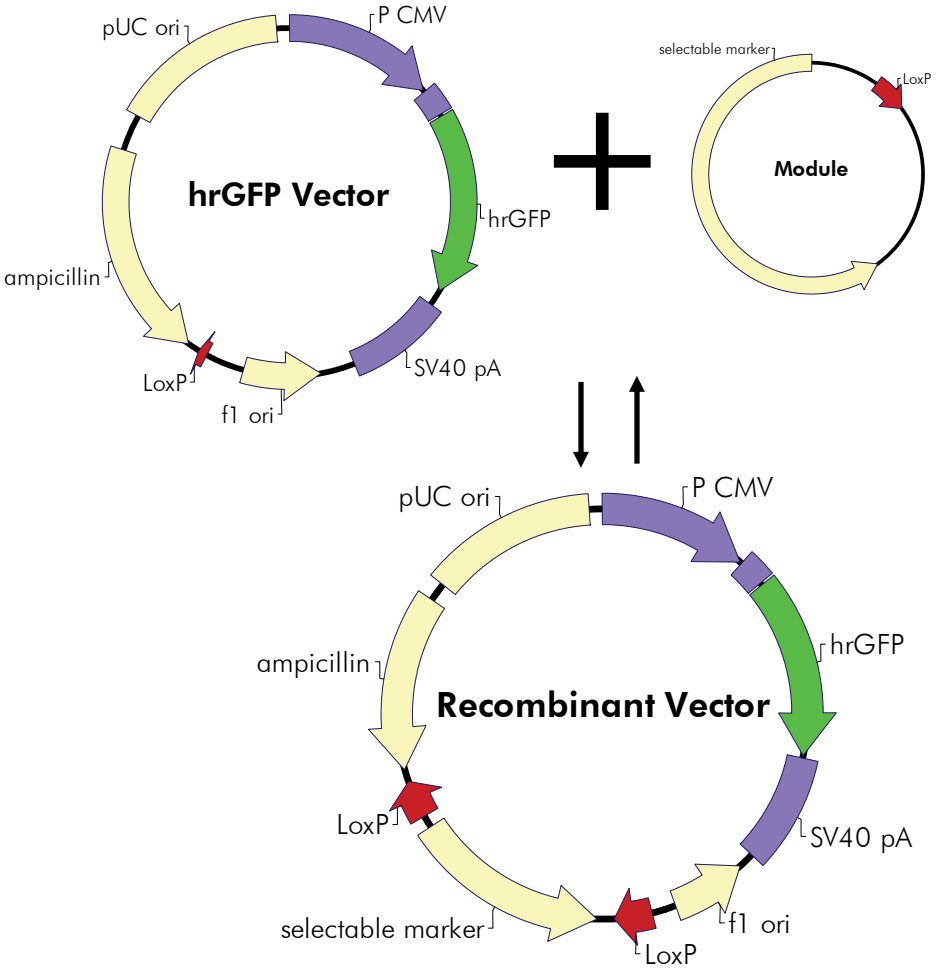


FIGURE 5 *Cre*-mediated recombination for introduction of new resistance cassettes. (*Note*: vector and module features are not drawn to scale.)

MODULE DESCRIPTIONS

The Vitality hrGFP vectors allow for the introduction of a desired eukaryotic resistance gene into the core expression vectors. Each module, shown in Figure 6, contains a *LoxP* site for insertion of either the hygromycin-, puromycin-, or neomycin-resistance module into the *LoxP* site of any of the Vitality hrGFP vectors. The hygromycin- and puromycin-resistance modules also have a chloramphenicol-resistance marker for selection. Further sequence information regarding the Exchange modules is available at *www.genomics.agilent.com*.

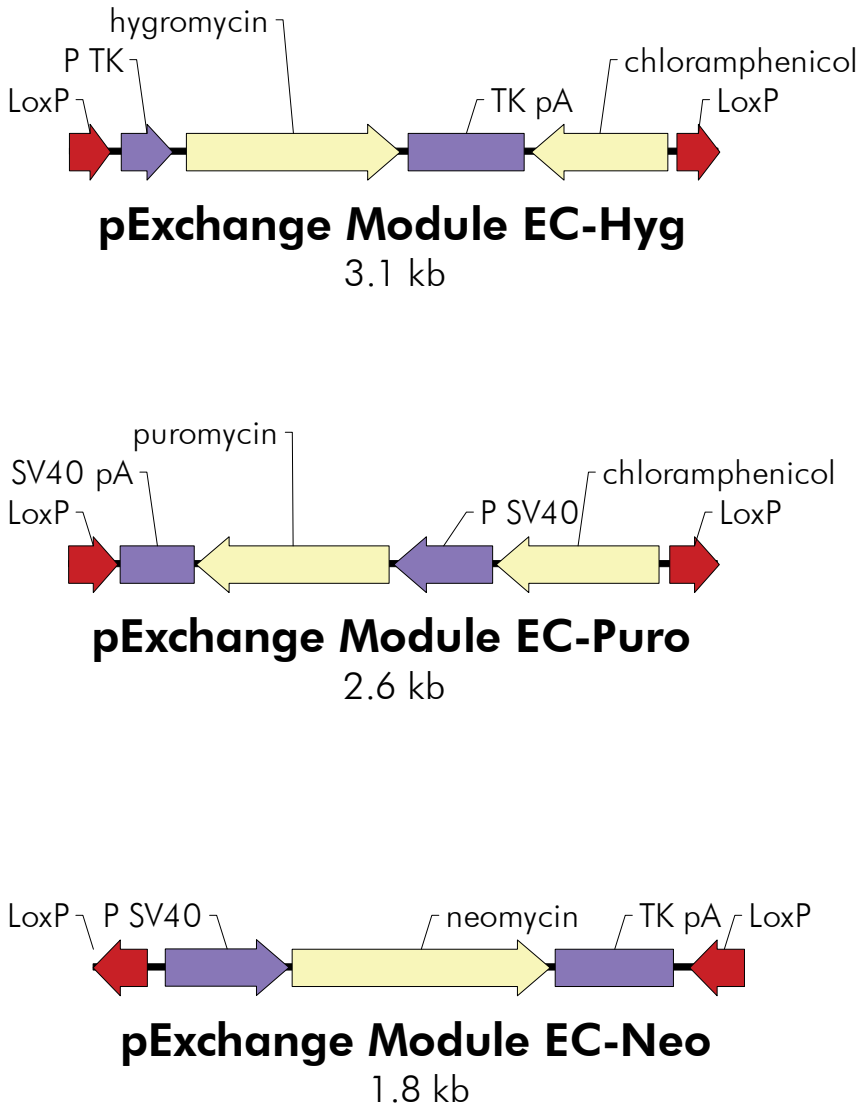


FIGURE 6 The three pExchange modules, available separately from Agilent. (*Note*: module features are not drawn to scale.)

1. To prepare the recombination reaction, add the following components to a 0.5-ml microcentrifuge tube:

   X µl of Vitality hrGFP vector containing the gene of interest (500 ng)
   X µl of pExchange module (100 ng)
   1 µl of *Cre* recombinase
   1 µl of 10× *Cre* recombinase reaction buffer
   X µl of distilled water (dH_2O) to a final volume of 10 µl

2. Incubate reaction for 30 minutes at 37°C. The reaction reaches equilibrium in ≤30 minutes.

3. Heat the reaction at 65°C for 20 minutes to denature the *Cre* recombinase. If not denatured, *Cre* recombinase will reduce the transformation efficiency.

Transform competent bacteria with 2.5 µl of the *Cre*-recombination reaction, and plate the transformed bacteria on LB agar plates containing the appropriate antibiotic. For the pExchange modules EC-Hyg and EC-Puro, the appropriate antibiotic is chloramphenicol. For the pExchange module EC-Neo, the appropriate antibiotic is kanamycin. Refer to reference 9 in *References* for a transformation protocol.

Note *Competent cells with transformation efficiencies ≥5 × 10^9 cfu/µg are available from Agilent.*

Confirming Recombination

The presence of recombinant plasmids can be confirmed by analyzing the miniprep DNA of a few colonies by restriction digestion. Ninety-five percent of the colonies are expected to contain recombinant plasmids.

MAMMALIAN CELL TRANSFECTION

Prepare enough DNA of appropriate purity for the mammalian cell transfection procedure to be carried out. Protocols for transfection of mammalian cell lines can be found in Sambrook, *et al*. (1989).[9]

The efficiency of transfection will vary depending on the host cell line used. In most cases, mammalian host cell lines transfected with plasmids should show expression of hrGFP 24–72 hours after transfection. Fluorescing cells growing in tissue culture dishes can be observed using an inverted fluorescence microscope. For subcellular visualization of organelles we recommend the use of high resolution fluorescent spectroscopy. Transfected cell monolayers may be grown on chambered slides (NUNC brand, Nalge Nunc International). Cells are prepared for examination by washing one time in PBS, fixing for 10 minutes in 3% paraformaldehyde in PBS, and rinsing three times in phosphate buffer. For best results, we recommend that cells be coverslipped using Fluoromount-G (Electron Microscopy Sciences). For digital imaging, a high resolution microscope such as the Olympus BX60 fluorescent upright microscope fitted with a Spot™ RT slider digital camera (Diagnostic Instruments Inc.) is recommended.

Images of HeLa cells expressing each of the Vitality subcellular localization vectors can be found in Figure 3 of reference 8 or visit *www.genomics.agilent.com* and navigate to the Vitality vectors product page.

SPECIFICATIONS FOR HRGFP AND EGFP EXCITATION AND EMISSION SPECTRA

GFP Form[a]	Excitation/Emission Spectra Maxima (nm)
hrGFP	500/506
EGFP	488/509[b]

[a] Both forms of GFP compared in this table have been codon-optimized for maximum expression in human cells.

[b] The emission spectrum for EGFP also shows a shoulder at 540 nm.

Note *Filter sets compatible with the detection of hrGFP and EGFP are sold by Omega Optical, Inc. (Phone: 802 254 2690, or see* www.omegafilters.com*):*

Exciter filter: *XF1073*

Emitter filter: *XF3084*

Beam splitter: *XF2010*

Microscope cube set with the exciter filter, emitter filter and beam splitter: *XF100-2*

TROUBLESHOOTING

Observation	Suggestion
Very few colonies following transformation with *Cre*-recombined vector	Transformation efficiency is inhibited by *Cre* recombinase that is not denatured by heat treatment. Heat the recombination reaction at 65°C for 20 minutes to denature the *Cre* recombinase
	Ensure that the *Cre*-recombinant transformations are plated on the appropriate agar plates
	Transformation is inefficient. Check transformation efficiency with a control plasmid
	DNA is degraded. Electrophorese the DNA to check its quality

PREPARATION OF MEDIA AND REAGENTS

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave
Cool to 55°C.
Add appropriate antibiotic.
Pour into petri dishes (~25 ml/100-mm plate)

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MSDS INFORMATION

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