Preparation of Standard Plasmids for Real-Time PCR

Materials

- 20% Lysol
- 70% Ethanol
- Plasmid
- Restriction Enzyme with Buffer
- 10X GeneAmp PCR Buffer (Applied Biosystems, Cat# N8080189)
- Sheared Salmon Sperm (SSS) DNA (Ambion, Cat# 9680)
- Nuclease-free Water (Ambion, Cat# 9937)
- 15mL polypropylene tube
- p20, p200, and p1000 pipettes with aerosol barrier tips
- 1.7mL microcentrifuge tubes
- 0.65mL microcentrifuge tubes
- Vortex mixer
- Biosafety cabinet with UV capabilities
- QIAquick PCR Purification Kit (Qiagen, Cat# 28104)
- QIAmp DNA Mini Kit (Qiagen, Cat# 51304)
- Spectrophotometer

Procedure

Note: Work inside the biosafety cabinet to avoid potential contamination from the laboratory environment. Real-time PCR is extremely sensitive to contamination.

- Clean the biosafety cabinet work surface and pipette shafts with 20% Lysol, followed by 70% Ethanol. Irradiate with UV light for 10-15 minutes.
- Linearize the standard plasmid with a restriction enzyme that cuts outside your intended PCR target.
- Purify the linearized plasmid using the QIAquick PCR purification kit if it is less than 10kb in size. For larger plasmids, use the QIAmp DNA mini kit. In both cases, pre-warm the elution buffer to 70°C before use. Also, pre-warm the columns loaded with elution buffer at 70°C for 5 minutes. Carry out two elutions in a minimum volume to maximize recovery and concentration of the plasmid. Note: Do not carry out a phenol:chloroform: isoamyl purification of the digest as any contamination with phenol will affect the accuracy of the spectrophotometric reading.
- Determine the concentration of the linearized plasmid by spectrophotometry and convert the concentration readout to grams per Liter (g/L). Note: Make sure the spec reading falls within the linear range of the instrument. Dilute the sample less if necessary. Blank the reading against an equal dilution of elution buffer and use the background correction feature of the instrument (read at 320nm).
- Calculate the formula weight (F.W.) of your standard plasmid: F.W. = Plasmid size (in base pairs) x 662 grams/mole.bp
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- Calculate the molar concentration (M) of your linearized plasmid. \( M = \text{mole/L} = \frac{\text{mass (in grams)/F.W.}}{1\text{L}}. \)
- Determine copy number per microliter of your linearized plasmid based on molar concentration. 1M is equivalent to about 6.0221415 \( \times 10^{23} \) copy numbers.
- Make the first dilution, typically with a final concentration of 1\( \times 10^{10} \) copies per 5µL* (2\( \times 10^9 \) copies/µL). Use the \( C_1V_1=C_2V_2 \) relationship, where \( V_1 \) is the unknown volume, \( C_1 \) is the stock linearized plasmid concentration (copies/µL), \( V_2 \) is the final volume (typically 100µL) and \( C_2 \) is the final concentration (2\( \times 10^9 \) copies/µL). Add \( V_1 \) volume of stock linearized plasmid to 10µL of 10X GeneAmp PCR Buffer + 10µL of 1x GeneAmp PCR Buffer with 20ng/µL of SSS DNA + enough nuclease-free water to make a 100µL solution.
  *Note: In our lab, we add 5 µL of standard (or test article or control) per PCR reaction( or per well). If a different volume of standard is used, change the calculations accordingly.
- Carry out a serial dilution of 1\( \times 10^{10} \) copies/µL of the linearized standard. Use 1X GeneAmp PCR Buffer with 2ng/µL of SSS DNA as diluent. A volume of 90µL of diluent is used per dilution. See the diagram below as reference. Discard the last 10µL. Use the 1\( \times 10^7 \) copies/µL through the 10 copies/µL dilutions as standards. Store at -20°C.

Making Standards

1. Pipet 90µl of 1X PCR Buffer 2ng/µl SSS DNA into the 8 standard tubes.
2. Then pipet 10µl of 10e9 into the 10e9 standard tube and vortex for 20 seconds.
3. Again pipet 10µl of 10e8 into the 10e8 standard tube and vortex for 20 seconds.
4. Repeat this process of transferring 10µl of a greater diluted standard to a lesser along with vortexing until 10e1 is made.