

## PROTOCOL- Calculation of Copy Number Standards for PCR Screening Assays

The following is based on the calculation given on the University of Michigan website at <http://www.med.umich.edu/tamc/spike.html>

The general calculation makes two assumptions: 1) the haploid content of a mouse genome is  $3 \times 10^9$  bp and 2) the concentration of DNA in the "stock" (PCR template) solution is known (i.e. one well of a 96-well ES cell culture contains ~2 ug of DNA/150 uL elution; the normal range will be 5 ug +/- 3 ug when gDNA is isolated using the Promega SV 96 Genomic DNA Purification System). The calculation will determine the dilution necessary to make a single copy, hemizygous gene-equivalent solution from the available plasmid construct DNA (the "long" version made for assay development) for dilution into the known "stock" DNA. This is the relative amount of the hemizygous gene available for template in a transfected ES cell culture when present as the result of homologous recombination. Please note that the amount of non-homologous recombination can exceed that of homologous recombination by several gene copies to several orders of magnitude in a typical ES cell electroporation experiment. The screening assay must contain at least one primer outside one arm of the construct used for transfection in order to be specific for homologous recombination events. To design a homologous recombination PCR screening assay a "test" plasmid with one extended arm should be constructed to validate the PCR screening assay. The PCR reaction will include one primer within the inserted DNA and one primer on the "extended" region of the "test" plasmid. To prepare a 1-copy per genome standard for assay development make the following calculation:

$$\frac{\text{mass of transgene DNA}}{\text{ug gDNA}} = \frac{\text{N bp transgene DNA}}{3 \times 10^9 \text{ bp gDNA}}$$

1. Determine the bp of the transgene. This will be the size of the construct plus ARMS plus vector (since this is the amount of plasmid DNA that contains one copy of the neo cassette that will be used for spiking). The size should be reduced accordingly if a digested fragment is used.
2. Calculate the mass of transgene DNA based on the above equation:

$$\text{mass of transgene DNA} = \frac{(\text{bp transgene}) \times (\text{ug DNA})}{3 \times 10^9 \text{ bp gDNA}}$$

$$\text{i.e. for a 12000 bp insert: mass} = \frac{(12000 \text{ bp}) \times (1 \text{ ug gDNA}^*)}{(3 \times 10^9 \text{ bp})} = 0.000004 \text{ ug DNA} \quad (4 \text{ pg DNA})$$

*\*NOTE: While the total gDNA in the "stock" that will be spiked with plasmid DNA is 2 ug (in 150 uL volume) the transfected gene is hemizygous so only 1/2 of the total DNA in a genuine transfected ES cell will contain the transfected gene so ( $2 \times \frac{1}{2} = 1$ ) 1 ug should be used for this calculation.*

3. To prepare a 1-copy standard for the positive control sample add 4 pg of transgene DNA (for the 12 kp plasmid example) to 2 ug of ES cell gDNA. If the "test" plasmid is 1ug/uL dilute it to give a 10 pg/uL solution ( $1:10^5$ ). Spike 150 uL ES cell gDNA (i.e. the entire content of one well of a 96-well dish) with 4 uL of "test" plasmid DNA dilution to give a 10-copy ES cell gDNA standard. Additional dilutions of the 10-copy "test" plasmid DNA with additional ES cell gDNA can be used to make a dilution series: 1-copy (1:10), 0.5-copy (1:20), 0.1-copy (1:100), etc to help assess the sensitivity of the screening assay. The amount of the gDNA standard used as template in your PCR assay will normally be in the 1-5 uL range (~13-65 ng gDNA) per 50 uL PCR reaction, the optimum template concentration is a function of the template capacity of your PCR assay, it should be relatively broad to accommodate varying gDNA concentrations in the ES cell samples but generally needs not be optimized for screening. The "spiked" plasmid DNA concentration in the same reaction will range from ~0.003 ng~1.3 ng (depending on the size of the construct) well above the detection limit of most PCR assays. The assay specificity will be assessed using "unspiked" gDNA as a negative control sample, lack of signal with the negative control and generation of signal with the "spiked" controls indicates an assay with the requisite specificity.

*If the PCR assay does not generate the predicted product for the single copy control sample then the assay sensitivity is too low to use for screening ES cell gDNA samples. The sensitivity must be increased to the single copy level before it can be used to screen ES clones. To increase assay sensitivity try optimizing the PCR reaction using a different polymerase, a range of  $Mg^{2+}$  levels, addition of DMSO or betaine to the reaction (or other commercially available additives), adjustment to the time and/or temperature of cycling, etc. If all else fails repeat the reaction with a different primer set until an assay with the requisite sensitivity is achieved.*

