

Escherichia coli Plasmid Copy Number Assay

INTRODUCTION

Measurement of plasmid copy number can be achieved using sequence-specific DNA assays on the Threshold[®] System. Plasmid copy number is essentially the number of copies of a given plasmid per cell. To overcome inconsistencies in the efficiency of DNA extraction from cells between experiments, the quantity of chromosomal DNA extracted is generally used to normalize the plasmid measurements. Copy number is therefore usually expressed as a ratio of the number of plasmid molecules per chromosome (or chromosomal equivalent). To determine plasmid copy number from a given sample of cells, it is necessary to prepare genomic DNA (total DNA, plasmid and chromosome), and measure the ratio of plasmid to chromosomal DNA. This can be done using the Threshold system by performing two sequence-specific DNA assays, one for a chromosomal marker sequence, and one for a plasmid marker sequence. The sequence-specific DNA assay is described in the ILA application note, "Sequence-Specific DNA Assay".

For sequence-specific assays two oligonucleotide probes are required, one biotinylated and the other fluoresceinated. They should be about 30 bases long and must both hybridize to the same strand of the target molecule. Larger DNA fragments containing the target site generally give lower signals, so for greatest sensitivity the sample or standard DNA should be digested with a restriction enzyme that yields the smallest possible fragment containing the binding sites for both probes. In addition, because the signal is dependent on this fragment length, the same restriction enzyme should be used for standards and unknowns to achieve accurate quantitation.

For the plasmid copy number assay, two pairs of probes are required, one pair for the plasmid determination and one pair for the chromosome determination. It is convenient and desirable for the plasmid and chromosomal target fragments to be released by the same restriction enzyme. This will enable use of aliquots of the same digest for determination of both plasmid and chromosome, and eliminate concerns over differences due to performing separate restriction digestions for chromosome and plasmid.

This application note provides a preliminary protocol for a plasmid copy number assay. The development and characterization of an assay for *Escherichia coli* pMB1 based replicons (such as pBR322) is used as a model system. This application note is intended as a guide and does not represent a validation of this assay, nor necessarily the optimal performance parameters for all probe and target DNA combinations.

Note: One chromosomal equivalent is an amount of DNA equivalent to the mass of one chromosome. Owing to the size and fragility of the chromosome, and the preparation techniques used, one chromosomal equivalent will not contain one single complete chromosome, but will contain fragments from many chromosomes. For conversions from mass of chromosomal DNA to chromosomal equivalents, the size of the E. coli chromosome was taken as 4.64×10^6 base pairs¹, and one base pair was taken as having a molecular weight of 650 Daltons.

MATERIALS

- 1 **Threshold[®] System** from Molecular Devices Corporation (catalog #0200-0500), 1311 Orleans Drive, Sunnyvale, CA 94089, tel: 408-747-1700 or 800-635-5577.
- 2 **Immuno-Ligand Assay Detection Kit** from Molecular Devices Corporation (catalog #R9003).
- 3 **Restriction enzyme *HinP1 I*** from New England Biolabs (catalog #139S), used with the supplied 10X restriction buffer.
- 4 **Biotinylated and Fluoresceinated oligonucleotide probes** are available from a variety of sources. The probes used were purchased from Applied Biosystems (plasmid probes) and Operon Technologies, 1000 Atlantic Avenue, Alameda, CA 94501, tel: 510-865-8644 (Chromosomal probes). Probes were biotinylated or fluoresceinated at the 5' end by adding a biotin or fluorescein in these positions during the synthesis.

Plasmid probes

The probes used are complementary to a 114 bp region of the pMB1 origin of replication (ORI), which is common to many *E. coli* plasmids. This target region is flanked by *HinP1 I* restriction sites. The probe sequences are (written 5' to 3'):

Biotinylated:

b-CCAGTTACCTTCGGAAAAAG

Fluoresceinated:

f-TAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT

Chromosomal probes

The probes used are complementary to a 140 bp conserved region of the 16S rRNA genes (*rrs* genes, of which there are seven copies in the *E. coli* chromosome). This target region is flanked by *HinP1* I restriction sites. The probe sequences are (written 5' to 3'):

Biotinylated:

b-GCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCC

Fluoresceinated:

f-GGGGATGACGTCAAGTCATCATGGCCCTTACGACC

- 5 ***Qiagen columns*** were purchased from Qiagen Inc. (Qiagen-tip 500, catalog #10063, Qiagen Genomic-tip 500/G, catalog #10262, Qiagen Genomic-tip 20/G, catalog #10223). Buffers for running the columns were made according to the Qiagen Plasmid and Qiagen Genomic Handbooks, with exceptions as noted below.
- 6 ***BSA, fraction V*** was purchased from Sigma Chemical Co. (catalog #A7888) and dissolved at 25 mg/mL in sterile water for injection.
- 7 ***Hybridization Buffer***. The formulation for Hybridization Buffer is:

Stock (1 Liter)	Quantity	Final Concentration
NaH ₂ PO ₄ .H ₂ O	20.7g	150 mM
NaCl	26.3g	450 mM
0.5 M EDTA	6 mL	3 mM
Triton-X-100	2.5 mL	0.25% v/v
25 mg/mL BSA	1 mL	25 µg/mL

Stir to dissolve in 900 mL of deionized water, then adjust to pH 7.4 with 10 M NaOH (approximately 10 mL) before making up to final volume. Filter sterilize through a 0.2 µm filter.

*** BSA was added to the hybridization buffer used for the generation of the data in this applications note, however it may cause problems with filtration, especially with extended denaturing times, and can be omitted.

METHODS

Standard molecular biology techniques can be used for generation of DNA stocks, restriction digestion and agarose gel electrophoresis. There are several comprehensive laboratory manuals covering these basic techniques^{2,3} including details of the reagents required. In general when handling DNA, aseptic technique and sterile disposables should be used.

Preparation of stock DNA

Plasmid DNA

Plasmid stocks were prepared using Qiagen-tip 500 columns following the instructions in the Qiagen Plasmid Handbook, and digested with the restriction enzyme *HinP1* I or its isoschizomer *Hha* I. For further details see the ILA application note, "Sequence-Specific DNA Assay".

Genomic DNA

Genomic DNA (total DNA: chromosomal DNA for plasmid-free strains, or chromosome and plasmid for plasmid-bearing strains) was prepared using Qiagen Genomic columns. In order to improve the recovery of representative levels of chromosomal DNA relative to the plasmid DNA, a modification of the Bacterial DNA Isolation Protocol given in the Qiagen Genomic DNA handbook was used. One important factor is to take care not to overload the columns. For this reason the number of cells used was reduced as detailed. In order to calculate the volume of culture to use, the number of cells per mL should be determined using a microscope and bacterial counting chamber. For convenience, a calibration curve can be constructed relating cell numbers to optical density at 590nm, by measuring dilutions of the culture in a spectrophotometer. The modifications to the Qiagen method are detailed below:

For a Qiagen Genomic-tip 500/G, 10mL of an overnight culture of the *E. coli* strain was grown in Luria Broth, with appropriate antibiotic supplement for plasmid-bearing strains. Approximately 3×10^{10} cells (4 to 10 mL of the culture), was used per Genomic-tip 500/G.

For the lysis step the level of Lysozyme was increased by a factor of 4 (to 120mg per preparation). The Proteinase K was not added at this stage. The lysis incubation time at 37°C was extended to 1.5 to 2 hours.

The Proteinase K was added at the beginning of the clearing step with Guanidine Hydrochloride (Buffer B2). The level of Proteinase K was increased by a factor of 3 (to 30mg per preparation). This 50 °C incubation was extended to 1 hour, or until clear.

Prior to loading the columns with the bacterial lysate, the lysate was vortexed vigorously until viscosity was reduced, improving flow on the columns.

The column elution buffer (Buffer QF) was modified by increasing the concentration of NaCl to 1.6M. The column elution was performed by adding 5mL aliquots of QF pre-warmed to 55°C. The column may in addition be placed in a warm room (37°C), to aid the recovery of chromosomal DNA.

ASSAY PROTOCOL

The copy number determination is performed in the following steps:

- 1 Prepare total DNA from sample (cells).
- 2 Digest DNA with *HinP1* I.
- 3 Test appropriate dilutions of digests for plasmid (against a plasmid standard curve, with the plasmid probes), and for chromosomal DNA (against a chromosomal standard curve, with the chromosomal probes).
- 4 From these results calculate the ratio of plasmid to chromosome.

Sample DNA Preparation

DNA was prepared from samples using Qiagen genomic tips, using the method described above. Smaller scale columns are generally more appropriate for samples. Qiagen Tip 20Gs have been used successfully for samples, using the modifications to the Qiagen protocol listed above, scaled down as appropriate. The sample preparations were made using approximately 1.8×10^9 cells per Genomic-tip 20/G. These smaller columns gave similar performance to the large columns with respect to relative recovery of chromosomal and plasmid DNA.

Restriction Digestion of Samples

Bulk digests of standard plasmid and chromosomal DNAs with *HinP1* I were set up as described in the ILA application note, "Sequence-Specific DNA Assay". Each digest provided enough material to run several chromosomal standard curves or many plasmid standard curves. *HinP1* I was used instead of the isoschizomer *Hha* I (as used in the ILA application note, "Sequence-Specific DNA Assay") because *HinP1* I does not require BSA in the digest. At the high loading of sample required for the top chromosomal standards, the denatured BSA from the *Hha* I digests blocked filtration of the samples.

Sample DNA was also digested with the enzyme *HinP1* I. After digestion the digest was diluted to a suitable concentration for the chromosomal measurement. From this dilution aliquots were taken for the chromosomal determination, and further dilutions were made for the plasmid determination. The chromosomal measurement was typically made at a loading of 1-2 μ g of total DNA per test replicate (i.e. 2 μ g/100 μ L), and the plasmid measurement was typically made at 1:10 to 1:100 dilutions from this concentration, depending on the plasmid content of the sample.

Threshold Assay

Two Threshold Assays were run side by side, one set of chromosomal standards and sample sticks for the chromosomal determination, using chromosomal probes, and a second set of plasmid standards and sample sticks for the plasmid determination, using the plasmid probes. All hybridizations were performed in Hybridization Buffer in 2mL sterile Sarstedt screwcap tubes and detection was performed with the Threshold System and standard ILA reagents as follows:

- Step 1** Dispense 100 μ L of target DNA (Standards and digested samples in Hybridization Buffer) to each 2 mL Sarstedt tube.
- Step 2** Prepare a probe cocktail containing both probes in Hybridization Buffer, plasmid probes each at 5nM concentration for the plasmid determination, OR chromosomal probes each at 5nM concentration in Hybridization Buffer for the chromosomal determination.
- Step 3** Add 100 μ L of probe mix to each tube. Vortex briefly to mix.
- Step 4** Incubate tubes in a heating block at 105°C for 10 minutes (denaturing step).
- Step 5** Transfer all tubes to a heating block at 55°C, incubate for 10 minutes (annealing step).
- Step 6** Transfer all tubes to ice, incubate for 5 minutes.
- Step 7** Add 1 mL of reconstituted ILA Capture reagent diluted 1:10 in ILA Assay Buffer to each tube. Cap the tubes and invert several times to mix in the Capture reagent.
- Step 8** Transfer the entire volume from the tubes to the filtration units and filter all samples onto Threshold sticks on low vacuum.
- Step 9** Add 2 mL of ILA Wash Buffer to each sample well, then filter on high vacuum.
- Step 10** When all the Wash Buffer has filtered through, change to SPECIAL vacuum. It is important that all the wells are totally empty before adding the Enzyme reagent, otherwise the Enzyme will be diluted which will result in reduced signals for those wells.
- Step 11** Add 100 μ L of reconstituted ILA Enzyme reagent to each well. Check carefully for bubbles on the surface of the membrane. These will prevent the filtration of the enzyme. If a bubble is present it can be dislodged by sharply tapping the filter block with your hand.
- Step 12** When the wells are empty, add 1 mL of ILA Wash Buffer to each sample well, then filter on high vacuum.
- Step 13** When all the wells are empty turn off the vacuum and read the sticks.

Note: Reagent additions in steps 3, 7, 9, 11, and 12 can be made using an Eppendorf Repeater Pipettor with a Combitip[®]. The 100 μ l additions are more precise if a micropipette tip is fitted onto the Combitip.

Preparation of standard curves

In order to quantitate plasmid copy number and compensate for stick to stick variation it is necessary to generate plasmid and chromosomal standard curves and mid-calibrators to run in the # position on each stick. Plasmid standards were typically run as a twofold dilution series of the digested pGEM3 in hybridization buffer, in the range 15000×10^6 to 235×10^6 molecules/test (45 to 0.7 ng/test). A mid-calibrator of 1875×10^6 molecules/test and a zero calibrator of hybridization buffer was run on each stick in the # and * positions respectively. Chromosomal standards were typically run as a twofold dilution series of the digested *E. coli* chromosomal DNA in hybridization buffer, in the range 2000×10^6 to 31.3×10^6 chromosomal equivalents/test (approximately 10 to 0.16 μg /test). A mid-calibrator of 250×10^6 chromosomal equivalents (approximately 1 μg /test) and a zero-calibrator of hybridization buffer was run on each stick in the # and * positions respectively. One hundred microliters of each of these standards, and multiple 100 μL aliquots of the zero- and mid-calibrators (one for each stick) were prepared and then treated with the samples according to the assay protocol. Typical standard curves are shown superimposed in Figure 1. The power equation was generally found to give the best fit for both plasmid and chromosomal standard curves.

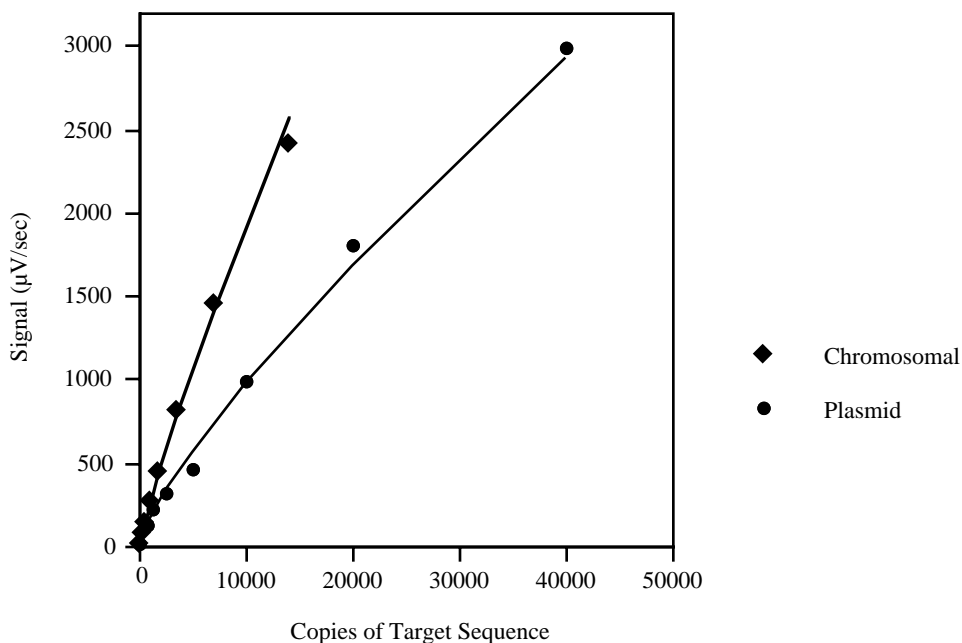


Figure 1: Chromosomal and Plasmid Standard Curves Superimposed

ASSAY CHARACTERIZATION

Plasmid assay

The plasmid assay characterization is described in the "Sequence-Specific DNA Assay" ILA application note. General characteristics of the sequence-specific assay are also described there.

Chromosomal Assay

Probe loading study

A probe loading study was performed to determine the appropriate concentration of probes to use in the assay. Probe mixes containing 0.5, 1.25, 2.5, 5, and 10nM of each probe were tested with 0, and 250×10^6 molecules/test of Chromosomal DNA. The probe mix with a concentration of 5 nM of each probe was chosen because it gave a combination of low background, high signal and economy of use of the probes.

Hybridization kinetics

The time required for hybridization (annealing) of the oligonucleotide probes to the single stranded target DNA was determined using 0, and 250×10^6 molecules/test of target DNA, and annealing times of 0, 2.5, 5, 10, 15, and 30 minutes. Signals were consistently good between 2.5 and 15 minutes. The signal decreased at 30 minutes, consistent with the plasmid assay results. It is believed that the decrease in signal with long hybridization times is due to the complementary strand re-annealing to the target strand and displacing the oligonucleotide probes. Ten minutes was selected as the optimum hybridization time for the routine protocol.

Hybridization temperature

The hybridization (annealing) temperature for the chromosomal probes was investigated in two experiments covering the range from 35°C to 65°C. The best annealing temperature was found to be 55°C. This means that both annealing time and temperature were the same for plasmid and chromosomal assays, so the tubes for the two assays could be easily processed in parallel.

Specificity

Specificity of the chromosomal probes was demonstrated in two ways. Firstly, the interference due to plasmid DNA was investigated. Chromosomal DNA at 400×10^6 and 100×10^6 chromosomal equivalents was assayed in the presence of 0, 0.25, and 1 μ g of plasmid (approximately 80×10^9 and 325×10^9 copies of plasmid). The raw signals are given below. The apparent slight decrease in signal at the highest loading of plasmid was not statistically significant:

Plasmid (μ g/test)	Chromosomal 100×10^6 copies μ V/sec	Chromosomal 400×10^6 copies μ V/sec
0	385.5	1639.6
0.25	403.1	1678.7
1.0	415.8	1519.6

Secondly, the specificity was demonstrated by digesting the chromosomal DNA with the restriction enzyme *Mnl* I. This enzyme cuts in the middle of the target sequence, separating the binding sites for the two probes which should abolish

the assay signal as shown in Figure 2. When chromosomal DNA preparations were digested separately with *HinP1* I and with *Mnl* I they gave a strong signal with the *HinP1* I digest and no signal in the assay with the *Mnl* I digest.

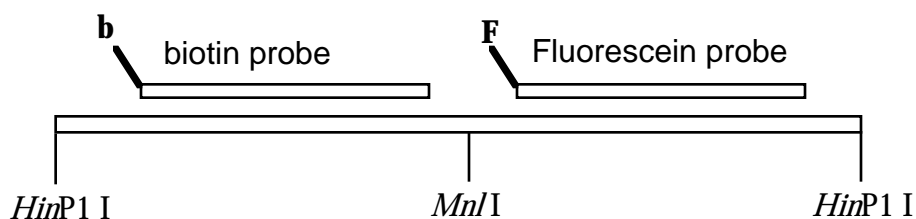


Figure 2: The 140bp *rrs* gene chromosomal target region showing probe alignment and restriction sites.

Copy Number Assay

The copy number assay was characterized with respect to reproducibility and accuracy in two different ways: firstly using mixtures of purified plasmid and chromosomal DNA; and secondly using DNA purified from mixtures of cells with and without plasmid.

Accuracy

Purified DNA: Separate DNA preparations were made of plasmid and of chromosomal DNA. These were quantitated by spectrophotometry and the concentration of plasmids and chromosomal equivalents determined using their size in base pairs and molecular mass. The DNAs were then mixed at fixed plasmid to chromosome ratios to simulate various plasmid copy numbers. The results are given below.

Theoretical Copy Number	Measured Copy Number
1000	1020
250	280
100	109

Mixtures of cells: Cells with a very high plasmid copy number were mixed with plasmid-less cells in varying ratios. The copy number was measured from DNA prepared from these cell mixtures and the results compared to the theoretical copy number for each mixture of cells. These results are shown below.

Theoretical Copy Number	Measured Copy Number
1827	1967
735	770
266	287
24	25

Reproducibility

Purified DNA: A mixture of chromosomal and plasmid DNA was restriction digested and assayed 12 times in one assay for within day precision and in 5 separate assays for between day precision. The results in terms of number of copies of plasmid or chromosomal equivalents are given in the table below. The final column gives the mean of the derived copy numbers calculated on each of the 5 days.

	Within Day (n=12)		Between Day (n=5)		
	Plasmid	Chromosome	Plasmid	Chromosome	Copy Number
Mean	37252	224	42228	194	217
St. Dev.	3168	11	4915	10	20
% C.V.	8.5%	4.8%	11.6%	5.3%	9.1%

Mixtures of Cells: A mixture of cells containing 25% plasmid bearing cells was prepared. On each of two days, three DNA preparations were made from this cell mix. Each DNA preparation was digested with *HinP1* I, and assayed in the plasmid copy number assay. The plasmid copy number results (number of plasmid copies per chromosome equivalent) are given below.

	Day 1 (n=3)	Day 2 (n=3)	Overall (n=6)
Mean	889	885	887
St. Dev.	19	11	14
% C.V.	2.1%	1.3%	1.6%

SUMMARY

The data presented in this application note was generated using *E. coli* K12 strains with pMB1 based plasmids digested with *HinP1* I, and a sequential ILA protocol. Good linearity was seen with standard DNA preparations, and the assay specificity, accuracy and reproducibility was good. The plasmid copy number assay described here should have applications in fermentation monitoring and cell banking of microbial strains.

ACKNOWLEDGEMENT

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