TITLE:

The Lambda Select *cII* Mutation Detection System

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Keywords:
Bacteriophage, *cII* transgene, embryonic mouse fibroblasts (EMF), mutation, shuttle vector.

SUMMARY:
We describe a detailed protocol for the Lambda Select *cII* mutation assay in cultured cells of transgenic rodents or the corresponding animals treated with a chemical/physical agent of interest. This approach has been widely used for mutagenicity testing of carcinogens in mammalian cells.

Abstract:
A number of transgenic animal models and mutation detection systems have been developed for mutagenicity testing of carcinogens in mammalian cells. Of these, transgenic Big Blue mice and the Lambda Select *cII* Mutation Detection System have been employed for mutagenicity experiments by many research groups, worldwide. Here, we describe a detailed protocol for the Lambda Select *cII* mutation assay, which can be applied to cultured cells of transgenic mice/rats or the corresponding animals treated with a chemical/physical agent of interest. The protocol consists of the following steps: (1) isolation of genomic DNA from cells or organs/tissues of transgenic animals treated *in vitro* or *in vivo*, respectively, with a test compound; (2) recovery of the lambda shuttle vector carrying a mutational reporter gene (*i.e.*, *cII* transgene) from the genomic DNA; (3) packaging of the rescued vectors into infectious bacteriophages; (4) infecting a host bacteria and culturing under selective conditions to allow propagation of the induced *cII* mutations; and (5) scoring the *cII*-mutants and DNA sequence analysis to determine the *cII* mutant frequency and mutation spectrum, respectively.

INTRODUCTION:

A wide range of transgenic animal models and mutation detection systems have been developed for mutagenicity testing of carcinogens in mammalian cells. Of these, transgenic Big Blue mice and the λ Select *cII* Mutation Detection System have been employed for mutagenicity experiments by our group and many other research groups, worldwide [1-9]. For the past 16 years, our laboratory has investigated the mutagenic effects of various chemical and/or physical agents using these transgenic animals or their corresponding embryonic fibroblast cell cultures treated with a test compound and subsequently analyzing the phenotype and genotype of the *cII* transgene by the λ Select *cII* assay and DNA sequencing, respectively [10-24]. The genome of these transgenic animals contains a bacteriophage λ shuttle vector (λLIZ) integrated on chromosome 4 as a multi-copy head-to-tail concatemer [1,2,25]. The λLIZ shuttle vector carries two mutational reporter genes, namely the *lacI* and *cII* transgenes [1,2,25-47]. The λ Select *cII* assay is based on the recovery of the λLIZ shuttle vectors from the genomic DNA of cells derived from organs/tissues of transgenic animals [1,2,25]. The recovered λLIZ shuttle vectors are then packaged into λ phage heads capable of infecting an indictor host *Escherichia coli*. Subsequently, the infected bacteria are grown under selective conditions to allow for scoring and analysis of mutations in the *cII* transgene [1,3]. Here, we describe a detailed protocol for the λ Select *cII* assay, which consists of isolation of genomic DNA from cells/organs of transgenic animals treated *in vitro*/*in vivo* with a test compound, retrieval of the λLIZ shuttle vectors from the genomic DNA, packaging of the vectors into infectious λ phages, infection of the host *E. coli* with the bacteriophages, identification of the *cII*-mutants under selective conditions to determine the *cII* mutant frequency, and DNA sequence analysis to establish the *cII* mutation spectrum. The protocol can be applied to transgenic mouse/rat cell cultures treated *in vitro* with a chemical/physical agent of interest, or tissues/organs of the corresponding animals treated *in vivo* with the test chemical/agent [1,2,4,48-52]. A schematic presentation of the λ Select *cII* assay is shown in Figure 1.

PROTOCOL:

1. Genomic DNA isolation from mouse embryonic fibroblasts

Note: Primary mouse embryonic fibroblasts are isolated from embryos derived from Big Blue mouse on C57BL/6 genetic background, according to our published protocol [53]. The starting material for this protocol consists of 1 x 10^6 to 1 x 10^7 embryonic fibroblast cells treated with a test compound *versus* control. Harvesting and counting of the cells using standard methods are described in refs. [10,54,55].

1.1. Prepare buffer A (0.3 M Sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris-HCl, pH 8.0, 0.5 mM spermidine, 0.15 mM spermine, and 2 mM EDTA), buffer B (150 mM NaCl and 5 mM EDTA, pH 7.8), and buffer C (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 20 mM EDTA, 1% SDS) in advance, and preserve at room temperature (RT) for up to one year [54,55].

1.2. Resuspend cells in 2-4 mL buffer A in a 15-mL conical centrifuge tube by repeatedly pipetting up and down using a wide bore 1000 µL pipette tip.

1.3. Add one volume (2-4 mL) buffer A containing 1% octylphenoxypolyethoxyethanol (e.g. Nonidet P40) using a glass serological pipette.

1.4. Incubate the tube on ice for 5 minutes.

1.5. Centrifuge the tube at 1000 x *g* for 5 minutes at RT.

1.6. Discard the supernatant and wash the pellet with 10-15 mL buffer A using a glass serological pipette.

1.7. Resuspend the pellet in 2-4 mL buffer B.

1.8. Add one volume (2-4 mL) buffer C containing 600 µg/mL proteinase K.

1.9. Incubate the tube for 3 hours at 37 °C.

1.10. Add RNase A to a final concentration of 100 µg/mL.

1.11. Incubate the tube for an additional 1 hour at 37 °C.

1.12. Add one volume (2-4 mL) phenol (saturated in 0.1 M Tris-HCl, pH 8.0):chloroform:isoamyl alcohol (25:24:1 vol/vol).

Note: Phenol can pose a severe health hazard and must be handled with extreme caution. Phenol is highly corrosive to the skin and readily absorbed through it, among other effects. When handling phenol, always use double gloving, wear protective eyewear, and work in a chemical fume hood.

1.13. Mix well by inverting the tube for 5 minutes on a tube rotator at 4 °C.

1.14. Centrifuge the tube at 1000 x*g* for 5 minutes at 4 °C.

1.15. Remove aqueous phase (top layer) to a new tube using a glass serological pipette.

1.16. Repeat phenol:chloroform:isoamyl alcohol extraction 1 to 3 times until the aqueous phase is clear and the interface is no longer turbid.

1.17. Add 1/10 volume (200-400 µL) 3M Sodium acetate, pH 5.2.

1.18. Add 2.5 volume (5-10 mL) 100% ethanol (chilled) and invert the tube gently by hand for 2-3 minutes.

1.19. Spool the DNA with a glass hook and transfer it to a new tube containing 1-5 mL 70% Ethanol and wash it thoroughly. Alternatively, centrifuge the tube at high speed (3,500 x*g*) at 4 °C to pellet the DNA and wash it thoroughly with 1-5 mL 70% Ethanol.

1.20. Centrifuge the tube at high speed (3,500 x*g*) at RT, remove the supernatant, and air-dry the DNA for 10-15 minutes.

1.21. Dissolve the DNA in 10-100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and store at 4 °C (for short storage of up to one month) or at −20 °C (for longer storage period). Optimal concentration of DNA for the λ Select *cII* assay is 0.5-1.0 µg/µL (in TE buffer) [56].

2. *In vitro* packaging reaction

2.1. Per one packaging reaction, add ~5 µg genomic DNA (final volume: 8-12 µL, genomic DNA was isolated in **Section 1** from mouse embryonic fibroblasts treated *in vitro* with a test compound or control) to a microcentrifuge tube containing the first reaction mix (~10 µL).

Note: In-house prepared or commercially available λ packaging extracts are used in different laboratories. In the commercial packaging extract kit used here [56] (see the Table of Materials), red tubes contain the first reaction mix (~10 µL) and blue tubes contain the second reaction mix (~ 70 µL for, at least 5 reactions).

2.2. Incubate the tube for 90 minutes at 30 °C.

2.3. Add the required volume (~ 12 µL) of the second reaction mix to the tube.

2.4. Incubate the tube for an additional 90 minutes at 30 °C.

2.5. Add 1.1 mL of SM buffer to the tube.

Note: One milliliter of this solution (*i.e.*, packaging reaction mixture) will be used for screening λ *cII*-mutants. The remainder will be used for titering.

2.5.1. Prepare **SM buffer** by mixing 5.8 g NaCl, 2.0 g $MgSO_4.7H_2O$, 50 mL 1 M Tris-HCl (pH 7.5), and 5 mL 2% (w/v) gelatin. Add dH_2O to a final volume of 1 liter, autoclave for 30 minutes. Store at room temperature for up to 1 year.

2.6. Vortex the tube containing the packaged DNA sample for 10 seconds at RT (vigorous vortexing).

2.7. Pulse spin the tube in a microcentrifuge and store on ice until ready to use. If the sample is not going to be used within the same day of packaging, add 50 µl of chloroform per mL of packaged DNA sample, vortex gently, and store at 4 °C for up to 2 weeks.

3. Preparing the *E. coli* G1250 bacterial culture

3.1. At least two days before plating, make a few bacterial streak plates from *Escherichia coli* (*E. coli*) G1250 on TB1-kanamycin agar plates.

3.1.1. Prepare TB1-kanamycin agar plates as follows. Mix 5.0 g NaCl, 10.0 g Casein peptone, and 12.0 g agar. Add 800 mL dH_2O. Add 1 mL 0.1% Thiamine hydrochloride. Adjust pH to 7.0 with NaOH or HCl. Add dH_2O to a final volume of 1 liter.

3.1.2. Mix well and autoclave for 30 minutes. Allow to cool to 55 °C. Add 50.0 mg Kanamycin and mix. Pour into sterile 100-mm petri dishes (20-25 mL per dish). Store plates at 4 °C for up to two weeks.

3.2. Incubate the bacterial streak plates in a stationary 30 °C incubator for at least 24 hours.

3.3. One day before plating, combine 10 mL of TB1 liquid medium with 100 µl of 20% (w/v) maltose-1 M $MgSO_4$ solution in a sterile 50-mL screw-cap conical tube.

3.3.1. Prepare TB1 liquid medium as follows. Mix 5.0 g NaCl and 10.0 g Casein peptone => Add 800 mL dH_2O => Add 1 mL 0.1% Thiamine hydrochloride => Adjust pH to 7.0 with NaOH or HCl => Add dH_2O to a final volume of 1 liter => Mix well and autoclave for 30 minutes => Store medium at room temperature for up to three months.

3.3.2. Prepare 20% (w/v) Maltose-1 M $MgSO_4$ as follows. Mix 20.0 g Maltose and 24.6 g $MgSO_4 . 7H_2O$ => Add dH_2O to a final volume of 100 mL => Filter sterilize => Store at 4 °C for up to 6 months.

3.4. Inoculate the liquid medium with several colonies from the bacterial streak plate using a sterile inoculating loop [56].

3.5. Incubate the liquid culture overnight in a 30 °C shaking incubator with vigorous shaking (250-300 rpm).

3.6. On the day of plating, centrifuge the conical tube containing the G1250 liquid culture at 1500 x*g* for 10 minutes at RT to pellet the bacterial cells.

3.7. Discard the supernatant and resuspend the cell pellet in 10 mL of 10 mM $MgSO_4$.

3.8. Measure the absorbance of a 1:10 dilution of the cell suspension at wavelength 600 nm using a UV-Vis spectrophotometer (*e.g.*, 100 µl cell suspension + 900 µl 10 mM $MgSO_4$) [56].

3.9. Dilute the cell suspension to a final OD600 of 0.5 with 10 mM $MgSO_4$. The prepared suspension of G1250 *E. coli* with OD600 = 0.5 is referred to as 'the G1250 plating culture'.

3.10. Store the G1250 plating culture on ice and use within 1-2 hours.

4. Plating the packaged DNA samples

4.1. Per one packaged DNA sample, prepare sixteen sterile 14 x 100-mm round-bottom tubes and sixteen TB1 agar plates, ten from each set will be used for screening and six for titering (three for Titer 20 and three for Titer 100).

4.1.1. Prepare TB1 agar plates as follows. Mix 5.0 g NaCl, 10.0 g Casein peptone, and 12.0 g Agar => Add 800 mL dH_2O => Add 1 mL 0.1% Thiamine hydrochloride => Adjust pH to 7.0 with NaOH or HCl => Add dH_2O to a final volume of 1 liter.

4.1.2. Mix well and autoclave for 30 minutes => Allow to cool to 55 °C => Pour into sterile 100-mm petri dishes (20-25 mL per dish) => Store plates at 4 °C for up to two weeks.

Note: TB1 agar plates should be prepared at least 24 hours prior to use.

4.2. Aliquot 200 µl of the G1250 plating culture into each round-bottom tube.

4.3. **For titering**, make a 1:100 dilution of the packaged DNA sample and mix well by vortexing (*i.e.*, 10 µl packaged DNA sample + 990 µl SM buffer).

4.4. Add 20 µL of the 1:100 dilution to each of the three Titer 20 tubes.

4.5. Add 100 µl of the 1:100 dilution to each of the three Titer 100 tubes.

4.6. **For screening**, add 100 µl of the '*undiluted*' packaged DNA sample to each of the ten screening tubes.

4.7. Process all titering and screening tubes (2 x 3 + 10 = 16 tubes), as follows: mix well by vortexing for approximately 10 seconds, and then incubate at room temperature for 30 minutes to allow the host *E. coli* to adsorb the phages.

4.8. Add 2.5 mL microwaved molten TB1 top agar (cooled to 55 °C) to each titer or screening tube, mix immediately by vortexing (gently) and pour into the appropriate titer or screening TB1 agar plates.

4.8.1. Prepare TB1 top agar as follows. Mix 5.0 g NaCl, 10.0 g Casein peptone, and 7.0 g Agar => Add 800 mL dH_2O => Add 1 mL 0.1% Thiamine hydrochloride => Adjust pH to 7.0 with NaOH or HCl => Add dH_2O to a final volume of 1 liter.

4.8.2. Mix well and autoclave for 20-25 minutes => Store at room temperature for up to three months.

4.8.3. Prior to use, melt the prepared TB1 top agar in a microwave, mix well, and allow to cool to 55 °C in a water bath.

Note: The molten and cooled TB1 top is added to the content of each titer or screening tube, and after mixing, is poured into the appropriate titer or screening TB1 agar plates.

4.9. Let the plates stand for 15-30 minutes at room temperature, with lid ajar to prevent condensation.

4.10. Invert the plates and place the ten screening plates in a stationary 24 °C incubator and incubate for 46-48 hours (*i.e.*, selective conditions) and the six titer plates in a stationary 37 °C incubator and incubate for 24 hours/overnight (*i.e.*, non-selective conditions).

Note: For quality assurance and standardization of the results, commercially available control phage solutions, containing a mixture of wildtype and mutant *cII* with known mutant frequency, are plated alongside the packaged DNA samples and included in all assay runs [56].

5. Examining the titer and screening plates to determine the *cII* mutant frequency

5.1. Following the 24 hours/overnight incubation at 37 °C, count the number of plaques formed in each of the three Titer 20 plates and Titer 100 plates. To more easily identify the plaques, hold the plate next to a white light box and against a dark background with lid removed (*see*, Fig. 2).

5.2. Make an average (mean) of the number plaques counted in each set of Titer 20 plates and Titer 100 plates (triplicate, each).

5.3. Choose the average number from the set of titer plates that falls closest to the range of 50-200 plaques per plate set.

5.4. Calculate the total number of plaques screened in the ten screening plates as follows:
Total plaques screened = (Mean number of plaques in the chosen set of titer plates ÷ Number of µl of dilution used per chosen titer plate) x Dilution factor x Number of µl of packaged DNA sample per screening plate [100 µl/plate] x Number of screening plates [10].

Note: For example, if the mean number of plaques per plate in the set of Titer 20 plates is 128 and the corresponding number in the set of Titer 100 plates is 478, the number 128 will be used for calculation of the total number of plaques screened, as follows:

(128 ÷ 20) x 100 [dilution factor] x 100 [µl/plate] x 10 [plates] = 640,000

This equals multiplying the mean number of plaques by 5,000 or 1,000 if the average number is based on counts from Titer 20 plates or Titer 100 plates, respectively.

5.5. Following the 46-48 hours incubation at 24 °C, count the total number of plaques formed in the ten screening plates (follow the instructions provided in Section 5.1).

Note: The *cII* mutant frequency is calculated by dividing the total number of plaques formed in the screening plates to the total number of plaques screened. Using the above example, if the total number of plaques counted in the ten screening plates is 112, the *cII* mutant frequency for this DNA sample will be $112 \div 640{,}000 = 17.5 \times 10^{-5}$.

6. Verification of the putative λ *cII* mutants, PCR amplification, and DNA sequencing

6.1. Core the plaque in question with a sterile wide-bore pipet tip and expel it (by pipetting up and down) into a sterile microcentrifuge tube containing 500 µl of sterile SM buffer.

6.2. Incubate for at least 2 hours at room temperature or at 4 °C overnight to allow the phage particles to elute from the agar plug.

6.3. In a sterile 14 x 100-mm round-bottom tube, mix 200 µl of the G1250 plating culture with 1 µl of the cored phage solution and incubate for 30 minutes at room temperature.

6.4. Plate the sample using 2.5 mL of 55 °C molten TB1 top agar and incubate the plate at 24 °C for 46-48 hours (selective conditions), as described in Section 4.8-4.10.

6.5. Once the secondary plaques are formed, use a pipette tip to carefully pick a single well-isolated verified λ *cII*-mutant plaque (avoid touching the bottom agar).

Note: Replating of the putative *cII* mutant plaques serves two purposes: (i) plating artifacts may sometimes be mistaken for small-size plaques; and (ii) an agarose core taken from a screening plate may contain non-mutant phage(s) together with a mutant phage. Secondary plaques from a low-density replating will provide an uncontaminated mutant template for PCR and subsequent DNA sequence analysis.

6.6. Transfer the plaque to a microcentrifuge tube containing 25 µl double-distilled water by pipetting up and down.

6.7. Place the tube in boiling water for 5 minutes.

6.8. Centrifuge at maximum speed (18,000 x*g*) for 3 minutes at RT.

6.9. Transfer 10 µl of the supernatant immediately to a new microcentrifuge tube containing 40 µl of a PCR mastermix in which the final concentrations of the reagents are 1x *Taq* PCR buffer, 10 pmol each of the forward and reverse primers, 12.5 nmol of each dNTP, and 2.5 U of Taq DNA polymerase.

Note: The forward and reverse primers are as follows: 5'-CCACACCTATGGTGTATG-3' (positions -68 to -50 relative to the *cII* start codon) and 5'-CCTCTGCCGAAGTTGAGTAT-3' (positions +345 to +365 relative to the *cII* start codon), respectively.

6.10. Amplify the template using the following cycling parameters: a 3-minute denaturation at 95 °C, followed by 30 cycles of 30 seconds at 95 °C, 1 minute at 60 °C, and 1 minute at 72 °C, with a final extension of 10 minutes at 72 °C.

6.11. Purify the 432-bp PCR product containing the *cII* gene and flanking regions using commercially available PCR purification kits according to the manufacturer's instructions.

6.12. Perform DNA sequencing using an appropriate sequencing platform, and analyze the resulting DNA sequences to detect mutations in the *cII* transgene (see, Note below).

Note: This is best achieved by alignment with the reference *cII* sequence using software programs, such as the web-based T-Coffee sequence alignment server. For instructions on how to use the program, visit: http://tcoffee.crg.cat/.

REPRESENTATIVE RESULTS:

Depending on data distribution, parametric or non-parametric tests are used to determine the significance of difference in the *cII* mutant frequency between treatment and control groups (*i.e.*, induced *versus* spontaneous mutant frequencies). Comparison of the induced *cII* mutant frequencies across different treatment groups is made by various (pairwise) statistical tests, as applicable. The hypergeometric test of Adams and Skopek is commonly used to compare the overall induced- and spontaneous mutation spectra [57], although other tests, such as χ^2 test or Analysis of Variance (ANOVA), can also be used to compare the frequency of each specific type of mutation (*e.g.*, transition, transversion, insertion, or deletion) between the induced- and control mutation spectra, or among various mutation spectra induced by different chemicals/agents or varying doses of the same chemical/agent.

Figure 3 is a compilation of mutant frequency data from our published studies in which we have demonstrated that the extent of increase in relative *cII* mutant frequency in mouse embryonic fibroblasts treated with various chemicals and/or physical agents may vary from a few- to several hundred-fold, depending on the mutagenic '*potency*' of the test compound. Statistically significant fold-increases in the *cII* mutant frequency are shown for mouse embryonic fibroblasts treated with acrylamide [12], glycidamide [14], aflatoxin B1 (AFB1) [22], tamoxifen [18], δ-aminolevulinic acid (δ-ALA) plus low dose ultraviolet light A (UVA: λ > 320–400 nm) [15], benzo(a)pyrene diol epoxide (B(a)PDE) [19], and equilethal doses of UVA, UVB (λ = 280–320 nm), and simulated sunlight UV (SSL) [21] (*see*, Fig. 3).

Figure 4 is a demonstration of the '*sequence-specificity*' of mutations in which we have shown the induction of specific types of mutation in the *cII* transgene in mouse embryonic fibroblasts irradiated with UVB relative to control [23]. The UVB-induced mutation spectrum is

characterized by significant increases in relative frequency of single- or tandem C→T transitions at pyrimidine dinucleotides.

FIGURE LEGENDS:

Figure 1. Schematic presentation of the λ Select *cII* assay. The assay is based on the retrieval of the λLIZ shuttle vectors, containing the *cII* transgene as a mutational reporter gene, from the genomic DNA of cultured cells derived from transgenic rodents treated *in vitro* with a test compound or tissues/organs of the corresponding animals treated *in vivo* with the tested chemical/agent **(Panels A and B)**. The rescued vectors are packaged into λ phage heads that can infect an appropriate host *E. coli* **(Panels C and D)**. The infected bacteria are then grown under selective conditions to allow for scoring and analysis of mutations in the *cII* transgene [1-3,25,52] **(Panel E)**. Determination of the induced *cII* mutant frequency and establishment of the mutation spectrum by DNA sequencing are outlined in **Panels F and G**. The induced- and spontaneous mutation spectra are visualized in different formats. For illustration purposes, we have highlighted a format in which the induced *cII* mutations are typed above the reference sequence, whereas the spontaneous mutations (control) are typed below the reference sequence **(Panel H)**. The height of a mutated base represents its frequency of mutations (*i.e.*, the higher the base, the more frequently mutated). Numbers above a mutated base indicate the percentage frequency of mutations in that base. Deleted bases are underlined. Inserted bases are shown with an arrow. Numbers below the bases are reference nucleotide positions. Data are from our published study [23].

Figure 2. Counting plaques in titer plates. To more easily identify the plaques, plates are held next to a white light box and against a dark background with lids removed. Titer 20 plate **(Panel A)** and Titer 100 plate **(Panel B)**.

Figure 3. Mutant frequencies of the *cII* transgene in mouse embryonic fibroblasts treated with various chemicals and/or physical agents in comparison to controls. Data are from our published studies on acrylamide [12], glycidamide [14], aflatoxin B1 (AFB1) [22], tamoxifen [18], δ -aminolevulinic acid (δ-ALA) plus low dose ultraviolet light A (UVA: λ > 320−400 nm) [15], benzo(a)pyrene diol epoxide (B(a)PDE) [19], and equilethal doses of UVA, UVB (λ = 280−320 nm), and simulated sunlight UV (SSL) [21]. To efficiently metabolize tamoxifen in mouse embryonic fibroblast cells, we used the S9-activation system (S9 mix) consisted of Aroclor 1254-induced rat liver preparations and cofactor reagents [22]. All differences between treated- and control samples are statistically significant at $P \leq 0.05$.

Figure 4. Mutation spectra of the *cII* transgene in mouse embryonic fibroblasts irradiated with UVB relative to control. Data are from our published study [23]. The strand mirror counterparts of all transitions (*e.g.*, G→A and C→T) and transversions (*e.g.*, G→T and C→A or G→C and C→G) are combined. Ins = insertion; Del = deletion. The UVB-induced mutation spectrum is characterized by significant increases in relative frequency of single- or tandem C→T transitions at pyrimidine dinucleotides.

DISCUSSION:

The λ Select *cII* assay is used for detection of mutations in the *cII* transgene recovered from the genomic DNA of cells derived from organs/tissues of Big Blue rodents [3]. The genome of these transgenic animals contains multiple tandem copies of the chromosomally integrated λLIZ shuttle vector, which carries the *cII* (294 bp) and *lacI* (1,080 bp) transgenes, as the mutational reporter genes [1,2,25]. The λ Select *cII* assay is based on the retrieval of the λLIZ shuttle vectors from the genomic DNA of cells/tissues of the transgenic animals, followed by packaging of the rescued vectors into λ phage heads that can infect an appropriate host *E. coli*. Subsequently, the infected bacteria are grown under selective conditions to allow for scoring and analysis of mutations in the *cII* transgene (*see*, Fig. 1) [1,3]. The λ Select *cII* assay has been extensively used for mutagenicity testing of a wide range of chemicals and/or physical agents (reviewed in refs. [2,49]). The assay has been successfully applied to transgenic mouse/rat cell cultures treated *in vitro* with various chemicals and/or physical agents, and tissues/organs of the corresponding animals treated *in vivo* with different test chemicals/agents [4-23,24 ,34,58-75].

The λ Select *cII* assay in cultured cells of transgenic rodents treated with a test compound represents, in many ways, a viable alternative to *in vivo* mutagenicity experiments in the corresponding animals treated with the tested chemical/agent [3]. As a general rule, the *in vitro* models offer significant advantages over their counterpart *in vivo* animal models as they are much less labor intensive and costly, require far less time to be completed, and most importantly, do not involve direct use of the animals [2,50,52]. At the same time, the *in vitro* models may not fully recapitulate all aspects of mutagenesis due to differences in pharmacokinetic and pharmacodynamic properties of chemicals between the cultured cells *in vitro* and experimental animals *in vivo* [2,3]. For example, chemicals whose route of exposure is inhalation (*e.g.*, cigarette smoke or e-cig vapor) can only be made amenable to *in vitro* testing in cell cultures after they are converted from gaseous or vapor forms to liquid or condensate, which complicates their pharmacokinetics. Also, incomplete or absent metabolic capacity of cultured cells *in vitro* to convert certain chemicals into DNA-reactive species may not represent DNA-damage driven mutagenicity in animals exposed *in vivo* to genotoxic chemicals [2,3]. Though this drawback may be compensated, to varying extents, by the addition of an external metabolic activation system (*i.e.*, S9 mix) to the *in vitro* cell culture models [22].

Furthermore, replication of real life human exposure to genotoxic chemicals/agents is more limited in *in vitro* cell culture models than in experimental animals *in vivo* [3]. Generally, humans are exposed to chronic doses of genotoxic agents over a span of several years to a few decades [76-78]. The finite lifespan of cells in culture as compared to relatively longer lifetime of rodents (*i.e.*, days/weeks *versus* a few years) makes modeling of human exposure to genotoxins more challenging in the former models [2,3]. Nonetheless, mutagenicity analysis in *in vitro* cell culture models can provide an initial indication of the genotoxic potential of a given chemical/agent(s), and the results can be used as a guide to design 'refined' *in vivo* experiments with 'reduced' number of animals [2,3].

In conclusion, the λ Select *cII* assay in cultured cells of transgenic rodents treated with a test compound or the corresponding animals treated with the tested chemical/agent is a valuable approach for mutagenicity testing. The approach has been successfully used by our group and many other research groups throughout the world [4-23,24 ,34,58-75]. More recently, we have expanded the applications of this approach by developing a new technique in which a modification of the λ Select *cII* assay together with next-generation sequencing enables high throughput analysis of mutations in a time-, cost-, and labor effective manner [23].

ACKNOWLEDGMENTS

We would like to acknowledge the contributions of all colleagues and collaborators to our original studies whose results have been referred to in this manuscript (for illustration purposes). Work of the authors is supported by grants from the National Institute of Dental and Craniofacial Research of the National Institutes of Health (1R01DE026043) to AB and from the University of California Tobacco-Related Disease Research Program to AB (TRDRP-26IR-0015) and ST (TRDRP-25IP-0001). The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit for publication.

CONFLICT OF INTEREST STATEMENT

All the authors declare no conflict of interest.

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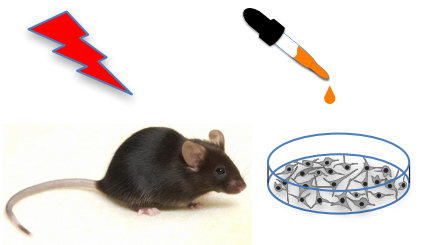
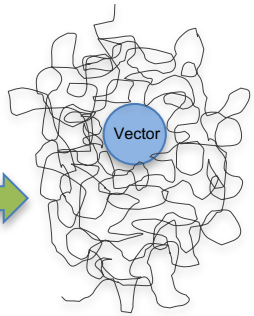
A

Treatment with test chemical / agent



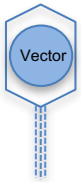
B

Isolation of genomic DNA



C

Recovery of λLIZ shuttle vector & packaging into bacteriophage



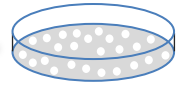
D

Infection of *E. coli* bacteria

E

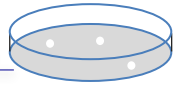
Incubation for phenotypic expression

Non-selective
37 °C ~ 24h



Titering

Selective
24 °C ~ 48h



Mutant selection

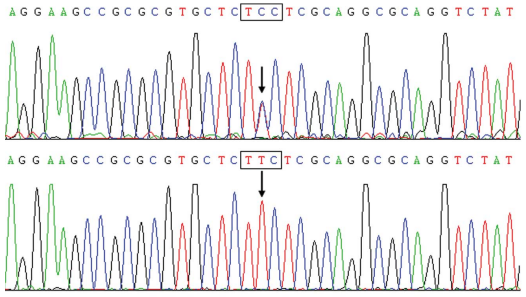
Mutant frequency = $\frac{\text{\# \textit{cII} mutant plaques}}{\text{Total plaques plated}}$

F

DNA sequencing

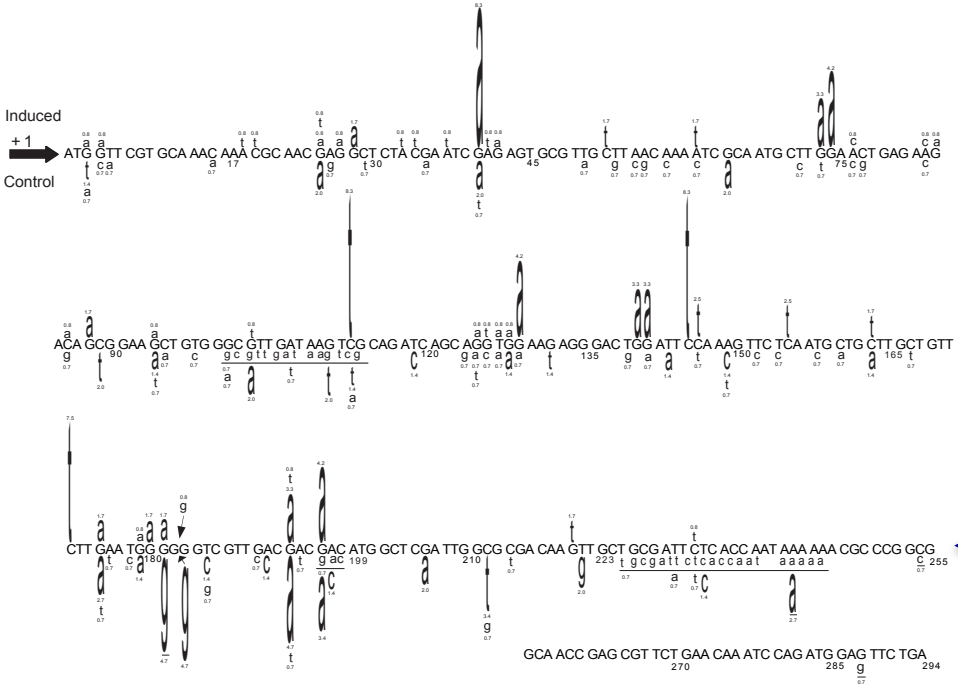
G

Sequence alignment

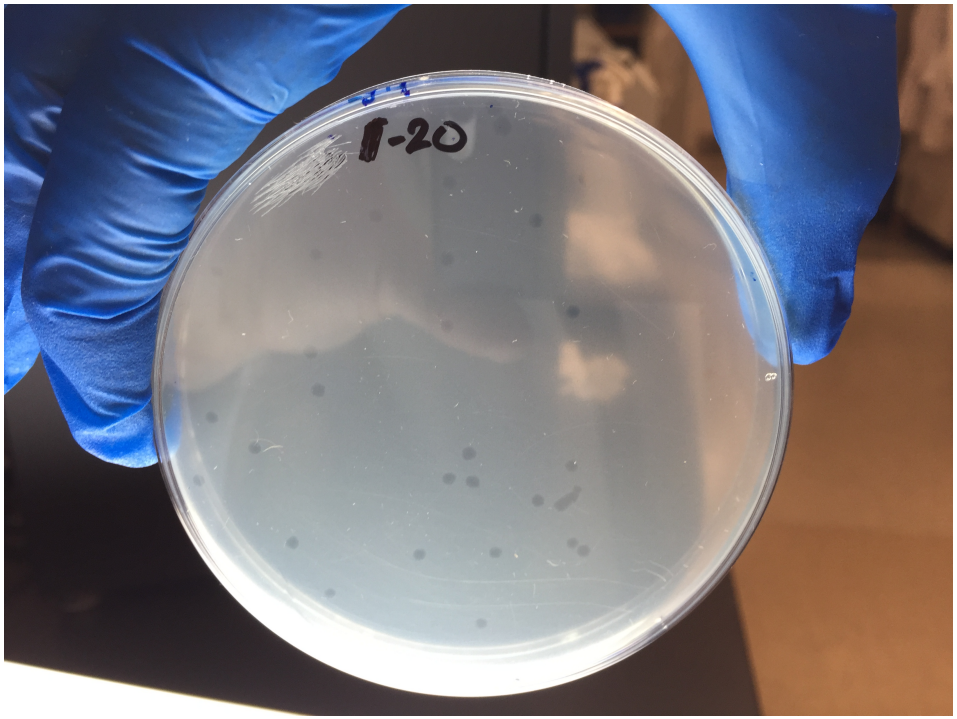


H

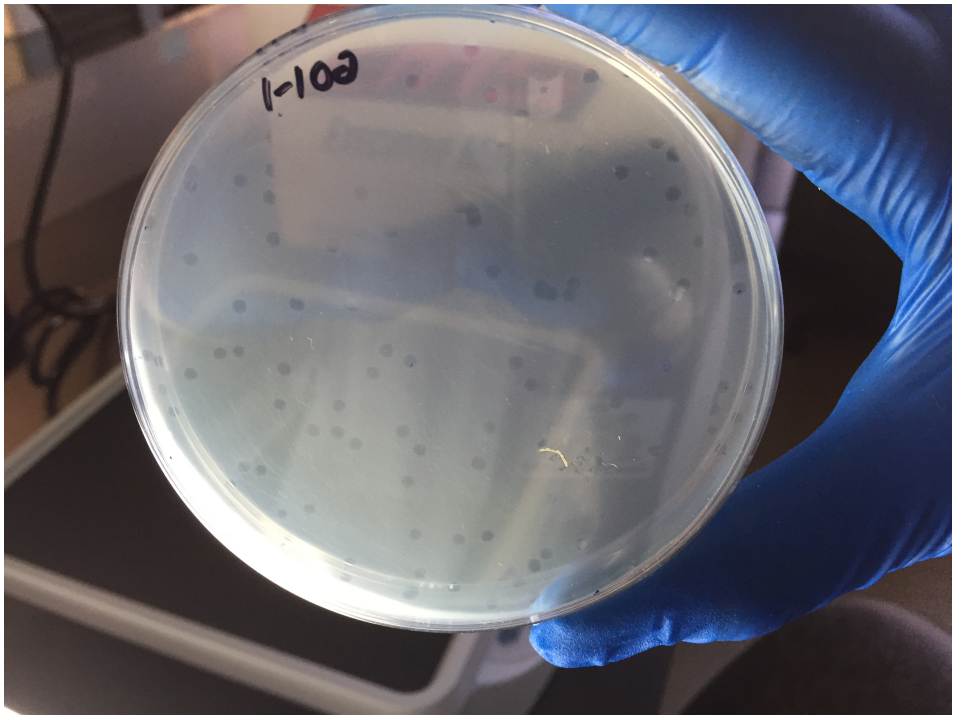
Mutation spectra

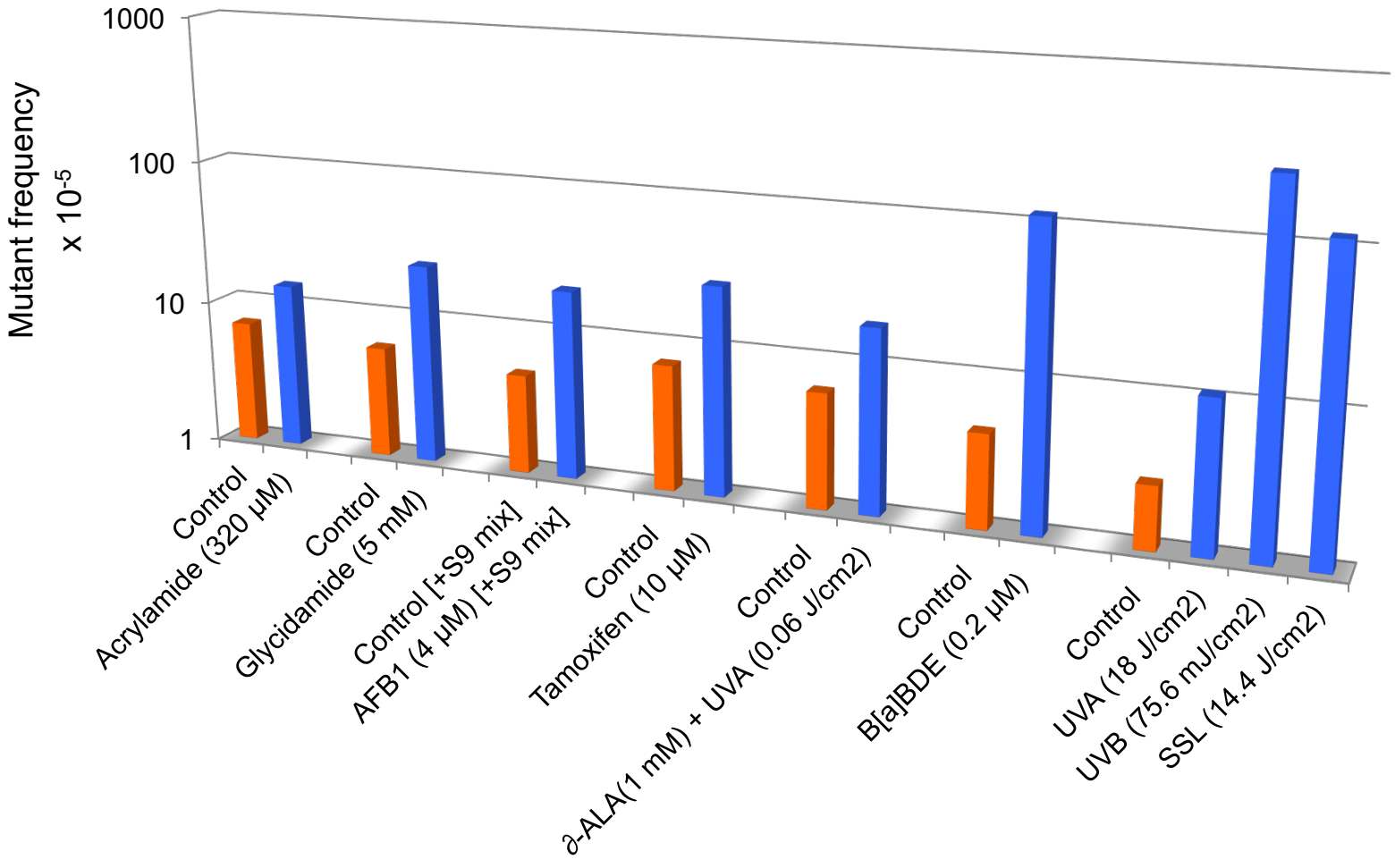


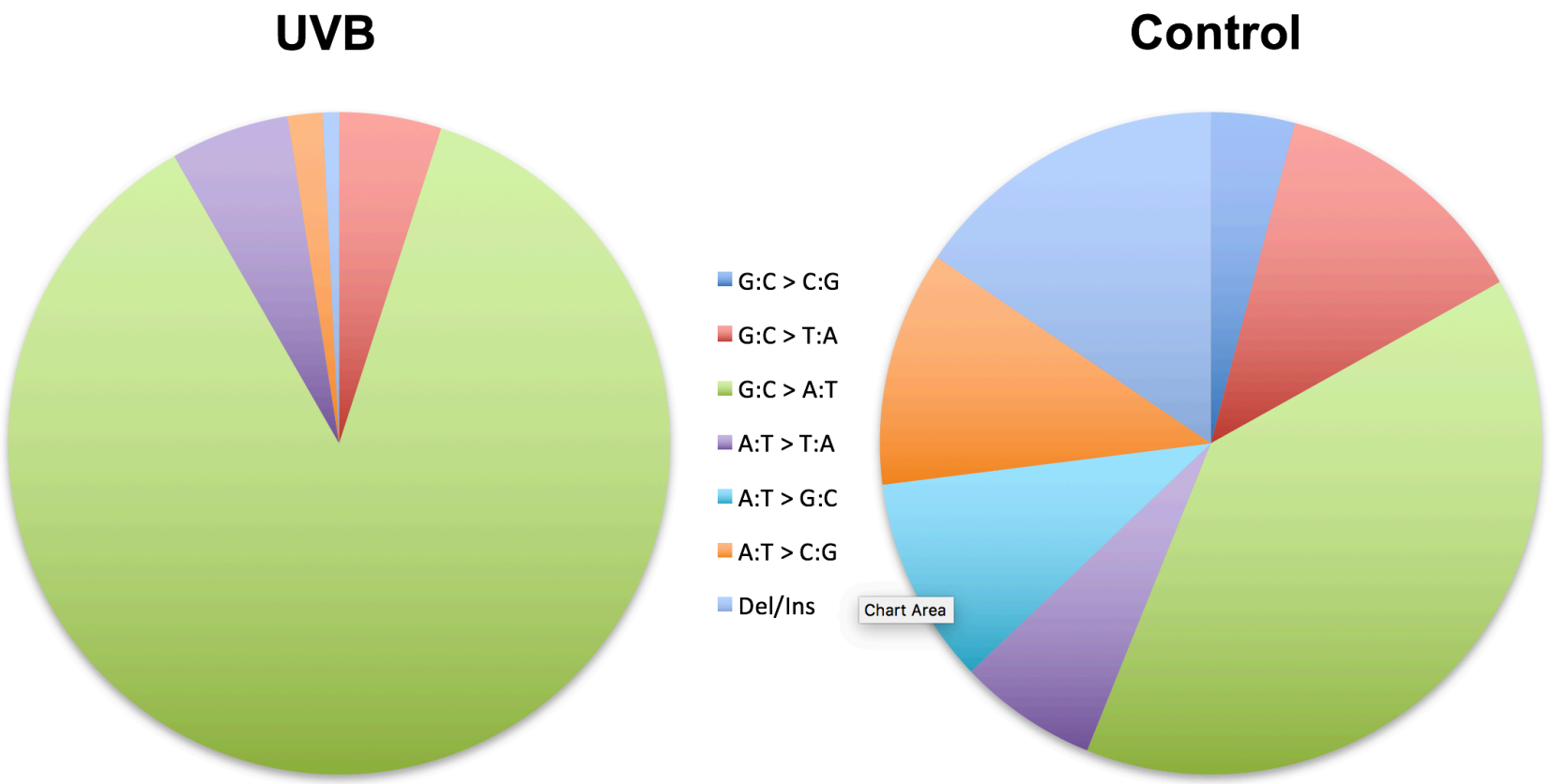
A



B




Mutant frequency
x 10-5
1000
100
10
1
Control
Acrylamide (320 µM)
Control
Glycidamide (5 mM)
Control [+S9 mix]
AFB1 (4 µM) [+S9 mix]
Control
Tamoxifen (10 µM)
Control
∂-ALA(1 mM) + UVA (0.06 J/cm2)
Control
B[a]BDE (0.2 µM)
Control
UVA (18 J/cm2)
UVB (75.6 mJ/cm2)
SSL (14.4 J/cm2)


UVB
Control
G:C > C:G
G:C > T:A
G:C > A:T
A:T > T:A
A:T > G:C
A:T > C:G
Del/Ins
Chart Area