INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 14.2)

Issued by: the Validation Management Team (VMT)

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A. PURPOSE OF THIS DOCUMENT

This document is provided to clarify the conduct of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet assay to identify genotoxic carcinogens, as a potential replacement for the *in vivo* rodent hepatocyte unscheduled DNA synthesis (UDS) assay. This document represents the final study protocol developed as a result of the collaboration efforts of the participating testing facilities and the VMT. Each testing facility will develop a study protocol based on the information provided in this document.

B. ASSURANCE OF DATA QUALITY

The study will be conducted in facilities that are Good Laboratory Practice compliant. Consistency between raw data and a final report is the responsibility of each testing facility. The VMT may review the data for accuracy, if deemed necessary.

C. ANIMAL WELFARE AND 3Rs

Appropriate national and/or international regulations on animal welfare should be followed. The 3Rs-principle for experimental animal use should be considered for determining the experimental design.

D. TESTING PROCEDURE

1. MATERIALS AND METHODS

1.1. Test substances and positive/negative controls

1.1.1. Test substance

With the exception of ethyl methanesulfonate (EMS), test substances will be supplied to each testing facility by the VMT. When coded substances are supplied, appropriate safety information will be provided in a sealed envelope to be opened only by an appropriate individual within the organization who is not involved in the study and/or in the case of

an emergency. If opened, appropriate documentation and justification will need to be provided to the VMT.

1.1.2. Test substance preparation

Each test substance will be dissolved or suspended with an appropriate solvent/vehicle just before administration (see section 1.1.4.).

1.1.3. Positive control

EMS (CAS No. 62-50-0); the source and lot number to be used will be provided by the VMT. EMS will be dissolved in physiological saline just before administration (within 2 hours).

1.1.4. Negative control (solvent/vehicle)

Solvents/vehicles for test substance preparation will be used as negative controls. An appropriate solvent/vehicle for a test substance may be indicated by the VMT. In the absence of instruction from the VMT, an appropriate solvent/vehicle will be chosen for each test substance by the testing facility in the following order: physiological saline, 0.5% w/v sodium carboxymethylcellulose aqua solution, corn oil.

1.2. Test animals

1.2.1. Species

Although either rats or mice can be used in this assay, the validation study will use rats. The rat is the species most commonly used in toxicological studies and is the preferred species in the *in vivo* rodent hepatocyte UDS assay.

1.2.2. Sex

In order to allow for a direct comparison with the rat hepatocyte UDS assay, males will be used.

1.2.3. Strain

Rat: Crl:CD (SD)

1.2.4. Source

Charles River Laboratories, Inc.

1.2.5. Age

At the time of purchase: 6-8 weeks of age (body weight 150 g - 320 g)

At the time of dosing: 7-9 weeks of age

1.2.6. Body weight

The weight variation of animals should be +/- 20% of the mean weight at the time of

dosing.

1.2.7. Number of animals in each dose group at each sampling time

Five males (see note 1).

1.2.8. Animal maintenance

Animals will be reared under appropriate housing and feeding conditions according to the standard operating procedures (SOP) in each testing facility, consistent with Section C "Animal Welfare".

1.2.8.1. Diet

Animals will be fed *ad libitum* with a commercially available pellet diet.

1.2.8.2. Water

Animals will be given free access to tap water *ad libitum* .

1.2.9. Animal quarantine and acclimation

Animals will be quarantined and acclimated for at least 5 days prior to the start of the study, according to SOPs in each testing facility. Only healthy animals approved by the Study Director and/or the Animal Facility Veterinarian will be used.

1.2.10. Animal identification and group assignment

Animals will be identified uniquely and assigned to groups by randomization on the basis of body weight according to the SOP in each testing facility.

1.3. Preparation of Comet assay solutions

The following solutions will be prepared, consistent with laboratory SOPs, unless otherwise specified (see note 2).

1.3.1. 1.0-1.5% (w/v) standard agarose gel for the bottom layer (if used)

Regular melting agarose will be dissolved at 1.0-1.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave.

1.3.2. 0.5 % (w/v) low-melting agarose (Lonza, NuSieve GTG Agarose) gel for the cell-containing layer and, if used, a top layer

Low-melting agarose will be dissolved at 0.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave. During the study this solution will be kept at 37-45°C and discarded afterward.

1.3.3. Lysing solution

The lysing solution will consist of 100 mM EDTA (disodium), 2.5 M sodium chloride, and 10 mM tris hydroxymethyl aminomethane in purified water, with the pH adjusted to

10.0 with 1 M sodium hydroxide and/or hydrochloric acid. This solution may be refrigerated at <10°C until use. On the same day of use, 1 % (v/v) of triton-X100 and 10 % (v/v) DMSO will be added to this solution and the complete lysing solution will be refrigerated at <10°C for at least 30 minutes prior to use.

1.3.4. Alkaline solution for unwinding and electrophoresis

The alkaline solution consists of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water, pH >13. This solution will be refrigerated at <10°C until use. The pH of the solution will be measured just prior to use.

1.3.5. Neutralization solution

The neutralization solution consists of 0.4 M tris hydroxymethyl aminomethane in purified water, pH 7.5. This solution will be either refrigerated at <10°C or stored consistent with manufacturer's specifications until use.

1.3.6. Mincing buffer

The mincing buffer consists of 20 mM EDTA (disodium) and 10% DMSO in Hank's Balanced Salt Solution (HBSS) (Ca^{++}, Mg^{++} free, and phenol red free if available), pH 7.5 (DMSO will be added immediately before use). This solution will be refrigerated at <10°C until use.

1.3.7. Staining solution

The fluorescent DNA stain is SYBR Gold (Invitrogen-Molecular Probes), prepared and used according to the manufacturer's specifications.

1.4. Comet assay procedure

1.4.1. Experimental design

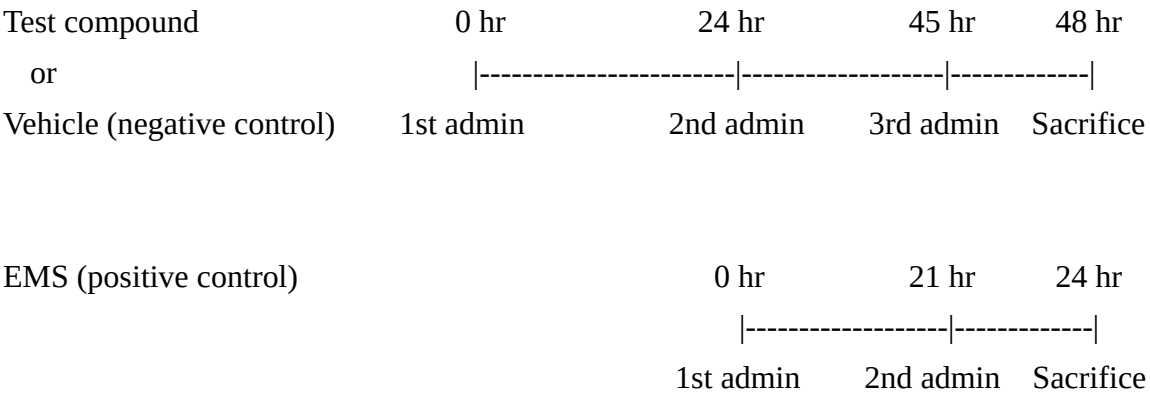
Compound	Dose (mg/kg/day)	Number of animals (see note 1)
Vehicle (negative control)	0	5
EMS (positive control)	200	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

*High dose selection (see note 3): in general, in the absence of VMT directions, the high dose level of a test compound will be selected as the dose producing signs of toxicity such that a higher dose level, based on the same dosing regimen, would be expected to

produce mortality, or an unacceptable level of animal distress. Selection of doses will be based on the toxicity of the test substance but will not exceed 2000 mg/kg/day.

1.4.2. Administration to animals

The test substance will be administered three times orally by gavage, 24 and 21 hours apart, i.e. the second administration is 24 hours after the first administration, and the third administration is 21 hours after the second administration (at 3 hours before animal sacrifice). EMS will be administered twice orally by gavage at 24 hours and 3 hours before animal sacrifice. The administration regimes are summarized in a figure below; this protocol enables us to integrate the comet and micronucleated erythrocyte assay into one assay (see note 4). The dosage volume will be 0.1 mL per 10 g body weight in rats on the basis of the animal weight just before administration.



1.4.3. Measurement of body weight and examination of animal conditions

Individual body weights will be measured in accordance with local SOPs and just prior to administration (the weight at this time will be used to determine the volume of each substance administered) and at the time of termination. The clinical signs of the animals will be observed from just after dosing to just before tissue removal with an appropriate interval according to the SOP in each testing facility.

1.4.4. Tissue sampling

Animals will be humanely killed at 3 hours after third administration of a test substance and at 3 hours after second treatment of EMS, consistent with Section C “Animal Welfare and 3Rs”. The stomach and the liver will be removed (see note 5). Tissues will be placed into ice-cold mincing buffer, rinsed sufficiently with the cold mincing buffer to remove residual blood (more rinses would likely be needed if exsanguination is not used), and stored on ice until processed. For histopathology, samples will be obtained from the same

liver lobe, and from a minimal possible area of stomach.

1.4.5. Preparation of single cells

Single cell preparation should be done within one hour after animal sacrifice (see note 6). The liver and the stomach will be processed as follows:

Liver: A portion of the left lateral lobe of the liver will be removed and washed in the cold mincing buffer until as much blood as possible has been removed (see note 7). The portion will be minced with a pair of fine scissors to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained through a Cell Strainer to remove lumps and the remaining suspension will be placed on ice), and the supernatant will be used to prepare comet slides.

Stomach: The stomach will be cut open and washed free from food using cold mincing buffer. The forestomach will be removed and discarded. The glandular stomach will be then placed into cold mincing buffer and incubated on ice for from 15 to 30 minutes. After incubation, the surface epithelia will be gently scraped two times using the a scalpel blade or a Teflon scrapper. This layer will be discarded and the gastric mucosa rinsed with the cold mincing buffer. The stomach epithelia will be carefully scraped 4-5 times (or more, if necessary) with a scalpel blade or Teflon scrapper to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained with a Cell Strainer to remove clumps and the remaining suspension will be placed on ice), and samples of the supernatant used to prepare comet slides.

1.4.6. Slide preparation

Slide preparation should be done within one hour after single cell preparation (see note 6). Comet slides will be prepared using laboratory specific procedures. The volume of the cell suspension added to 0.50% low melting agarose to make the slides will not decrease the percentage of low melting agarose by more than 10% (i.e., not below 0.45%) .

1.4.7. Lysis

Once prepared, the slides will be immersed in chilled lysing solution overnight in a refrigerator under a light proof condition (see note 6). After this incubation period, the slides will be rinsed in purified water or neutralization solution to remove residual detergent and salts prior to the alkali unwinding step.

1.4.8. Unwinding and electrophoresis

Slides will be randomly placed onto a platform of submarine-type electrophoresis unit

and the electrophoresis solution added. A balanced design will be used (see note 8). The electrophoresis solution will be poured until the surfaces of the slides are completely covered with the solution. The slides will be left to be unwind for 20 minutes. Next, the slides will be electrophoresed at 0.7 V/cm for at least 20 minutes, with a constant voltage at approximately 300 mA (see note 9). The current at the start and end of the electrophoresis period should be recorded. The temperature of the electrophoresis solution through unwinding and electrophoresis should be maintained at a constant temperature <10°C . The temperature of the electrophoresis solution at the start of unwinding, the start of electrophoresis, and the end of electrophoresis should be recorded.

1.4.9. Neutralization and dehydration of slides

After completion of electrophoresis, the slides will be immersed in the neutralization buffer for at least 5 minutes. All slides will be dehydrated by immersion into absolute ethanol (≥99.6%) for at least 5 minutes if slides will not be scored soon, allowed to air dry, and then stored until scored at room temperature, protected from humidity > 60 %. Once scored, slides should be retained and stored under low humidity conditions (e.g., in a desiccator) for potential rescoring.

1.4.10. DNA staining, comet visualization and analysis

Coded slides will be blind scored according to laboratory specific SOPs. The slides will be stained with SYBR Gold according to manufacturer's specifications. The comets will be measured via a digital (e.g. CCD) camera linked to an image analyzer system using a fluorescence microscope at magnification of 200X. For each sample (animal/tissue), fifty comets per slide will be analyzed, with 2 slides scored per sample (see note 10). Approximately 10 areas/slide should be observed at 5 cells or less/field (see note 11), taking care to avoid any selection bias, overlap counting of cells, and edge areas of slides. Heavily damaged cells exhibiting a microscopic image (commonly referred to as hedgehogs) consisting of small or non-existent head and large, diffuse tails will be excluded from data collection if the image analysis system can not properly score them (see note 12). However, the frequency of such comets should be determined per sample, based on the visual scoring of 100 cells per sample. The comet endpoints collected will be % tail DNA, tail length in microns measured from the estimated edge of the head region closest to the anode (see note 13), and, if possible for a particular image analysis system, Olive tail moment [= a measure of tail length (a distance between a center of head mass and a center of tail mass; microns) X a measure of DNA in tail (% tail DNA/100): Olive et al., 1990]. (see note 14)

1.4.11. Histopathology

When a positive Comet assay response is obtained for a tissue, a sample histopathological assessment will be conducted to evaluate for the presence of apoptotic and/or necrotic cells according to the SOP in each testing facility.

2. STATISTICS

Different approaches for data analysis have been proposed for comet data generated across a range of test substance dose levels (Lovell et al. 1999; Hartmann et al. 2003; Wiklund and Agurell 2003). The primary endpoint of interest for DNA migration is the % tail DNA. In addition, the distribution of migration patterns among cells within an animal will be considered. The percentage of "hedgehogs" will also be evaluated as a function of treatment. The unit of analysis for a specific tissue is the individual animal.

In data analysis process of this validation study, three conceptual key terms, i.e. "Endpoint", "Estimate", and "Effect" are defined and used. Briefly, "Endpoint" is defined as individual observed values for a parameter such as % DNA in tail. "Estimate" is defined as a mean calculated with values of a particular "Endpoint" in each animal. "Effect" is defined as difference of an average of "Estimate" between a negative control group and a treatment group (see note 15). Dunnett's test (two-sided, $P<0.05$) and linear Trend test (two-sided, $P<0.05$) will be applied to "Effect" to judge positive or negative as assay results. For the positive control group, Student's t-test (one-sided, $P<0.025$) will be applied to the "Effect".

3. DATA AND REPORTING

3.1.1. Treatment of results

Individual animal data and group summaries will be presented in a fixed tabular form that will be provided from the VMT.

3.1.2. Evaluation and interpretation of results

A positive response is defined as a statistically significant change in the % tail DNA in at least one dose group in comparison with the vehicle control value using Dunnett's test (two-sided, $P<0.05$) as well as a statistically significant linear Trend test (two-sided, $P<0.05$). A negative response is defined as the statistically nonsignificant change in both Dunnett's test and the linear Trend test, and an equivocal response is defined as the statistically significant change in either of Dunnett's test or the linear Trend test. The

positive control should produce a statistically significant increase in Student's t-test (one-sided, $P<0.025$), and if not, the study data will not be acceptable. Where a positive response is obtained in a test substance group, the investigator(s) will assess the possibility that a cytotoxic rather than a genotoxic effect is responsible based on the percentage of "hedgehogs" and histopathology (see note 16). Positive results indicate that the test substance induce DNA damage in the target tissue(s) investigated. Negative results indicate that, under the test conditions used, the test substance does not induce DNA damage *in vivo* in the tissue(s) evaluated.

3.1.3. Study report

The study report from each testing facility will at least include the following information:

3.1.3.1. Test substance and positive/negative controls

Identification; Chemical Abstracts Service Registry number (when available); supplier, lot number and purity (when available); physiochemical properties relevant to the conduct of the study, if known; justification for choice of vehicle; and solubility and stability of the substances in the solvent/vehicle, if known.

3.1.3.2. Test animals

Species/strain used; number, age and sex of animals; source, housing conditions, quarantine and acclimation procedure, and animal identification and group assignment procedure; individual weight of the animals on the day of receipt, at the end of the acclimation period, and before administration (at the time of grouping), including body weight range, mean and standard deviation for each group; and choice of tissue(s) and justification.

3.1.3.3. Reagents to prepare reagent solutions

Identification; supplier; lot number; and time limit for usage if known.

3.1.3.4. Test conditions

Data from range-finding study, if conducted; rationale for dose level selection; details of test substance preparation; details of the administration of the test substance; methods for verifying that the test substance reached the general circulation or target tissue, if applicable; details of food and water quality; detailed description of treatment and sampling schedules; method of measurement of toxicity, including histopathology; detailed methods of single cell preparation; method of slide preparation, including duration between tissue sampling and slide preparation, agarose concentration, lysis conditions (duration for lysis, etc.), alkali conditions and pH, alkali unwinding time and

temperature, electrophoresis conditions (pH, V/cm, mA, and temperature at the start of unwinding and the start and the end of electrophoresis) and staining procedure; criteria for scoring comets and number of comets analyzed per slide, per tissue and per animal; evaluation criteria; criteria for considering studies as positive, negative or equivocal.

3.1.3.5. Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual and mean values for DNA migration (and ranges) and % hedgehogs in individual tissue, animal, and group; concurrent positive and negative control data; and statistical evaluation.

3.1.3.6. Discussion of the results and/or conclusion, as appropriate.

4. ARCHIVES AND REVIEW

The study report and all raw data (including slide samples and image data) from this study will be retained according to the SOP in each testing facility. All raw data will be submitted to the management team for review if required.

5. NOTES

1) We evaluated the data of the 3rd phase validation studies as to whether or not fewer (two, three or four) animals were sufficient in the positive control group to show a statistically significant increase in the Effect (difference) with a one-tailed student's t-test ($P<0.025$). The analysis results were presented and discussed at the Florence meeting held on August 25-26, 2009, and the participants felt that the reduction of animal number would be possible but the slight decrease in the statistic power might require additional experiments and result in the increase in animal usage. Thus the VMT decided to continue using five animals as the positive control in this validation effort. We may need to further investigate the appropriate number of animals/group afterwards based upon power calculation.
2) We will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols.
3) The VMT extensively discussed at the Osaka meeting held on Feb. 4-6, 2009 how a preliminary dose-finding study should be done to choose an appropriate high dose level, because selection of a suitable high dose would be closely related to the sensitivity/specificity of genotoxicity assays in general. The VMT decided to request each facility to submit its own protocol for dose-selection, and the VMT will review them and then direct each facility to use its own protocol as it is or to follow a

dose-finding study protocol recommended by the VMT.

4) When following the regimen for EMS as a positive control, micronucleus (MN) induction will be detected in bone marrow but not in peripheral blood. To also detect MN induction in peripheral blood, it would be necessary to administer EMS as well as the other test chemicals three times. It was also pointed out at the Florence meeting (August, 2009) that four times administration of test chemicals excluding the positive control, EMS, would be needed if we expect to detect micronuclei in the peripheral blood.
5) In this validation study, Comet analysis for the liver and the stomach will be conducted. Comet analysis along with MN for the bone marrow and/or the peripheral blood are optional in this validation study.
6) At the Florence meeting, it was pointed out that the duration of tissue sampling should be kept to a set time (e.g. within 10 min) and the duration for lysis should be controlled, in order to obtain more stable negative control values. The VMT considers that such action would be preferred and recommended but not required of participant laboratories because the feasibility would depend on the performance of each laboratory. To further address this issue, the duration of tissue sampling and the duration for lysis should be recorded in the study report of each facility.
7) The size of the liver portions will be at the discretion of the laboratory, because there is no recommendation for standardizing this step.
8) In each electrophoresis run, there should be the same number of slides from each animal in the study; see Attachment 1, an example of how to keep track of each slide during each electrophoresis run. Each laboratory will need to provide its own electrophoresis box chart, as different boxes can accommodate different numbers of slides.
9) Under those electrophoresis conditions, it is expected that an average DNA migration obtained in the negative control group will be 1-8% tail DNA for the liver, and 1-20% tail DNA for the stomach. These ranges were set based on the analysis with negative control data from the 2nd and 3rd phase validation studies, i.e. the average +/- 3XS.D. values were as follows in the 2nd and 3rd phase validation studies, respectively: 3.8+/-4.8 (n=15 from 5 labs) and 3.1+/-3.9 (n=12 from 4 labs) in the liver, and 12.5+/-6.9 (n=12 from 4 labs) and 8.8+/-9 (n=10 from 4 labs) in the stomach. The reason why the lowest value is set at 1 is to be able to detect a significant decrease in % DNA in the tail. The decrease in DNA migration is expected for cross-linkers, and if such agents are intended to be detected using the Comet assay then a decrease

in migration would be easier to detect when the negative control value is at the higher end of the acceptable range. If the negative control average deviates from the range, the duration of electrophoresis will be adjusted to achieve this range.

10) An investigation was conducted to compare with two slides/animal and three slides/animal about some data of the 3rd phase validation study, and the result was presented and discussed at the Florence meeting. As there was no difference between them as far as the present analysis method was used, the VMT decided to use two slides/animal.
11) In order to obtain suitable areas for observation, dilution of cell suspension may be required during the single cell preparation process.
12) This instruction indicates that if a comet is analyzable by the software program then it should be analyzed. However the following cases will be excluded from the analysis: a) analyzable but the recognition by software is considered incorrect (e.g. the automatic recognition of nucleus center is shifted); and b) the staining of nucleus and/or migration is considered poor. At the Florence meeting, more detailed analysis methods were discussed and agreed to, i.e. cells should be classified into three categories, scorable, non-scorable and hedgehog, and also scorable cells with a 90% or more DNA in the tail should not be adopted as part of the data for analysis. The VMT will prepare a color atlas to instruct how to distinguish comet and hedgehog.
13) 'Tail length' is defined as 'Tail migration' in some image analyzers such as Comet IV.
14) At the Atagawa meeting held on March 13-14, 2008, there was discussion about the need to collect data on tail length and Olive tail moment in this validation study. Again, there was brief discussion about this point at the Osaka meeting. The consensus was that % DNA in tail seems to be a sufficient endpoint for validation and therefore these parameters would no longer be analyzed statistically. However, data on tail length and tail moment will continue to be collected in this validation study in case there is a reason to analyze these data in the future.
15) Effect (difference) seems to be more suitable for revealing variation between labs than Effect (ratio), which was pointed out at Osaka meeting in the discussion of the data of 3rd phase validation study.
16) At the present moment, there is no evident data on the consistency between the percentage of "hedgehogs" and histopathology. In this validation study, histopathology will be used as a primary endpoint to evaluate cytotoxicity, although both of the data will be collected for further analysis on the consistency between the percentage of "hedgehogs" and histopathology.

6. REFERENCES

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Collins AR, et al., Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis, 14, 1733-1735, 1993.

Hartmann A, et al., Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis, 18(1), 45-51, 2003.

Lovell DP, G Thomas G, R Dubow., Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. Teratog Carcinog Mutagen. 19(2), 109-119, 1999.

Olive PL, et al., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the “comet” assay. Radiat. Res., 122, 86-94, 1990.
Tice RR et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., 35, 206-221, 2000.

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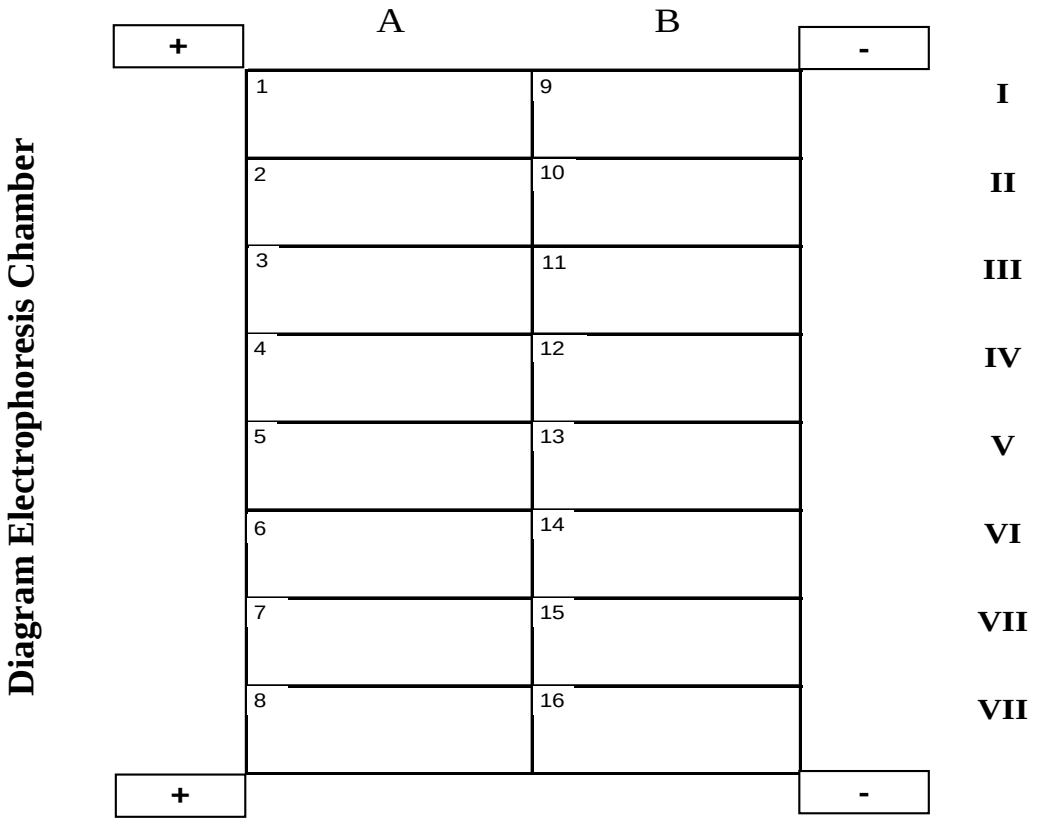
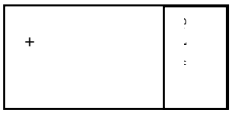
Attachment 1:

SLIDES UNWINDING & ELECTROPHORESIS RECORDING SHEET

Electrophoresis Run #				**Initials & Date**
Approximate alkaline electrophoresis buffer volume in chamber				
Unwinding				
Time	Total	Start	End	
Buffer Temperature				
Electrophoresis				
Running time	Total	Start	End	
Volts				
Milliamperes				
Buffer Temperature				
Thermometer No.				
Electrophoresis chamber No.				
Power supply No.				





Position of slide in