OCULAR IRRITECTION® ASSAY PROTOCOL

OECD TG 496: *In vitro Macromolecular Test Method for Identifying Chemicals Inducing Serious Eye Damage and Chemicals not Requiring Classification for Eye Irritation or Serious Eye Damage*

I. Introduction

The Ocular Irritection® assay system is a standardized and quantitative *in vitro* test that predicts the ocular irritation potential of surfactant and non-surfactant based chemical, petrochemical, consumer products and cosmetic samples. The assay is based on the knowledge that the ability of a chemical to irritate the cornea is related to its propensity to promote denaturation and disruption of corneal proteins. Consequently, the assay has been developed as *in vitro* tests that mimic these biochemical phenomena making use of a protein reagent. When the test is performed, the changes in protein structure that are induced by the test material are then quantified by measuring the resulting changes in the turbidity (optical density) of the reagent solution at a wavelength of 405 nm using a spectrometer. The results of past comparative studies have been utilized to develop what is termed an "Irritection Draize Equivalent" (IDE) score which is a value derived from the *in vitro* studies that is equivalent to the predicted *in vivo* irritancy potential (Draize rabbit eye test).

This assay protocol provides a detailed description of the experimental procedures that are required to perform the Ocular Irritection® Assay. The protocol consists of five steps:

1. Preparation of the samples
2. Preparation of the protein reagent solution
3. Set-up of the assay
4. Incubation for 24 hours to permit reaction of the samples and protein reagent.
5. Determination and interpretation of the assay results.

The first three steps may be completed in approximately two hours. The final step, to be performed on the following day, may be completed in approximately one hour.

Prior to beginning the assay, place the Hydrating Solution in a 25˚C incubator for 1 to 2 hours to ensure that it is approximately 25˚C. Remove the 24-well assay plates filled with membrane discs from the refrigerator. Allow the membrane discs to equilibrate to room temperature (approximately 30-45 minutes) before being used in Step IV.B.2.

II. Instrumentation, Reagents and Materials

A. Kit Contains:

- Store at room temperature

1. Ocular Reagent Powder (1 Bottle)
2. Ocular Hydrating solution (1 Bottle)
3. Ocular Blanking Buffer (1 Bottle)
4. Ocular Inhibition Check (1 Vial)
5. Ocular Activator (1 Vial)
6. Four Ocular Calibrator Solutions: Cal 0, Cal 1, Cal 2, Cal 3
7. Two Ocular Quality Control Solutions: QC 1, QC 2
8. 96-well Reading Plate (1)
9. Wooden Stirring Sticks (12)
10. Whatman #1 Filter Paper, 12.5 cm diameter (1)
11. Range Specification Data Sheet

- **Store at 2-8˚C**

12. 24-well Assay Plate(s) Filled with Membrane Discs
    For 4-sample kit – 48 clear discs
    For 3-sample kit – 38 clear discs
    For 2-Sample kit – 28 clear discs
    For 1-Sample kit – 18 clear discs

B. Additional Materials Required:

1. Plate Reader (Instruments such as Modified Cambridge 7520 Microplate reader, Dynex MRX, Molecular Devices and Tecan Sunrise can be integrated with the Irritection® Assay System software. Prospective users are recommended to try the software with their reader before planning any experiments)
2. Windows PC Compatible with Irritection® Assay System Software
3. Incubator Maintained at 25˚C ± 1˚C
4. Balance (110 g Capacity)
5. pH Meter
6. Vortex Mixer
7. Positive Displacement Micro Pipettor (5 – 200 µl capacity) and compatible pipette tips

8. Multichannel Pipettor (such as Matrix 6 Channel EXP Impact Pipettor 15-1250 µl) and compatible Pipet tips
9. 100 ml Graduated Cylinder
10. 100 ml Beaker
11. Funnel (such as Oxford Vented Disposable Polystyrene Funnel, 12.5 cm diameter)
12. Plastic Forceps
13. Plastic Wrap
14. Repeating pipettor (250 – 1250 µl capacity) and 12.5 ml Combi-Syringes tips

III. Preparation - before testing

A. Test Compounds

It is important to characterize a test sample before Irritection® testing is started. First, it is necessary to determine that the pH of the sample is within the applicability domain range of ≥ 4 and ≤ 9. Second, in order to achieve optimal results, it is important to test surfactants with the proper procedure. Therefore, sample providers should clearly identify known surfactants to insure that they are tested using the proper application procedure. For unknown or blend samples, foam testing is performed in an attempt to determine whether the compound should be tested utilizing the surfactant or non-surfactant application procedure. However, since some surfactants may not be successfully identified with this test, sample providers should attempt to define the chemical nature of the submitted material to aid in sample characterization and selection of the appropriate assay procedure.

B. Determination of Sample pH:

Employ the following method to determine the pH range of the test sample:

1. Record the pH of distilled water that was used to dissolve the samples. The pH of distilled water should be in the approximate range of 5.5 to 7.0.
2. Add 2 ml of distilled water into a 7 ml tube.
3. If the sample is a liquid, add a 200 µl aliquot of the sample to be tested to the 2 ml of distilled water.
4. If the sample is a solid, add 200 mg of the sample to be tested to the 2 ml of distilled water.
5. Cap tube, invert four times, and vortex mix for 5 seconds.

6. Place the tube in a test tube rack on bench top and place the pH probe into the liquid. Gently swirl the probe in the liquid to insure that is surrounded by the sample solution and there are no bubbles adhering to the probe surface. Hold the probe still when the reading of the pH meter begins to slow down.
7. Measure the pH:
   a. For samples that have completely dissolved in water, record the pH after a stable reading is maintained for a minimum 10 seconds.
   b. For samples that do not appear to completely dissolve in water, but show a stable reading within the first 5 minutes, record the pH after the meter maintains a stable reading for a minimum 10 seconds.
   c. For the samples that do not completely dissolve in water and do not display a stable pH reading within the first 5 minutes, allow them to stand for 5 more minutes before recording the final pH reading. For these types of samples, put a notation in the MDS to indicate that the final pH reading was not stable.
8. Record the pH result.
   If the pH is ≥4 or ≤9, the sample is suitable for Irritection testing.
   If the pH is less than 4 or greater than 9, it is not suitable for Irritection testing.

C. Determination of Sample Property - Surfactant or Non-Surfactant

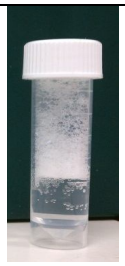
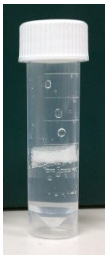
The Irritection test method application procedures for surfactants differ from those that are used for non-surfactants. In order to achieve optimal results, it is important to test higher concentration surfactants with the proper procedure. Therefore, sample providers should clearly identify known surfactants to insure that they are tested using the surfactant application procedure. For unknown and blend samples, foam testing is suggested to be performed in an attempt to determine whether the compound should be tested utilizing the surfactant or non-surfactant application procedure. For this purpose, the following method is suggested to characterize the surfactant-like properties of a test sample:

1. Add 2 ml of distilled water into a 7 ml tube.
2. If the sample is a liquid, add a 200 µl aliquot of the sample to be tested to the 2 ml of distilled water.
3. If the sample is a solid, add 200 mg of the sample to be tested to the 2 ml of distilled water.

4. Cap the tube and place it horizontally on the top of Vortex mixer to maximum the surface area of the liquid. Vortex mixes the sample for 10 seconds.
5. Place the tube in a test tube rack on the bench top and allow it to stand for 5 minutes.
6. Examine the sample to see if there is a persistent layer of bubbles (see below). Record the height of the solution phase and the bubble layer.

The following criteria are suggested to characterize the surfactant-like properties of a test sample:

1. If there is a persistent layer of bubbles with a column height at least equal to the height of the solution phase, the sample should be handled as a surfactant. See examples 1 and 2 in the photographs (see below).
2. If there is not a persistent layer of bubbles with a column height at least equal to the height of the solution phase, the sample should be handled as a non-surfactant. See examples 4 and 5 in the photographs (see below).
3. For a sample which has a layer of bubbles slightly less than the height of solution, the sample should be handled as a surfactant if the height of the bubble layer was greater than the height of the solution phase immediately after performing the vortex mixing (see below).

	Example 1	Example 2	Example 3	Example 4	Example 5
After 10 second vortex					
After 5 minutes Stand					
	Surfactant	Surfactant	Surfactant	Non-Surfactant	Non-Surfactant

IV. Method

A. Preparation of Test Substances

Different types of chemicals require different types of handling procedures in order to perform the Irritection assay properly. **Sample preparation should be completed prior to proceeding to Step IV.B.**

1. Handling of Non-Surfactant Chemicals - No further sample preparation

Chemical sample may be tested without further dilution.

2. Handling of Solid Surfactant Chemicals - Additional dilutions are required

Additional dilutions are required before the Irritection assay can be performed. Obtain fine shavings from different locations on a solid material, e.g., a bar of soap.

2.1. Add 0.5 g of test substance to 10 ml distilled water to create a "5.0% working solution".

2.2. Mix on a magnetic stir plate or vortex mixer to achieve dissolution of the solid.

2.3. Perform a two-fold serial dilution of the "5.0% working solution" in the following manner:

Test Sample Number	Concentration of Surfactant (%)	Volume of Sample solution Required (ml)	Volume of Distilled Water Required (ml)
1	5%	2.5 ml sample 1	0.0
2	2.5%	2.5 ml sample 1	2.5
3	1.25%	2.5 ml sample 2	2.5
4	0.625%	2.5 ml sample 3	2.5
5	0.3125%	2.5 ml sample 4	2.5

3. Handling of Liquid Surfactant Chemicals - Additional dilutions are required

Additional dilutions are required before the Irritection assay can be performed.

3.1. Add 500 µl of test substance to 9.5 ml distilled water to create a "5.0% working solution".

3.2. Use a vortex mixer for 10 seconds to achieve dissolution of the sample.

3.3. Perform a two-fold serial dilution of the "5.0% working solution" in the following manner:

Test Sample Number	Concentration of Surfactant (%)	Volume of Sample solution Required (ml)	Volume of Distilled Water Required (ml)
1	5%	2.5 ml sample 1	0.0
2	2.5%	2.5 ml sample 1	2.5
3	1.25%	2.5 ml sample 2	2.5
4	0.625%	2.5 ml sample 3	2.5
5	0.3125%	2.5 ml sample 4	2.5

B. ROUTINE PROCEDURES

1. Reagent Preparation:

   Place the Hydrating Solution in a 25˚C incubator and remove the 24-well assay plates filled with membrane discs from the refrigerator. Allow the membrane discs to equilibrate to room temperature (approximately 30 – 45 minutes) before being used in the test.

2. Rehydration:
   - 2.1. Pour all of the Hydrating Solution into the Reagent Powder and gently swirl.
   - 2.2. Let the dissolved Reagent stand at room temperature for approximately 10 minutes before filtering into a graduated cylinder.

   Note: After rehydration, the Reagent must be used within 50 minutes.

3. Filtration:
   - 3.1. Fold the filter paper and place in a funnel.
   - 3.2. Pour the entire dissolved Reagent into the funnel and collect the filtrate in a graduated cylinder at atmospheric pressure (Do not use a vacuum pump). This will take approximately 15 to 20 minutes.
   - 3.3. Collect 40 ml filtered Reagent and pour into a 100-ml beaker.
   - 3.4. Record the initial pH and temperature verifying that it falls within the following specified ranges: pH: 7.91-8.19 and T: 23 – 25°C.

4. Labelling the 24-Well Assay Plates:
   - 4.1. Align the assay plate(s) with the square corners toward the left and label their lid(s).

     For 4-sample kit, a typical plate configuration is shown in Figure 1 on page 11
     For 3-sample kit, a typical plate configuration is shown in Figure 2 on page 12
     For 2-sample kit, a typical plate configuration is shown in Figure 3 on page 13
     For 1-sample kit, a typical plate configuration is shown in Figure 4 on page 14
   - 4.2. Remove the discs from the 24-well plate(s) and place them on the lid(s)

5. Activation:
   - 5.1. Slowly add **800 µl** of the Activator to the filtered Reagent solution and add **600 µl** of the Activator to the Blanking Buffer. Gently swirl to mix.
   - 5.2. Record the final pH of the Activated Reagent solution, verifying that it falls within the following specified range: pH: **6.42-6.74** and T: 23 – 25°C.
   - 5.3. It is not necessary to measure the pH or temperature of the Activated Blanking Buffer.

C. TEST MATERIAL EXPOSURE PROCEDURES

1. Fill Wells of 24-Well Plate with Activated Reagent solution and Blanking Buffer:
   1.1. Set the repeating pipettor (Such as Eppendorf 4780 Repeater Pipette Dispenser) at 1250 µl.
   1.2. Use the lid or plate configuration as a reference when filling the wells. Add 1250 µl Activated Reagent solution to the appropriate wells  . Discard the unused portion.
   1.3. Add 1250 µl Activated Blanking Buffer to the appropriate wells  . Discard the unused portion.
2. Add Calibrators and Quality Control Samples:
   Kit includes four Calibrators (Cal 0, Cal 1, Cal 2 and Cal 3) which are analyzed in each assay to ensure standardization. Two Quality Controls (QC 1 and QC 2) are also included to verify the performance of Ocular Irritection® assay. All Calibrators and Quality Controls have established ranges for optical density to ensure assay performance.

   Note: Avoid cross contamination among the separate Calibrator, Quality Control and sample wells by utilizing a clean pipette tip for each.
   2.1. Insert the membrane discs into the wells (B 0) which contain activated Blanking buffer for the Cal 0.
   2.2. Insert the membrane discs into the wells (Cal 0, Cal 1, Cal 2, Cal 3, QC 1 and QC 2) which contain activated Reagent solution for the Calibrators and Quality Control Samples.
   2.3. Use the single channel pipettor and apply 125 µl of Cal 0 onto the membrane discs which you placed into B 0 wells.
   2.4. Use the single channel pipettor and apply 125 µl of Cal 0, Cal 1, Cal 2, Cal 3, QC 1 and QC 2 onto the membrane discs which you placed into the corresponding wells of the assay plate(s).
3. Add Test Samples:
   3.1. **Handling of Non-Surfactant Liquid Chemicals**:
      3.1.1. Insert the membrane discs into the wells which contain activated Blanking buffer and Reagent solution.
      3.1.2. Use the single channel pipettor and apply 25, 50, 75, 100, and 125 µl of each test sample onto the membrane discs which you placed in the corresponding wells of assay plates.

3.1.3. For viscous or sticky chemicals, use a positive displacement pipette (such as Lab Industries Pipette™ Micropipettor Model P-250, 5 – 250 µl) to apply 25, 50, 75, 100, and 125 µl of each test sample onto the membrane discs which you placed in the corresponding wells of assay plates.

3.2. **Handling of Non-Surfactant Solid Chemicals**:

3.2.1. Weigh 25, 50, 75, 100, and 125 mg +/- 1 mg of each test sample onto the membrane discs and insert them into the corresponding wells which contain activated Blanking buffer.

3.2.2. Weigh 25, 50, 75, 100, and 125 mg +/- 1 mg of each test sample onto the membrane discs and insert them into the corresponding wells which contain activated Reagent solution.

3.3. **Handling of Non-Surfactant Waxy Solid (Pieces) Chemicals**:

3.3.1. Weigh 25, 50, 75, 100, and 125 mg +/- 1 mg of each test sample directly into the corresponding wells (without membrane discs) which contain activated Reagent solution and Blanking buffer.

3.3.2. Insert the membrane discs into the wells which contain activated Reagent solution and Blanking buffer after the test samples are applied.

3.4. **Handling of Surfactant Chemicals**:

3.4.1. Vortex mixes each dilute of the test substance before being used.

3.4.2. Pipette 125 µl of each sample dilution (from low to high concentration) directly into the corresponding wells (without membrane discs) which contain activated Reagent solution and Blanking buffer.

3.4.3. Insert the membrane discs into the wells which contain activated Reagent solution and Blanking buffer after the test samples are applied.

4. Incubation:

4.1. To prevent evaporation of the samples from the membrane discs, tightly wrap the assay plate with plastic wrap. Place the lid on the top of the wrapped plate.

4.2. Record the technician's name, date, and time on the lid of the assay plate.

4.3. Place the 24-Well assay plate in an incubator maintained at 25°C±1°C for 24 hours (± 30 minutes).

5. Removing Membrane Discs:

5.1. Remove the assay plates from the incubator.

5.2. Remove the lids from the assay plates and save them. They will be used as a reference when transferring from 24-well plate to the 96-well reading plate.

5.3. Carefully remove the plastic wrap from each assay plate.

5.4. Carefully remove each membrane disc (no dripping) individually with plastic forceps.

5.5. Check the membranes for damage. Verify that all membrane discs are intact. If membrane damage has occurred, it should be recorded.

5.6. Use the wooden stirring sticks to scrape Reagent solution and Blanking buffer wells to ensure that all of the precipitate is removed from the bottom of each well.

5.6.1. Use one stirring stick for B 0 wells.

5.6.2. Use one stirring stick for Calibrators, starting with Cal 0 first and then Cal 1, Cal 2 and Cal 3, respectively.

5.6.3. Use one stirring stick for QCs, starting with QC 1 and then QC 2.

5.6.4. Use two stirring sticks for each sample, one for Blanking buffer and one for Reagent solution. Start with the lowest dose/concentration wells first, progressing to wells of higher dose/concentration.

5.7. Note any wells with reduced volume. Reduced volumes may be indicative of hygroscopic effects or technical problems and should be recorded.

6. Transferring from 24-well Assay Plates to a 96-Well Reading Plate:

6.1. Set the multichannel pipettor to 260 µl for filling and 250 µl for dispensing.

6.2. Transfer 250 µl from each well of the 24-well assay plate to every other well of the 96-well reading plate by columns.

For 4-sample kit, a typical transferring layout is shown in Figure 5 on page 15
For 3-sample kit, a typical transferring layout is shown in Figure 6 on page 16
For 2-sample kit, a typical transferring layout is shown in Figure 7 on page 17
For 1-sample kit, a typical transferring layout is shown in Figure 8 on page 18

NOTE: When using multichannel pipettor, 260 µl is used for filling to ensure that no air bubbles are dispensed. The remaining 10 µl must be purged prior to refilling the multichannel pipettor.

6.3. Change pipette tips and repeat this process for each column.

6.4. To eliminate inaccurate readings produced by settling of the precipitated solution, the assay must be read immediately after transferring the solution to the reading plate.

Figure1. Suggested Place Configuration: Analysis of Four Samples at Five Doses/Concentrations

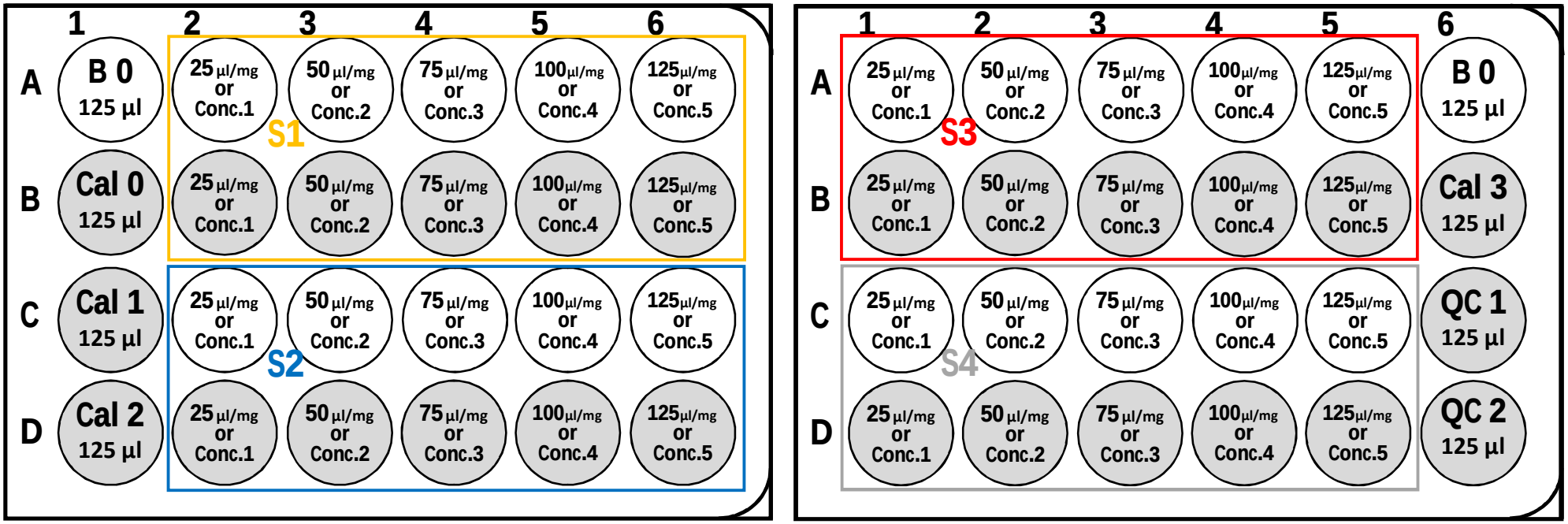


Figure Legend:

= well contains 1250 µl Activated Blanking Buffer

= well contains 1250 µl Activated Protein Reagent

S1 = Sample 1 S2 = Sample 2 S3 = Sample 3 S4 = Sample 4

Figure 2. Suggested Place Configuration: Analysis of Three Samples at Five Doses/Concentrations

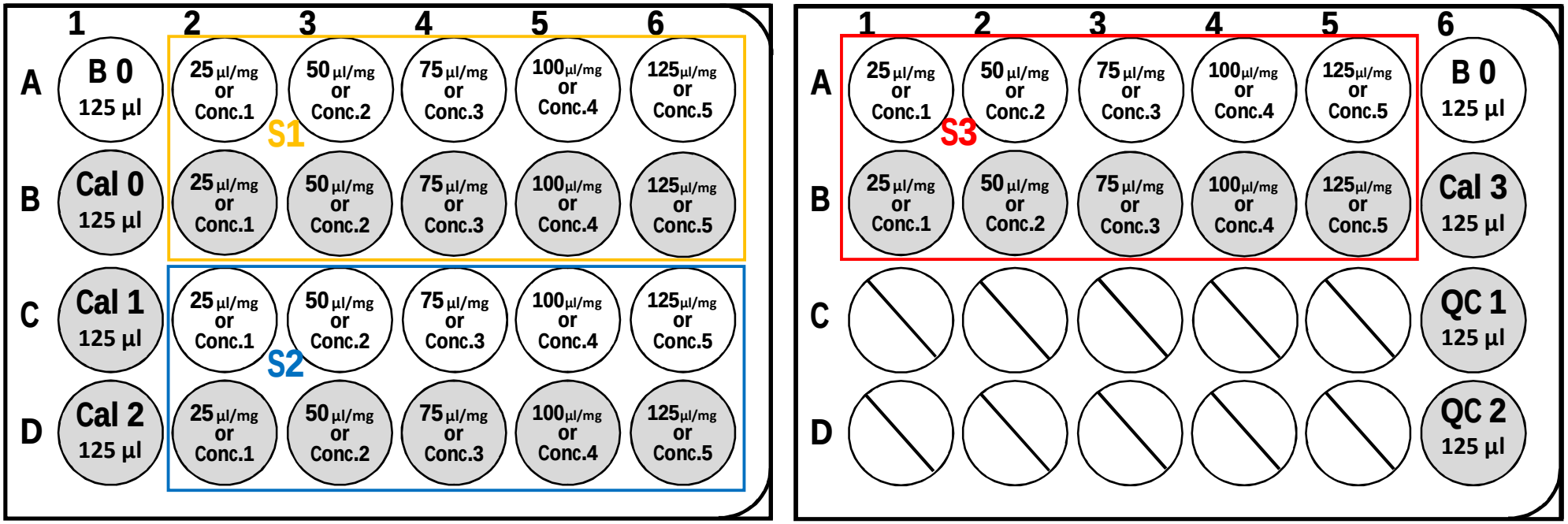


Figure Legend:

= well contains 1250 µl Activated Blanking Buffer

= well contains 1250 µl Activated Protein Reagent

= well is intended to be blank/empty

S1 = Sample 1 S2 = Sample 2 S3 = Sample 3

Figure 3. Suggested Place Configuration: Analysis of Two Samples at Five Doses/Concentrations

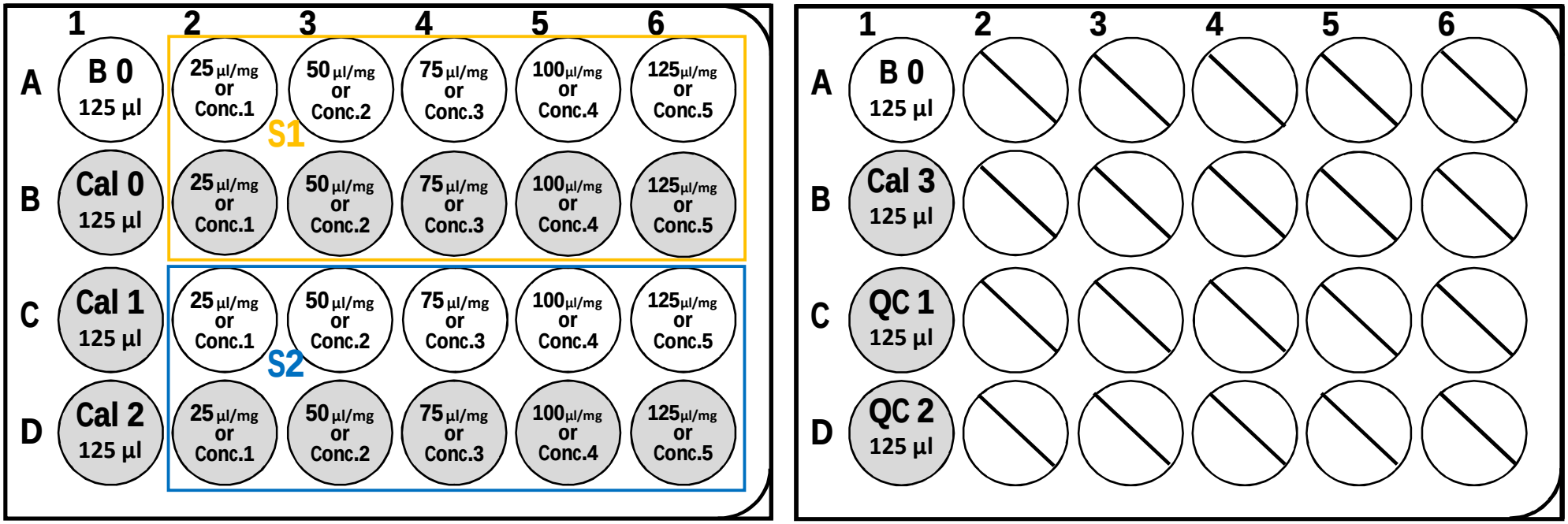


Figure Legend:

= well contains 1250 µl Activated Blanking Buffer

= well contains 1250 µl Activated Protein Reagent

= well is intended to be blank/empty

S1 = Sample 1

S2 = Sample 2

Figure 4. Suggested Place Configuration: Analysis of One Sample at Five Doses/Concentrations

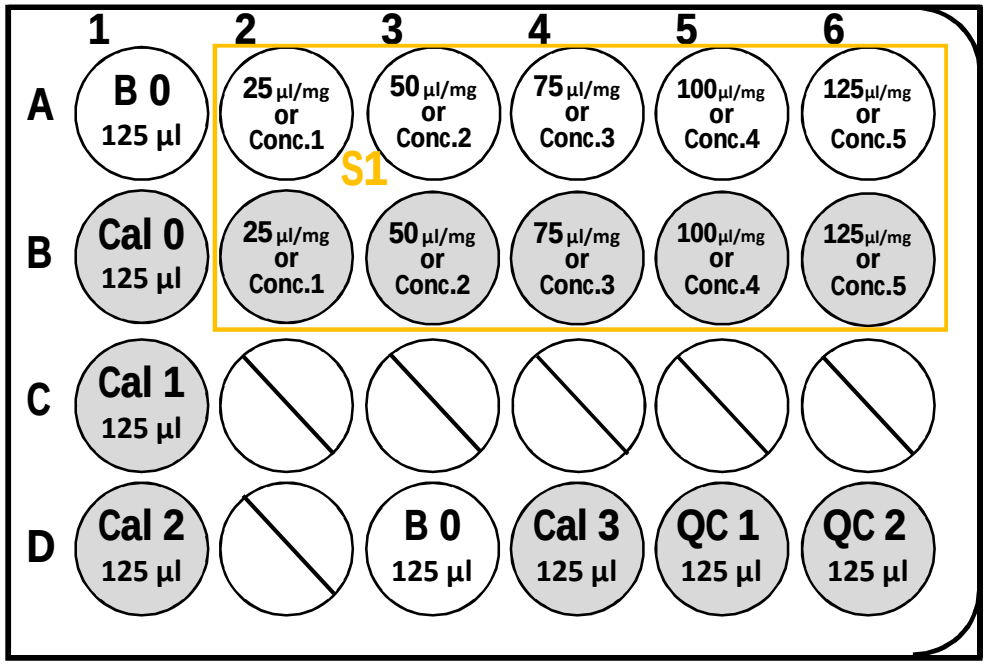


Figure Legend:

= well contains 1250 µl Activated Blanking Buffer

= well contains 1250 µl Activated Protein Reagent

= well is intended to be blank/empty

S1 = Sample 1

Figure 5. Suggested Transferring Layout for Four Samples at Five Doses/Concentrations

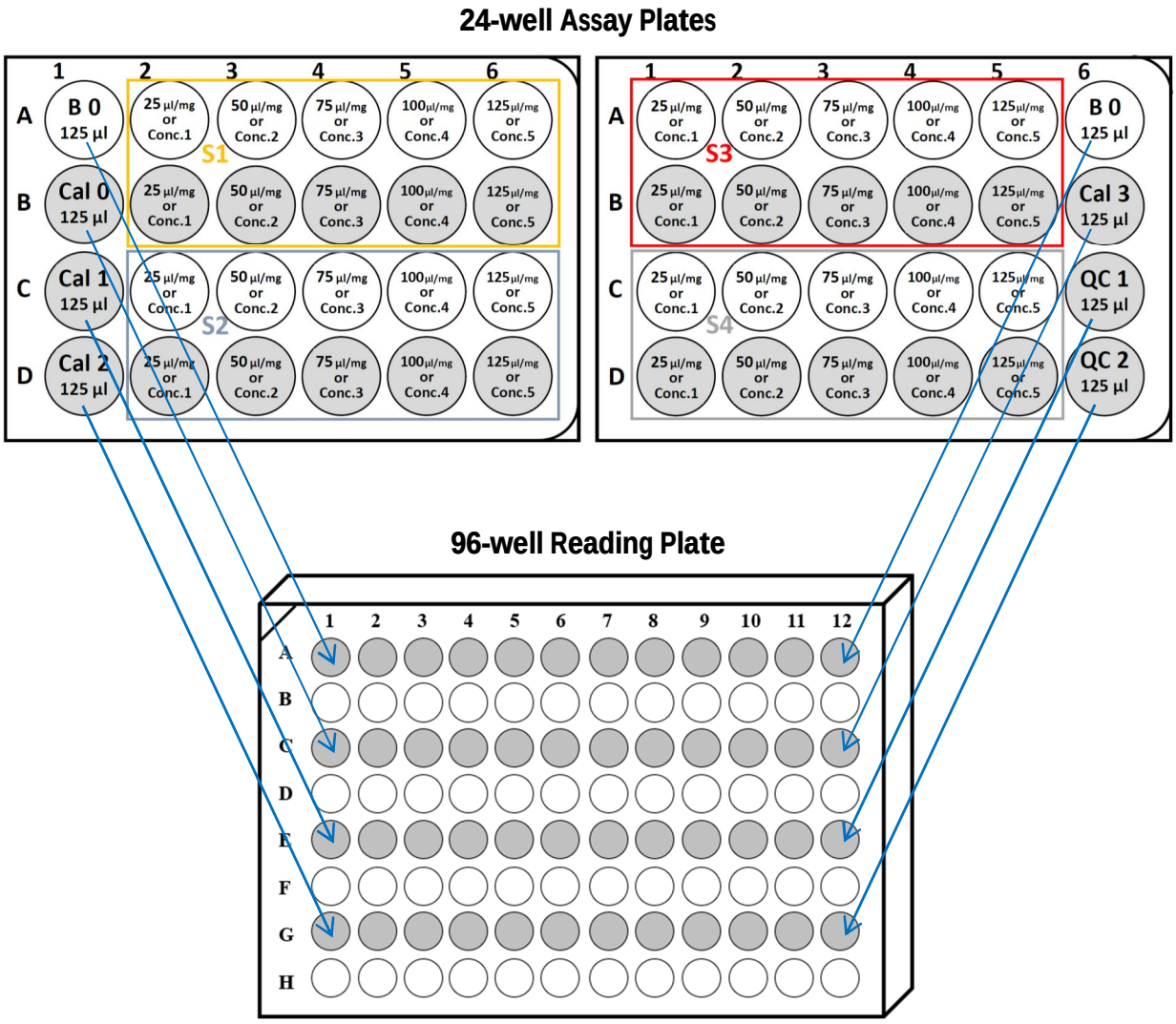


Figure Legend for 96-well Reading Plate:

= well with 250 µl Reagent solution or Blanking buffer from the 24-well assay plates

= empty well

Figure 6. Suggested Transferring Layout for Three Samples at Five Doses/Concentrations

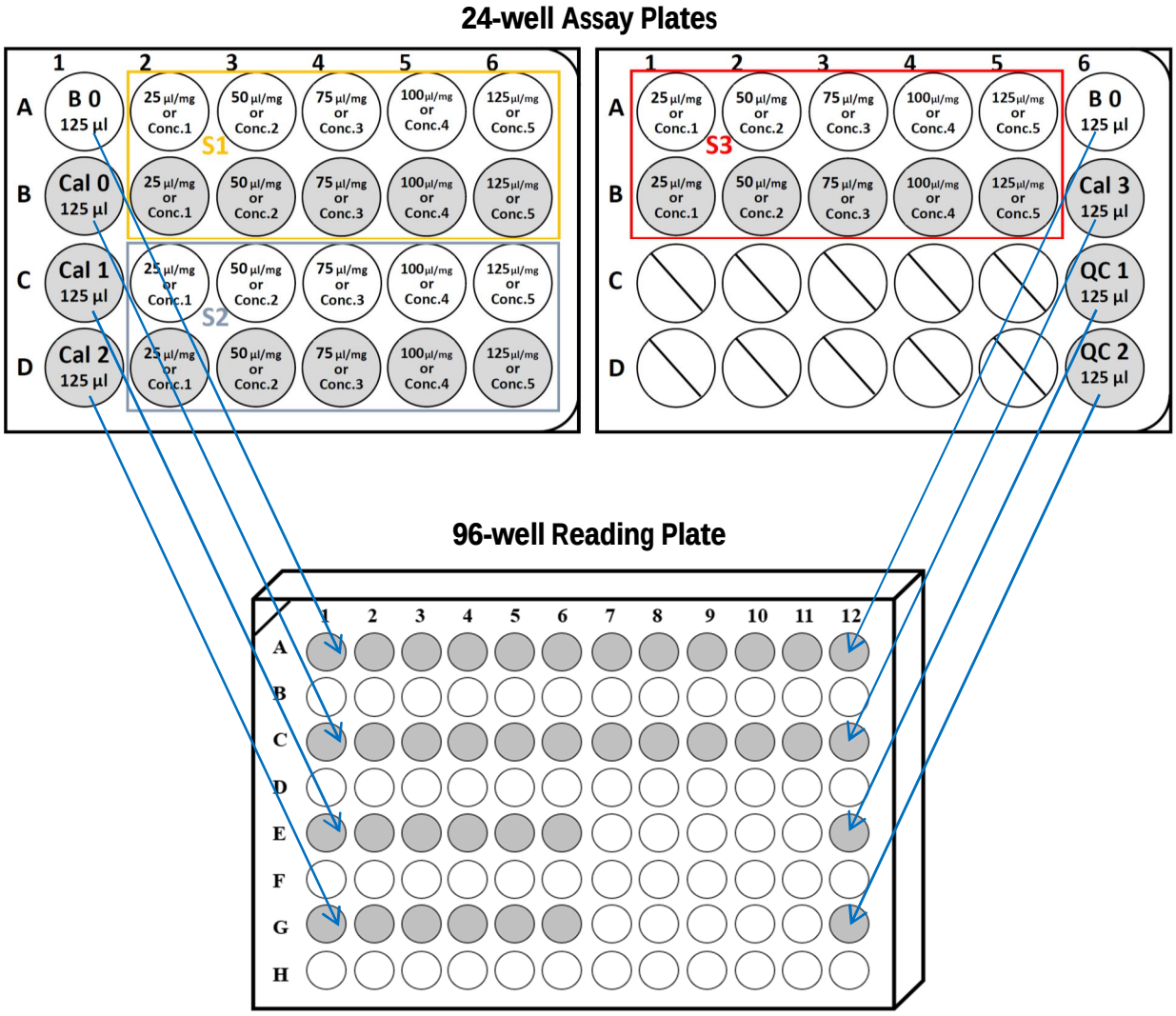


Figure Legend for 96-well Reading Plate:

= well with 250 µl Reagent solution or Blanking buffer from the 24-well assay plates

= empty well

Figure 7. Suggested Transferring Layout for Two Samples at Five Doses/Concentrations

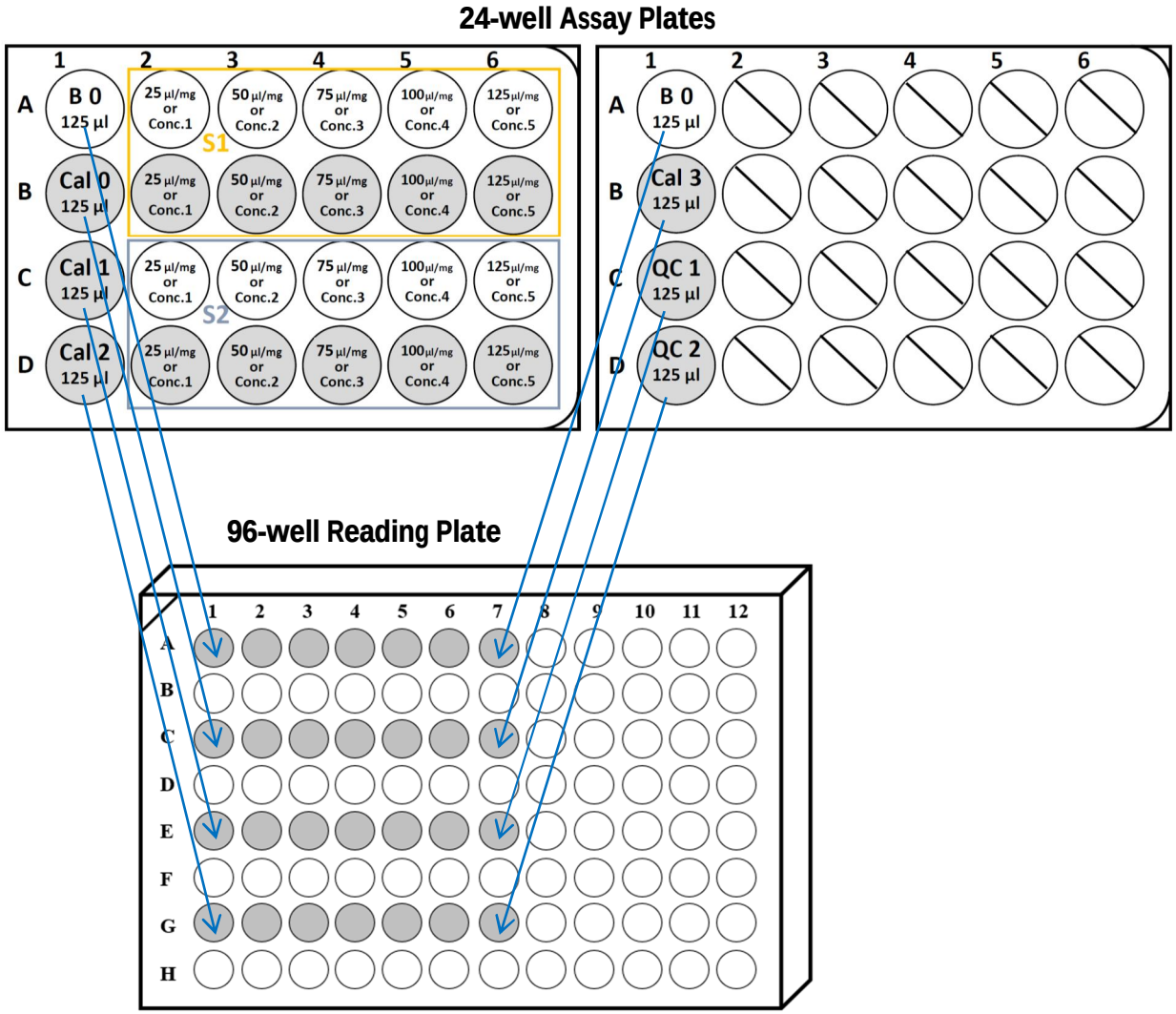


Figure Legend for 96-well Reading Plate:

= well with 250 µl Reagent solution or Blanking buffer from the 24-well assay plates

= empty well

Figure 8. Suggested Transferring Layout for One Samples at Five Doses/Concentrations

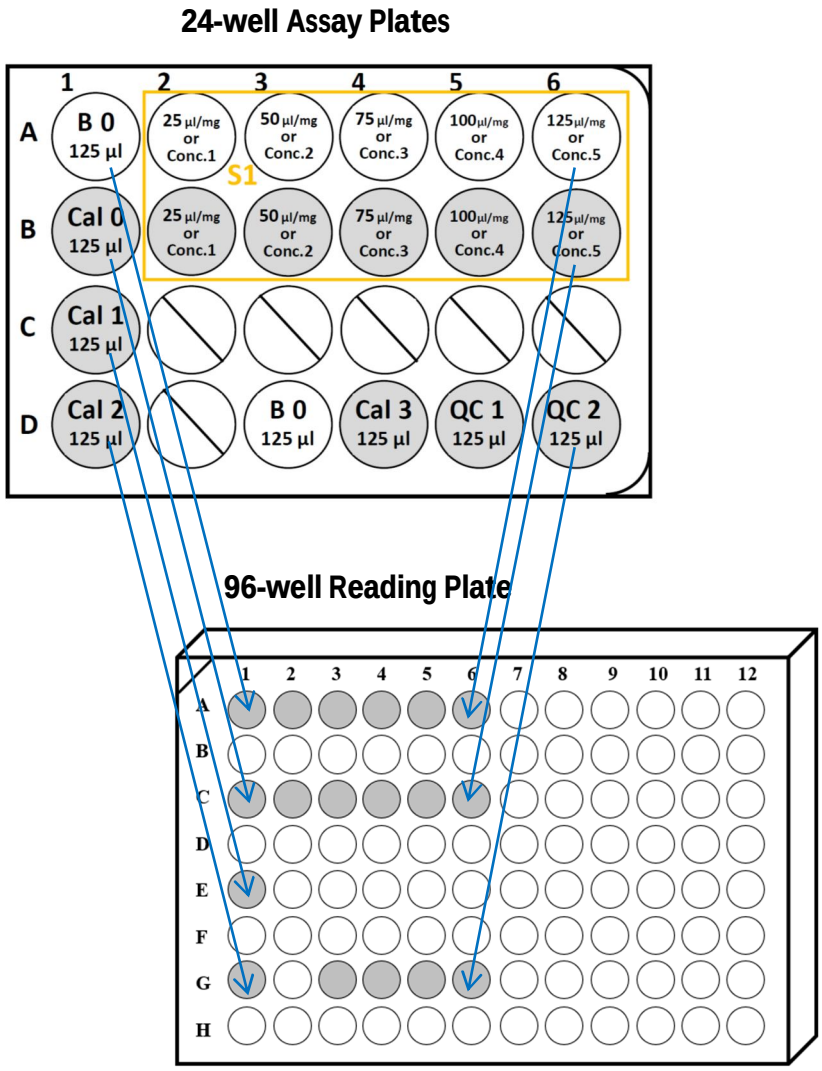


Figure Legend for 96-well Reading Plate:

= well with 250 µl Reagent solution or Blanking buffer from the 24-well assay plates

= empty well

D. ENDPOINT MEASUREMENT

1. Reading Assay on a compatible Plate Reader with Irritection® Software:
   The Irritection® Software program serves as the user interface to the plate reader. With the built in protocols, the program will automatically receive the optical density (OD) reading from plate reader and then convert the data to the Irritancy score. The program will determine assay performance by using the Calibrators and Quality Controls. The expected ranges for Calibrators and Quality Controls for each Irritection Assay are given on the Range Specification Data Sheet insert in the Kit. **You can also download the latest protocol and plate layouts information here (http://invitrointl.com/irritection-software/) and save the files under C:\IAS\OCULAR\.**
   1.1. Ensure that the Calibrator/Quality Control ranges in the Irritection Software are updated with the current kit.
   1.2. From the *Method* menu, choose *Select*. Select ***Ocular***. Click *OK*.
   1.3. From the *File* menu, choose *New*. Select *Assay***.**
   1.4. Select the appropriate protocol. ***New Ocular Irritection*** **(P-NO-1.RPT)**
   1.5. Select the appropriate plate layout.
   New 4 Samples at 5 Doses/Concs **for 4-sample kit (T-N4.RPT)**
   New 3 Samples at 5 Doses/Concs **for 3-sample kit (T-N3.RPT)**
   New 2 Samples at 5 Doses/Concs **for 2-sample kit (T-N2.RPT)**
   New 1 Sample at 5 Doses/Concs **for 1-sample kit (T-N1.RPT)**
   1.6. A series of screens will be displayed. You will need to enter the appropriate assay, and sample information.
   1.7. The plate reader will initialize. Remove the reading plate lid and insert the plate into the plate reader with the corner notches facing to the left. Click *Continue,* or press *ENTER.*
   1.8. After the data collection is completed, a dialog box will be displayed indicating if the assay was qualified or unqualified. Click *OK* to continue.
   1.9. If an Inhibition Check needs to be performed, a screen prompt will display the appropriate wells that require Inhibition Check.
   1.10. If an Inhibition Check is required, **add 25 µl Inhibition Check Reagent** to the appropriate wells.

2. Acceptance Criteria

   Data from the plate reader will be calculated by the Irritection software which will automatically perform the following qualification checks to ensure assay performance:

   2.1. Calibrator/Quality Control Check: Verifies that Calibrators are within the specified ranges (OD). The updated Range Specification Data Sheet comes with each kit.

   2.2. If all four Calibrators and two Quality Controls are within the ranges, assay performance is acceptable. When any one of six (four Calibrators and two Quality Controls) is not in range, the assay performance is still acceptable, but the unacceptable value will be assigned a substituted value by the software.

   2.3. Blanks in the range -50 to 500 can be processed. However, data derived from blanks below -50 and above 500 require careful consideration.

   2.4. Net OD Check: Verifies that Net OD ($OD_{reagent} – OD_{blank} = OD_{Net}$) is > -15.

   2.5. If the Net OD for the maximum qualified sample or for a selected sample dose or concentration is < Cal 2, then an Inhibition Check must be performed. With the Inhibition Check, the software verifies that the OD of the Inhibition Check sample is greater than the OD of Cal 2.

   2.6. Dose Response Check: Verifies that sample dose response curve is appropriate.

3. Upon acceptance of all qualification checks, the Irritection Software will generate a qualified assay report.

   3.1. Irritection Draize Equivalent (IDE) scores will be calculated based on the calibration curve. The irritancy of the test sample is judged to be defined by the highest IDE score, which is also termed the Maximal Qualified Score (MQS). A predicted *in vivo* class will be determined based on the following criteria:

Maximal Qualified Score (MQS) Range	Predicted Ocular Irritancy Classification	Predicted UN GHS Classification
0.0 – 12.5	**Minimal**	**Non-irritant (No Category)**
>12.5 – 30.0	**Mild**	**Irritant (Category 1/Category 2)**
>30.0 – 51.0	**Moderate**	
>51.0	**Severe**	

4. Clean Up:

   4.1. The Activator, Inhibition Check and all Calibrators should be disposed of after the assay has been completed.