DB-ALM Protocol n° 157 : Ocular Irritection® Assay System

Eye Irritation

This assay enables to assess the potential of a test compound to cause eye irritancy and corneal opacity by measuring the increase in optical density produced by the interaction of the test material with a protein matrix. The assay is currently available as a kit comprised of reagents and computer software that have been integrated with the instrumentation in the testing laboratory to provide an automated *in vitro* test.

Résumé

The Ocular Irritection® assay system, represents an optimized protocol of the former Eytex®, and it is a standardized and quantitative *in vitro* test that predicts the ocular irritation potential of cosmetics, consumer products, pharmaceuticals, and chemical raw materials. The Ocular Irritection® assay system evaluates the ocular hazard effects of test substances based on the premise that eye irritation and corneal opacity after exposure to irritating substances is the result of perturbation or denaturation of corneal proteins. The reduction in light transmission resulting from precipitate caused by the interaction of the test compound with a protein matrix is used to predict the potential of the test compound to cause eye irritancy and corneal opacity.

Experimental Description

Basic Procedure

The Ocular Irritection® (OI) assay system is based on the knowledge that the ability of a chemical to irritate the cornea is more likely related to its propensity to promote denaturation and disruption of corneal proteins. Consequently, the method has been developed as *in vitro* tests that mimic these biochemical phenomena making use of a protein reagent.

When the assay is performed, interactions between the irritant test material and reagent solution promotes protein denaturation and disaggregation of the macromolecular matrix. 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.

Analysis and interpretation of the resulting data are performed using the Irritection Assay System Software. This program compares the increase in optical density (OD$_{405}$) produced by the test material to a standard curve that is constructed by measuring the increase in OD$_{405}$ produced by a set of calibration substances.

The standard curve of the optical density measurement of the Ocular Irritection®. assay system relates to the ocular potential in the *in vivo* Draize rabbit eye test. The results of past comparative studies have been utilized to develop what is termed an “Irrition Draize Equivalent (IDE) score” which is a value derived from the *in vitro* studies that is equivalent to the predicted *in vivo* irritancy potential.

Discussion

The Ocular Irritection® test method represents a refinement of the former Eytex® method (Kelly, 1989, Gordon, 1992) following recommendations made by Balls et al. (1995). In 1996, the system underwent substantial revisions to take into account the recommendations made during the earlier multi-laboratory trials including the development of a single protocol, defined sample preparation and sample handling techniques (e.g. for surfactants and non-surfactants), and operating in a well-defined applicability domain. The non- surfactants are tested neat, over a range of doses and without any additional solvents. The list of the substances known to be incompatible with the assay is included in the Annex A. The experimental procedures of the Ocular Irritection® assay system are described in the Procedure Details section of this protocol.

These updates have led to use of the Ocular Irritection® assay system over the last 20 years in cosmetics, personal care products, chemical, textile, surfactant, and petrochemical products’ as well as in selected other markets. The assay is currently available, as a kit comprised of reagents and computer software that have been integrated with user instrumentation to provide a standardised and an automated *in vitro* test. Furthermore, because of its biochemical nature, Ocular Irritection® kit offers a long shelf-life (years), does not require sterile conditions and is readily available across the world.

Status

Participation in Validation Studies:

A prospective validation study was undertaken between 2009 and 2012 on the Ocular Irritection® assay system in order to obtain prospective data on its relevance (predictive capacity) and confirm its reliability (reproducibility within and between laboratories) using a challenging set of 56 coded test substances for which quality in vivo data were available. The prospective study was undertaken according to the principles and criteria documented in the OECD Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment (No. 34, OECD, 2005) and according to the ECVAM Modular Approach to validation (Hartung et al., 2004).

The obtained prospective data were then combined with existing data on the Ocular Irritection® collected from various sources, totalling 88 chemicals with parallel *in vivo* and *in vitro* data to obtain a comprehensive assessment of the test method performances, and its usefulness to be used in a tiered test strategy as defined in Scott et al. (2010), with the ultimate aim to replace the *in vivo* Draize rabbit eye test (OECD TG 405, 2012).

The Validation Management Group (VMG) concluded on the suitability of the Ocular Irritection® assay system to be used to predict chemicals not requiring classification (UN GHS Non-classified) for eye corrosion/irritation hazards according to the UN GHS / EU CLP classification systems (UN, 2013; EC, 2008). Furthermore the method was found also suitable to identify serious / irreversible eye damage (UN GHS Category 1) (Eskes et al., 2014).

The VMG recommends the Ocular Irritection® assay system to be used as a partial replacement either as an initial step of a bottom-up testing strategy to identify chemicals not requiring classification for eye corrosion/irritation hazards according to the UN GHS / EU CLP classification system, or in a top-down approach to identify UN GHS / EU CLP Category 1 chemicals.

Furthermore, VMG considered it useful to assess the usefulness of combining the Ocular Irritection® assay system with the currently regulatory accepted test methods into integrated testing strategies, to ultimately replace the *in vivo* rabbit Draize test (OECD TG 405, 2012).

The detailed documentation and results of these studies have been submitted to the EURL-ECVAM international validation centre for peer-review (Eskes et al., 2014).

Proprietary and/or Confidentiality Issues

There is a Registered Trade Mark for the Ocular Irritection® Assay System. In addition the manufacturing processes and certain chemical constituents of the test are considered to be trade secret, which are protected by contracts with the suppliers utilized to outsource those processes.

Abbreviations and Definitions

- CLP: Regulation on Classification, Labelling and Packaging
- EC: European Commission
- ECVAM: European Center for the Validation of Alternative Methods
- EU: European Union
- GHS: UN Globally Harmonized System
- MDS: Material Data Sheet
- OD: Optical Density
- OECD: Organisation for Economic Cooperation and Development
- OI: Ocular Irritection®
- SOP: Standard Operating Procedure
- TG: Test Guideline
- UN: United Nations
- VMG: Validation Management Group
The protocol presents the Standard Operation Procedure used in the Validation Study on the Ocular Irritection® Assay for Eye Irritation Testing conducted between 2009 and 2012. The Ocular Irritection® System represents a refinement of the former Eytex® assay following recommendations made in earlier validation studies (Balls et al., 1995). As a consequence, this SOP replaces the EYTEX™ protocol (DB-ALM Protocol No. 110).

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Materials and Preparations

Equipment

Fixed Equipment

- 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.
- Windows PC Compatible with Irritection Assay System software (Version 2)
- Incubator Maintained at 25°C ± 1°C
- Balance (110-g capacity)
- pH Meter
- Vortex Mixer
- Air Displacement Micro Pipettors (such as Denville Pipette XL3000i Single Channel Pipettes Model P-200, 20 – 200 µl)
- Positive Displacement Micro Pipettors (such as Thermo Labsystems Finnpipette PDP Model 200 µl, 20 – 200 µl)
- Multichannel Pipettor (such as Matrix 6 Channel EXP Impact Pipettor 15 – 1250 µl)*
- Repeating Pipettor (Such as Eppendorf 4780 Repeater Pipette Dispenser 250–1250 µl)*
- Disposable Reservoir Trays (2, such as 100-ml Matrix Disposable Reagent Reservoir)
- 100-ml Graduated Cylinder
- 100-ml Beaker
- Funnel (such as Oxford Vented Disposable Polystyrene Funnel, 12.5-cm diameter)
- Plastic Forceps
- Option: 8-channel manual pipettor, 0–250 µl capacity and a repeating pipettor, 250–1250 µl capacity.

Consumables

- Plastic Wrap
- 7- and 10-ml tubes
- 12.5 mL Combi-Syringes for Repeating Pipettor
- Compatible Pipet Tips

Media, Reagents, Sera, others

The following components are provided in the kit (sufficient for testing of four Samples in five doses):
Preparations

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 $\geq 4$ and $\leq 9$. 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 surfactant application procedure. For unknown 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.

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 template provided, or in your laboratory records, to indicate that the final pH reading was not stable.
8. Record the pH result in the MDS.

If the pH is $\geq 4$ or $\leq 9$, 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.
Surfactant and Non-Surfactant Application Procedures

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 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 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 maximize 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.
7. Record the height of the solution phase and the height of the bubble layer in the MDS.

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).
Method

Test Material Exposure Procedures

1. PREPARATION OF TEST SUBSTANCES

Different types of chemicals require different types of handling procedures in order to perform the Irritection assay properly.

- Handling of Non-Surfactant Chemicals:
  Chemical sample may be tested without further dilution. No further sample preparation is required.

- Handling of Surfactant Chemicals:
  Additional dilutions of the test material are required before the Irritection assay can be performed. These dilutions are described in detail below:

  1. For liquid chemicals, add 500 µl of the test substance to 9.5 ml distilled water to create a "5.0% working solution". For solid chemicals, add 500 mg +/- 10 mg of test material to 10.0 ml distilled water to create a "5.0% working solution".
  2. Cap tube and vortex mix for 10 seconds.
  3. Perform a two-fold serial dilution of the "5.0% working solution" in the following manner:
<table>
<thead>
<tr>
<th>Test Sample Number</th>
<th>Concentration of Surfactant (%)</th>
<th>Volume of Sample Solution Required (ml)</th>
<th>Volume of Distilled Water Required (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5%</td>
<td>2.5 ml sample 1</td>
<td>0.0 ml</td>
</tr>
<tr>
<td>2</td>
<td>2.5%</td>
<td>2.5 ml sample 1</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>3</td>
<td>1.25%</td>
<td>2.5 ml sample 2</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>4</td>
<td>0.625%</td>
<td>2.5 ml sample 3</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>5</td>
<td>0.3125%</td>
<td>2.5 ml sample 4</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

2. ROUTINE PROCEDURES

2.1 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.

2.2 Rehydration:

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

2.3 Filtration:

1. Fold the filter paper and place in a funnel.
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)
3. For four Samples/five volumes: Collect 40 ml filtered Reagent and pour into a 100-ml beaker.
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.

2.4 Labelling the 24-Well Assay Plates:

1. Align the assay plate(s) with the square corners toward the left and label their lid(s). A typical plate configuration that has been employed to evaluate four samples at five different volumes is shown below.
2. Remove the discs from the 24-well plate(s) and place them on the lid(s).

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**Figure Legend:**

- = well contains Blanking Buffer
- = well contains Reagent

B 0 1.25 ml Activated Blanking Buffer in well, 125 µl Cal 0 applied to membrane disc.
Cal 0 1.25 ml Activated Reagent in well, 125 µl Cal 0 applied to membrane disc.
Cal 1 1.25 ml Activated Reagent in well, 125 µl Cal 1 applied to membrane disc.
Cal 2 1.25 ml Activated Reagent in well, 125 µl Cal 2 applied to membrane disc.
Cal 3 1.25 ml Activated Reagent in well, 125 µl Cal 3 applied to membrane disc.
QC 1 1.25 ml Activated Reagent in well, 125 µl QC 1 applied to membrane disc.
QC 2 1.25 ml Activated Reagent in well, 125 µl QC 2 applied to membrane disc.
B 1.25 ml Activated Blanking Buffer in well, 25, 50, 75, 100, and 125 µl/mg sample applied to membrane discs. For surfactant samples, 125 µl of sample dilution (at concentrations 0.3125%, 0.625%, 1.25%, 2.5% and 5%) applied directly into corresponding blank buffer wells.
S 1.25 ml Activated Reagent in well, 25, 50, 75, 100, and 125 µl/mg sample applied to membrane discs. For surfactant samples, 125 µl of sample dilution (at concentrations 0.3125%, 0.625%, 1.25%, 2.5% and 5%) applied directly into corresponding blank buffer wells.

2.5 Activation:

1. For four Samples/five volumes: Add 800 µl Activator to the filtered Reagent and 600 µl Activator to the Blanking Buffer bottle. Gently swirl to mix and wait until pH is stable.
2. Record the final pH of the Activated Reagent solution, verifying that it falls within the following specified range: pH: 6.42 – 6.74.

3. TEST MATERIAL EXPOSURE PROCEDURES

3.1 Fill Wells of 24-Well Plate with Activated Reagent and Blanking Buffer:

1. Set the repeating pipettor (Such as Eppendorf 4780 Repeater Pipette Dispenser) at 1,250 µl.
2. Using the repeating pipettor, add 1,250 µl Activated Reagent to the appropriate wells (see the Figure above).
3. Repeat for Activated Blanking Buffer.
4. Insert the membrane discs into the wells which contain activated protein reagent or blanking buffer for the Calibrators and Quality Control Samples.

3.2 Add Calibrators and Quality Control Samples:

1. Pipette 125 µl Cal 0 onto the membrane disc which you placed into B 0 well. Repeat this procedure for the B 0 well of the second assay plate.
2. Pipette 125 µl Cal 1, Cal 2, Cal 3, QC 1, and QC 2 onto the membrane discs which you placed in the corresponding wells of the assay plates.

3.3 Add Test Samples:

3.3.1 Handling of Non-Surfactant Chemicals:

- Non-Surfactant Liquid Chemicals
  1. Insert the membrane discs into the wells which contain activated protein reagent or blanking buffer.
  2. Pipette 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. Use a positive displacement pipette (such as Lab Industries Pipette™ Micropipettor Model P-250, 5 – 250 µl) to apply the sample directly onto the membrane disc.

- Non-Surfactant Solid Chemicals
  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 blank and protein reagent wells of assay plates.

- Non-Surfactant Viscous or Sticky Chemicals
1. Insert the membrane discs into the wells which contain activated protein reagent or blanking buffer.

2. Pipette 25, 50, 75, 100, and 125 µl of each test sample into the membrane discs which you placed in the corresponding wells of assay plates.

3. Use a Positive Displacement Micro Pipettors (such as Thermo Labsystems Finnpipette PDP Model 200 µl, 20 – 200 µl) to apply the sample directly onto the membrane disc.

- **Non-surfactant Waxy Solid (Pieces) Chemicals**
  1. Weigh 25, 50, 75, 100, and 125 mg +/− 1 mg of each test sample directly into corresponding blank and the protein reagent wells of assay plates (without membrane discs).
  2. Insert the membrane discs into both blank and protein reagent wells after the samples are applied.

**3.3.2 Handling of Surfactant Chemicals:**

1. Vortex mixes each dilution of the test substance before being used.

2. Pipette 125 µl of sample dilution (at concentrations 0.3125%, 0.625%, 1.25%, 2.5% and 5%) directly into corresponding blank and the protein reagent wells of assay plates (without membrane discs).

3. Insert the membrane discs into both blank and protein reagent wells after the samples are applied.

3.4. Incubation:

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.

2. Record the technician’s name, date, and time on the lid of the assay plate.

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

3.5 Removing Membrane Discs:

1. Remove the assay plates from the incubator.

2. Remove the lids from the assay plates and save them. They will be used as a reference when transferring the Reagent and the Blanking Buffer to the clean 96-well reading plate.

3. Remove the plastic wrap from each assay plate.

4. Remove each membrane disc individually with plastic forceps.

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

6. Using the wooden stirring sticks, scrape the Reagent and Blank wells to ensure that all of the precipitate is removed from the bottom of each well. Use one stirring stick for each sample, starting with the lowest volume wells first, progressing to wells of higher volume. Use a separate stirring stick for each calibrator well.

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

3.6 Transferring from 24-Well Assay Plates to a 96-Well Reading Plate:

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

2. Transfer 250 µl of each well of the 24-well assay plate to every other well of the 96-well reading plate by columns (see below).

   **NOTE:** When using multichannel pipetter, 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 pipetter.

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

4. To eliminate inaccurate readings produced by settling of the precipitated Reagent, the assay must be read immediately after transferring the Reagent to the reading plate.
Figure Legend:

⊙ = cell with 250 μl Reagent or Blanking Buffer from the 24-well assay plates

⊙ = empty cell
Endpoint Measurement

Introduction:

The Ocular Irritection assay is a quantitative *in vitro* test method that mimics an acute ocular irritation test. When the assay is actually performed, controlled mixing of the test substance and the reagent solution during the assay incubation period promotes protein denaturation and disaggregation of the macromolecular matrix. Reaction of the test sample with these proteins and macromolecular complexes promotes conformational changes that may be readily detected as an increase in the turbidity of the protein solution. The turbidity may be detected spectrophotometrically at a wavelength of 405 nm. The irritancy potential of a test sample is expressed as an Irritection Draize Equivalent (IDE) score. This score is defined by comparing the increase in optical density (OD$_{405}$) produced by the test material to a standard curve that is constructed by measuring the increase in OD$_{405}$ produced by a set of Calibration substances. These Calibrators have been selected for use in this test because their irritancy potential has been previously documented in a series of *in vivo* investigations.

Irritection Software

The Irritection Software program serves as the user interface to the Plate Reader (such as Modified Cambridge 7520 Microplate reader, Dynex MRX, Molecular Devices and Tecan Sunrise). With the built in protocols, the program will automatically receive the optical density reading from plate reader and then convert the data to the Irritection Draize Equivalent (IDE) 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 Ocular Assay are given on the Range Specification Data Sheet insert in the Kit.

Reading Assay Results on a Plate Reader:

1. Ensure that the Calibrator/Quality Control ranges in the Irritection Software are updated with the current kit. The manufacturer will communicate directly with each OI test kit purchaser if ever a range change for OI Calibrators or Quality Controls is needed.
2. From the Method menu, choose Select.
3. Select Ocular. Click OK.
4. From the File menu, choose New.
5. Select Assay.
6. Select the appropriate protocol.
7. Select the appropriate plate layout.
8. A series of screens will be displayed. You will need to enter the appropriate assay, and sample information.
9. The plate reader will initialize. Remove the reading plate lid, wipe the bottom of the plate with a Kimwipe to remove any moisture and insert the plate into the plate reader with the corner notches facing to the left. Click Continue, or press ENTER.
10. After the data collection is completed, a dialog box will be displayed indicating if the assay was qualified or unqualified. Click OK to continue.
11. If an Inhibition Check (quality assurance step) needs to be performed, a screen prompt will display the appropriate wells that require Inhibition Check.
12. If an Inhibition Check is required, add 25 µl Inhibition Check Reagent to the appropriate wells.

Acceptance Criteria

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

1. Calibrator/Quality Control Check: Verifies that Calibrators are within the following specified ranges (OD): Cal 0: 62 – 262; Cal 1: 89 – 315; Cal 2: 351 – 945; Cal 3: 1277 – 2127 and Quality Controls are within the following specified ranges (IDE Score): QC1: 7.2 – 20.8; QC2: 23.6 – 35.6. The updated Range Specification Data Sheet comes with each kit.
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 Irritection Software.
3. Blanks in the range -50 to 500 can be processed. However, data derived from blanks below -50 and above 500 require careful consideration.

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

5. If the net OD for the maximum qualified sample or for a selected sample dose or concentration is $< Cal2$, 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.

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

B. Upon acceptance of all qualification checks, the Irritection Software will generate a qualified assay report (See example below).
ASSAY REPORT – ORIGINAL

Sample Description: Sample
Sample Number:  
Date:  
Time: 13:42:31
Product Type: Powder (White)
Technician Name: Amy
Assay Method: Ocular
Kit Lot Number: IO 090110
Protocol: Irritction Ocular_090110
Reagent temperature: 25.0
Incubation Time: 24.0 hours
Plate Layout: 4 Samples x 5 Volumes
Instrument Type: Dynex MRX
Wavelength: 405nm
Assay Number:  
Comment:  
Assay Qualification: Qualified

Sample Results:

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg</td>
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<td>-4</td>
<td>128</td>
<td>7.4</td>
<td>Minimal</td>
<td>Qualified</td>
</tr>
<tr>
<td>50 mg</td>
<td>114</td>
<td>-1</td>
<td>115</td>
<td>6.7</td>
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<tr>
<td>75 mg</td>
<td>140</td>
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<td>142</td>
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<td>Qualified</td>
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<tr>
<td>100 mg</td>
<td>151</td>
<td>-4</td>
<td>155</td>
<td>9.0</td>
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<td>Qualified</td>
</tr>
<tr>
<td>125 mg</td>
<td>134</td>
<td>-5</td>
<td>139</td>
<td>8.0</td>
<td>Minimal</td>
<td>Qualified</td>
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Calibrator Values:

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<thead>
<tr>
<th>Designation</th>
<th>OD</th>
<th>Irritancy Score</th>
<th>Range Limit (OD)</th>
<th>Qualification</th>
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<tbody>
<tr>
<td>Cal 0</td>
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<td>62-262</td>
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<td>Range qualified</td>
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<tr>
<td>Cal 2</td>
<td>665</td>
<td>30.0</td>
<td>351-945</td>
<td>Range qualified</td>
</tr>
<tr>
<td>Cal 3</td>
<td>1790</td>
<td>51.0</td>
<td>1277-2127</td>
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Quality Control Values:

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<th>Designation</th>
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<th>Irritancy Score</th>
<th>Range Limit (score)</th>
<th>Qualification</th>
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</thead>
<tbody>
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<td>241</td>
<td>13.5</td>
<td>7.2 - 20.8</td>
<td>Range qualified</td>
</tr>
<tr>
<td>QC 2</td>
<td>661</td>
<td>29.8</td>
<td>23.6 - 35.6</td>
<td>Range qualified</td>
</tr>
</tbody>
</table>

Sample Inhibition Check Results:

Dose / Inhibition Check OD
25 mg / 1686 50 mg / 1623 75 mg / 1694 100 mg / 1675 125 mg / 1648

* Mean value from assay data history
** Mean value from protocol defaults or adjusted value due to calibrator zero substitution
[] Value before substitution
Data Analysis

During each Irritection assay, the Irritection software employs an internal algorithm to assess the test results and compare them to those that would be expected for an ideal dose-response curve. In most cases, the results obtained with the test material are consistent with these model calculations. In these cases, the software print out provides a clear interpretation of the experimental results. The Sample Results section of the report lists the optical density data, calculated irritancy scores, and irritancy classifications. The statement “Qualified” in the Sample Results section means that the data for each of the tested samples obeys the expected behavior of typical dose-repose curve and no further analysis is required. The irritancy of the test sample is judged to be defined by the highest calculated irritancy score, which is also termed the Maximal Qualified Score (MQS).

Occasionally, the Qualification section of the report will contain a statement directing the user to “Examine Dose Curve” or “Examine Concentration Curve”. These messages are printed when the computer algorithm detects an unexpected assay result. The primary purpose of these messages is to cause the user to examine the printed graph of the test results to determine the source of the unexpected behavior (see Appendix C).

Prediction Model

IDE scores will be calculated by Irritection Software. The predicted in vivo classification will be determined based on the following criteria:

<table>
<thead>
<tr>
<th>Irritection Score (IDE) Range</th>
<th>Degree Ocular Irritancy</th>
<th>GHS classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 – 12.5</td>
<td>Minimal</td>
<td>Non-irritant</td>
</tr>
<tr>
<td>&gt;12.5 – 30</td>
<td>Mild</td>
<td>Irritant</td>
</tr>
<tr>
<td>&gt;30 – 51</td>
<td>Moderate</td>
<td></td>
</tr>
<tr>
<td>&gt;51</td>
<td>Severe</td>
<td></td>
</tr>
</tbody>
</table>
## Applicability and Limitations

Certain types of chemicals and chemical formulations are known to be incompatible with the protein reagents that are utilized in the Ocular Irritection assays. These materials are described below.

### Table A. Materials that are Incompatible with The Irritection Assay System

<table>
<thead>
<tr>
<th>Chemical or Chemical Formulation</th>
<th>Nature of Incompatibility</th>
<th>Solution for Problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic formulations, pH&lt;4.0</td>
<td>Assay interference</td>
<td>Additional controls and specific methods not included here.</td>
</tr>
<tr>
<td>Alkaline formulations, pH&gt;9.0</td>
<td>Assay interference</td>
<td>Additional controls and specific methods not included here.</td>
</tr>
<tr>
<td>Intensely colored materials</td>
<td>High OD readings for blanks and samples</td>
<td>Additional controls and specific methods not included here.</td>
</tr>
<tr>
<td>Oils and water-insoluble organic chemicals</td>
<td>Assay interference</td>
<td></td>
</tr>
<tr>
<td>Volatile ketones</td>
<td>Evaporation results in an under-estimation of irritancy</td>
<td></td>
</tr>
<tr>
<td>Nonionic Surfactants</td>
<td>Assay interference</td>
<td></td>
</tr>
<tr>
<td>Sorbitol at concentrations &gt;5%</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Urea at concentrations &gt;5%</td>
<td>False negative</td>
<td></td>
</tr>
<tr>
<td>Manganese violet</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Aluminum chlorohydrate</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Aluminum zirconium chlorohydrate</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Aluminum chloride</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Titanium oxide</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Silver salts</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>False positive</td>
<td></td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>False positive</td>
<td></td>
</tr>
</tbody>
</table>
Appendix B

Irritection Computer Software Instructions

IRRITEMOTION ® SOFTWARE INSTRUCTIONS FOR WINDOWS

A. Installing the Irritection software

1. Insert the Irritection Software installation disk.
2. From the Program Manager, go to the File menu, and select Run.
3. A Run dialog box will be displayed. Click on the Browse button.
4. From the Driver box, click the disk drive (a or b). From the File Name Box, click the setup.exe file, then click the OK button.
5. A Run dialog box will be displayed. Click the OK button.
6. The Irritection Software Setup will begin initialization. Click on the Continue button.
7. A Destination Path dialog box will be displayed. Click on the Continue button.
8. After the software has been installed, a message will appear stating, “Setup Succeeded.” Click the OK button.

Note: If the installation was not successful, please call your Technical Representative for assistance.

B. Setting up the instrument

1. Double-click on the Irritection Software icon to launch the program.
2. From the Instrument menu, choose Setup.
   You will need to enter the following system parameters (this needs to only be done once):

   **Comm Port:**
   Select the appropriate Comm Port.

   **Filters (nm):**
   The wavelength of each filter installed on the filter wheel of the plate reader will need to be entered in order for the program to choose the correct filter when reading. Enter zero to indicate an empty position.

   **Serial Number:**
   The serial number of the plate reader will need to be entered.
   **Note:** For the Cambridge plate reader, the serial number can be found inside the plate reader on the left corner panel. For the Molecular Devices or Dynex MRX plate reader, the serial number can be found on the rear panel.

   **Instrument Type:**
   Click the arrow in the Instrument Type box, and select the appropriate instrument type.

   **Mix Time:**
   Enter the desired seconds.

   **Manual Mode:**
   If enabled, user must manually enter all assay results.

   **Air Blank:**
   If enabled, all well reading will be zeroed against the air background.
   **Note:** The Baud Rate, Data Bits, Parity, and Stop Bits will correctly default for each instrument type. The Plate Type will default on Generic 96-Well.

3. Click the OK button, or press ENTER.

C. Selecting the assay method
1. From the Method menu, choose Select.
2. Select the desired assay method: Ocular, and click the OK button, or press ENTER.

D. Reading a new assay
1. From the File menu, choose New.
2. Select Assay, and click OK button or press ENTER.
3. Select the desired protocol, and click the OK button or press ENTER.
4. Select the desired plate layout, and click the OK button, or press ENTER.
5. A series of screens will be displayed. You will need to enter the appropriate assay, and sample information. When you have finished, click the OK button.
   **Note:** The Sample Number for each sample must be entered. The Sample Number is used as the file name for the data file that will contain the results of the assay.
6. The plate reader will initialize. Load the assay plate, and click on the Continue button or press ENTER.
7. After the data collection has been completed, a dialog box will be displayed indicating if the assay was qualified or unqualified. Press the OK button to continue. If an "Inhibition Check" prompt does not appear, proceed to Step E to print the processed report.

E. Performing an inhibition check
1. If an inhibition check is required, a series of screens will appear indicating the specific well(s) on the assay plate that require an inhibition check.
2. Add 25 µl of the inhibition check to the appropriate well(s).
3. Wait 5 minutes, load the plate, then click the Continue button.
4. A dialog box will indicate if the assay was qualified or unqualified. Remove the plate, and press the OK button to continue.

F. Performing an inhibition check, continued
1. A window, which displays the processed assay section of the report, for each sample will be displayed. To open additional windows (such as the protocol, plate layout, calibration curve, response curve or plate data), go to the View menu, and choose the desired window to be viewed.
2. To print the complete processed report, go to the File menu, and click the Print button. (The defaults have already been selected for you.)
3. To print additional sections of the report, select the section, and click on the Print button.
Interpretation of Irritection Data

During each Irritection assay, the Irritection software employs an internal algorithm to assess the test results and compare them to those that would be expected for an ideal dose-response curve. In most cases, the results obtained with the test material are consistent with these model calculations. In these cases, the software prints the statement “Range qualified” in the test report. This statement means that the data for each of the tested samples obeys the expected behavior of typical dose-repose curve and no further analysis is required.

Occasionally, the Qualification column of the test report will contain a different statement. These types of messages are printed when the computer algorithm detects an assay result that is not consistent with an ideal dose-response. The primary purpose of these messages is to cause the user to examine the printed graph of the test results to determine the source of the unexpected behavior.

The purpose of the guidelines given here is to provide information that can be utilized by the reviewer to interpret these atypical results. The reviewer should examine the first page of the Assay Report. It will be noted that this report contains three tables. These are labeled Sample Results, Calibrator Values, and Quality Control Values. The final column of each table is designated “Qualification.” This column of the tables will contain either the statement “Range qualified” or an alternative notation. A summary of these alternative messages and their interpretation will now be described.

First, there are some messages that are printed in the Qualification column of the Sample Results table if the computer algorithm has detected a single artifact or outlier. These messages include the following:

**Message 1: Dose eliminated**

**Message 2: Blank qualified sub**

In these cases, the algorithm has identified a single data point that is an outlier when compared to the expected value. The algorithm then substitutes an appropriate average value to permit correction of the Irritancy Score calculations. The irritancy values reported in the Sample Results table are acceptable as reported.

**Message 3: Examine concentration curve and/or examine dose curve**

Most commonly either of these two messages is printed when the tested material contains a compound that behaves somewhat like a surfactant or the chemical has a tendency to reduce the turbidity of the protein solution that is being measured by the microtiter plate spectrophotometer. These types of chemicals produce a declining dose-response profile, rather than the typical one that consists of an increase in the OD reading as the dose of the compound increases. The following table is a typical example:

<table>
<thead>
<tr>
<th>Dose (µl)</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl</td>
<td>159</td>
<td>-5</td>
<td>164</td>
<td>8.1</td>
<td>Minimal</td>
<td>Examine dose curve</td>
</tr>
<tr>
<td>50 µl</td>
<td>416</td>
<td>-6</td>
<td>422</td>
<td>18.7</td>
<td>Mild</td>
<td>Examine dose curve</td>
</tr>
<tr>
<td>75 µl</td>
<td>388</td>
<td>-4</td>
<td>392</td>
<td>17.6</td>
<td>Mild</td>
<td>Examine dose curve</td>
</tr>
<tr>
<td>100 µl</td>
<td>349</td>
<td>-2</td>
<td>351</td>
<td>16.1</td>
<td>Mild</td>
<td>Examine dose curve</td>
</tr>
<tr>
<td>125 µl</td>
<td>334</td>
<td>-2</td>
<td>336</td>
<td>15.6</td>
<td>Mild</td>
<td>Examine dose curve</td>
</tr>
</tbody>
</table>
The dose-response profile for this compound is shown in Figure 1.

![Dose-response profile](image)

**Figure 1.** Dose-response profile observed with a compound has the term “Examine dose curve” printed in the Irritection Sample Results table.

If the calibrator and quality control values are qualified, this dose-response profile is acceptable. The irritancy of the compound is judged to be defined by the highest calculated irritancy score, in this case 18.7. According to the criteria established for the ECVAM study, this compound would be considered to be an irritant because this score is greater than 12.5.

**Message: Inhibition failed**

This message is observed when some surfactant-like compounds are being analysed at relatively high concentrations. This message is printed because the chemical appears to dissolve the turbid protein solution that is normally produced when the inhibition test is performed. An example of this type of report is shown next.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.31%</td>
<td>137</td>
<td>-4</td>
<td>141</td>
<td>7.0</td>
<td>Minimal</td>
<td>Qualified</td>
</tr>
<tr>
<td>0.63%</td>
<td>305</td>
<td>0</td>
<td>305</td>
<td>14.4</td>
<td>Mild</td>
<td>Qualified</td>
</tr>
<tr>
<td>1.25%</td>
<td>651</td>
<td>-1</td>
<td>652</td>
<td>27.0</td>
<td>Mild</td>
<td>Qualified</td>
</tr>
<tr>
<td>2.5%</td>
<td>573</td>
<td>0</td>
<td>573</td>
<td>24.1</td>
<td>Mild</td>
<td>Examine concentration curve</td>
</tr>
<tr>
<td>5.0%</td>
<td>206</td>
<td>1</td>
<td>205</td>
<td>10.1</td>
<td>Minimal</td>
<td>Inhibition failed</td>
</tr>
</tbody>
</table>
The dose-response profile for this compound is shown in Figure 2.

Figure 2. Dose-response profile observed with a compound has the terms “Examine dose curve” and “Inhibition failed” printed in the Irritation Sample Results table.

Again, if the calibrator and quality control values are qualified, this dose-response profile is acceptable. The irritancy of the compound is judged to be defined by the highest calculated irritancy score, which is also termed the Maximal Qualified Score (MQS). In this case the MQS is 27.0. According to the criteria established for the ECVAM study, this compound would be considered to be an irritant because this score is greater than 12.5.

Message: Net min failed

This type of result is also seen when surfactant-like compounds are tested. Again, it results from the fact that a chemical, at elevated concentrations, will interfere with the assay by solubilizing the turbid protein solution that is serving as the tested substance. An example is shown next.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.31%</td>
<td>58</td>
<td>7</td>
<td>51</td>
<td>3.2</td>
<td>Minimal</td>
<td>Qualified</td>
</tr>
<tr>
<td>0.63%</td>
<td>136</td>
<td>3</td>
<td>133</td>
<td>8.3</td>
<td>Minimal</td>
<td>Qualified</td>
</tr>
<tr>
<td>1.25%</td>
<td>409</td>
<td>0</td>
<td>409</td>
<td>21.0</td>
<td>Mild</td>
<td>Qualified</td>
</tr>
<tr>
<td>2.5%</td>
<td>245</td>
<td>5</td>
<td>240</td>
<td>14.1</td>
<td>Mild</td>
<td>Examine concentration curve</td>
</tr>
<tr>
<td>5.0%</td>
<td>-18</td>
<td>1</td>
<td>-19</td>
<td>0.0</td>
<td>Minimal</td>
<td>Net min failed</td>
</tr>
</tbody>
</table>
The dose-response profile for this compound is shown in Figure 3.

![Dose-response profile](image)

**Figure 3.** Dose-response profile observed with a compound has the terms “Examine dose curve” and “Net min failed” printed in the Irritection Sample Results table.

As with the examples cited above, these results are acceptable if the calibrator and quality control values are qualified. The MQS would be 21.0 and this material would be considered to be an irritant.

**Message: Blank max failed and/or Blanks not flat**

These two error messages are printed when the algorithm detects results produced by interfering substances. It can be difficult to determine if these types of compounds should be included or excluded from further consideration.

As a rule, colored substances, particularly if they are brown or red, will produce these types of error messages because they absorb light at the same wavelength that is being utilized to measure turbidity in the Irritection test. A typical example of this is shown next.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg</td>
<td>2338</td>
<td>2053</td>
<td>285</td>
<td>14.3</td>
<td>Mild</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>50 mg</td>
<td>3060</td>
<td>2970</td>
<td>90</td>
<td>4.7</td>
<td>Minimal</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>75 mg</td>
<td>1784</td>
<td>1948</td>
<td>-164</td>
<td>0.0</td>
<td>Minimal</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>100 mg</td>
<td>1784</td>
<td>1948</td>
<td>-164</td>
<td>0.0</td>
<td>Minimal</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>125 mg</td>
<td>3105</td>
<td>1948</td>
<td>1157</td>
<td>39.2</td>
<td>Moderate</td>
<td>Blank max failed</td>
</tr>
</tbody>
</table>

Samples like this one that produce blank OD values greater than 1200 must be **excluded** from further consideration. They interfere with the test so strongly that they will not produce reliable results.
A solid white powder was analyzed and produced the results shown below:

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mg</td>
<td>2010</td>
<td>1581</td>
<td>429</td>
<td>21.0</td>
<td>Mild</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>50 mg</td>
<td>1959</td>
<td>1238</td>
<td>721</td>
<td>31.2</td>
<td>Mild/Moderate</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>75 mg</td>
<td>1921</td>
<td>738</td>
<td>1183</td>
<td>40.1</td>
<td>Moderate</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>100 mg</td>
<td>414</td>
<td>944</td>
<td>-530</td>
<td>0.0</td>
<td>Minimal</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>125 mg</td>
<td>677</td>
<td>706</td>
<td>-29</td>
<td>0.0</td>
<td>Minimal</td>
<td>Blank max failed</td>
</tr>
</tbody>
</table>

This example represents a slightly different situation and requires further analysis. In general, this analysis is conducted in two steps. First, even though it is apparent that this solid is causing some interference, there are three blank OD values that do not exceed 1200, so the sample should not be automatically eliminated from further study. Second, one should then examine the sample OD and blank OD values to determine if the signal to background ratio is greater than 2. If so, there may be sufficient discrimination between the protein reaction and the interference to permit an assessment of the irritancy of the test substance. In this example, only one of the doses (75 mg) displays a signal to background ratio that is greater than 2.0 (1921 divided by 738 = 2.60). Because there is only one data point that fulfills this criterion, there is insufficient data to make a determination and this sample should be excluded from the test data set.

In contrast, the following example presents a situation where it is possible to see a consistent discrimination between the Sample OD and the Blank OD.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Net OD</th>
<th>Irritancy Score</th>
<th>Irritancy Classification</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl</td>
<td>1916</td>
<td>470</td>
<td>1446</td>
<td>42.6</td>
<td>Moderate</td>
<td>Blanks not flat</td>
</tr>
<tr>
<td>50 µl</td>
<td>2292</td>
<td>573</td>
<td>1719</td>
<td>49.0</td>
<td>Moderate/Severe</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>75 µl</td>
<td>2270</td>
<td>800</td>
<td>1470</td>
<td>43.2</td>
<td>Moderate</td>
<td>Blank max failed</td>
</tr>
<tr>
<td>100 µl</td>
<td>2313</td>
<td>459</td>
<td>1854</td>
<td>&gt;51.0</td>
<td>Moderate/Severe</td>
<td>Blanks not flat</td>
</tr>
<tr>
<td>125 µl</td>
<td>2360</td>
<td>332</td>
<td>2028</td>
<td>&gt;51.0</td>
<td>Moderate/Severe</td>
<td>Blanks not flat</td>
</tr>
</tbody>
</table>

None of the Blank OD values are greater than 1200, so these data should be examined further. Next, the signal to background ratio for each of the tested doses can be calculated as follows:

<table>
<thead>
<tr>
<th>Dose</th>
<th>Sample OD</th>
<th>Blank OD</th>
<th>Signal to Background Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µl</td>
<td>1916</td>
<td>470</td>
<td>4.1</td>
</tr>
<tr>
<td>50 µl</td>
<td>2292</td>
<td>573</td>
<td>4.0</td>
</tr>
<tr>
<td>75 µl</td>
<td>2270</td>
<td>800</td>
<td>2.8</td>
</tr>
<tr>
<td>100 µl</td>
<td>2313</td>
<td>459</td>
<td>5.0</td>
</tr>
<tr>
<td>125 µl</td>
<td>2360</td>
<td>332</td>
<td>7.1</td>
</tr>
</tbody>
</table>

In each case, the signal to background ratio is greater than 2.0. This suggests that there is sufficient discrimination between the protein reaction and the interference produced by the test substance to permit an assessment of the irritancy of the test material. Since five data points are available for consideration, this test substance would be included in the study.

As a general rule, when performing this type of analysis, a minimum of three sequential data points that
fulfill both the criteria that the Blank OD is less than 1200 and the signal to background ratio is greater than 2.0 are required in order to include the results from the test substance in the study.

**Figure 4** below provides examples of typical volume-dependent assay profiles. They should be employed to assist in the interpretation of common assay findings. The maximum qualified score (MQS) that would be utilized to define the irritancy of the test substance is denoted in each of these graphs.

**Figure 4.** Common dose-response curves that are observed in the Irritection assay.
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