

A Spreadsheet for the Statistical Validation of Direct Transfer Sterility Testing

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The United States Pharmacopeia and the British Pharmacopeia detail procedures of direct transfer testing for the sterility of pharmaceuticals and solutions. All these regulations require validation of the procedure, and provide a qualitative method to assay for the ability of the test system to support growth. Bacteriostasis (and fungistasis) is an appropriate positive control for each assay, but not sufficient as validation of the procedure. A quantitative validation protocol is preferable, one that utilizes statistical comparisons of data to evaluate the neutralizer efficacy of a procedure. These comparisons are made using the spreadsheet described encompassing Student's t Test, the F test, and an estimation of the resolution of the data using a beta risk of 0.05. The spreadsheet, written in Lotus *Symphony*, utilizes a limited number of commands and should be easily written in leading spreadsheet programs.

INTRODUCTION

Validation of sterility testing protocols is required by United States Pharmacopeia (USP) XXII (Anon., 1990), although no procedures for quantitative validation are provided. The validation of a direct transfer sterility testing protocol requires demonstration of the ability to reproducibly recover a low inoculum of microorganisms from the appropriate ratio of test solution in the absence of the test solution. This is performed in a qualitative manner by the USP bacteriostasis and fungistasis tests.

These tests involve performing the direct transfer sterility test, using the determined dilution of solutions into media. The media is then inoculated with 10 - 100 colony forming units (CFU) of the test system, either *Bacillus subtilis* (ATCC #6633), *Candida albicans* (ATCC #10231), or *Clostridium sporogenes* (ATCC #11437). Growth is monitored by turbidity.

Validation of the sterility test should provide a reproducible, effective procedure for the recovery of small numbers of microorganisms from the media both in the presence and in the absence of the test solution. The quantitative demonstration of bacteriostasis and fungistasis is performed by pair-wise comparisons. Neutralizer efficacy is confirmed by comparison between the CFU in the agar medium in the absence, and presence, of the test solution. Reproducibility of the procedure is demonstrated by analysis of replicates. This demonstration is especially important in determining the sterility of microstatic or microbicidal solutions as residual amounts of the active agent may be bacteriostatic (Russell, 1981).

The spreadsheet described provides a statistical estimation of the reproducibility and adequacy of a neutralization procedure for use in testing antimicrobial solutions for sterility. It was written for ease of use on the part of the technician reading the results. The raw colony counts are entered into a restricted area of the spreadsheet. The calculations are performed automatically and the results provided through a series of nested @IF statements in the summary page, along with a test for the equivalence of variance (the F test) and an estimation of the resolution of the data. In addition, this provides population statistics for the replicates. The spreadsheet is written so that an initial, small number of samples can be used to forecast the adequacy of the procedure. This aspect can result in savings of time and test solutions.

MATERIALS AND METHODS

Spreadsheet Design and Functions

A schematic representation of the overall layout is presented in Figure 1.

Required Functions

Math Functions: Addition, subtraction, multiplication, division, antilog (i.e., 10^x)

@ Functions:

@ABS(x) - Calculates the absolute value of a number, equation, or cell reference.

@AVG(x) - Calculates the average value of a range of cells.

@COUNT(x) - Counts the number of entries in a

	A	B	C	D	E	F
30	*					
31	*	B. subtilis w/o TS				
32	*					
33	*		Log10		CFU	
34	*	Mean:	1.674		47.153	
35	*	Size:	8			
36	*	Variance:	0.002		23.411	
37	*	Stan Dev:	0.045		4.838	
38	*	Minimum:	1.591		39	
39	*	Maximum:	1.732		54	
40	*					
41	*					

Figure 3 Sample statistics for *Bacillus subtilis* in the absence of test solution.

The column headed Log10 contains the values used in the statistical tests outlined in Figure 2 and the text. These are the respective values for the transformed data set, which more closely approximates a normal distribution than the data set consisting of the CFU. The column headed CFU is the antilog of the first column, with the exception of the minimum and maximum values. The cell formulas are provided in the text.

```

F35: '|
A36: '*
B36: 'Variance:
C36: (@VAR(E76..E85)*C35)/(C35-1)
E36: (@VAR(D76..D85)*C35)/(C35-1)
The Lotus @VAR function calculates the popula-
tion variance; an adjustment is required to provide
the sample variance. Standard deviation of the
population should be defined as the square root of
this equation. The variance and standard devia-
tions for the data are calculated separately for the
transformed and the untransformed data.

F36: '|
A37: '*
B37: 'Stan Dev:
C37: @SQRT(C36)
E37: @SQRT(E36)
F37: '|
A38: '*
B38: ' Minimum:
C38: @MIN(E76..E85)
E38: @MIN(D76..D85)
F38: '|
A39: '*
B39: ' Maximum:
C39: @MAX(E76..E85)
E39: @MAX(D76..D85)
F39: '|
A40: '*
F40: '|
A41: '*
B41: \-
C41: \-
D41: \-
E41: \-
F41: '|-----

```

DISCUSSION

This spreadsheet allows for the quantitative validation of the United States Pharmacopeia Direct Transfer Sterility Test (Anon., 1990). If a significant reduction in CFU is seen in the presence of the test solution, a greater dilution may be necessary for growth. The validation procedure should be repeated using a greater ratio of growth medium to test sample.

The appropriate neutralization of microbiostatic agents in sterility testing is of critical importance to the safety of the solutions. Inadequate neutralization could result in false negative results, the appearance of sterility due to inhibition of the contaminating microorganism by residual active agent.

The described spreadsheet offers several advantages in this validation effort. First, it allows forecasting of results from a limited data set. This provides an opportunity to examine a protocol without committing a large volume of solution to the effort. Secondly, it provides a statistical check on the adequacy of the procedure by incorporating an assay for equivalence of variance between the populations. Finally, it provides an estimation of the resolving power of the data sets.

ACKNOWLEDGEMENTS

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REFERENCES

- ANON. (1990). *USP XXII, NF XVII*. United States Pharmacopeial Convention, Inc., Rockville, MD.
- DOWDY, S. & WEARDEN, S. (1983). *Statistics for Research*, pp. 193-194. New York: John Wiley & Sons.
- FREUND, J.E. & WEALPOLE, R.E. (1987). *Mathematical Statistics*, p. 422. Englewood Cliffs, NJ: Prentice-Hall.
- RUSSELL, A.D. (1981). Neutralization Procedures in the Evaluation of Bactericidal Activity. In *Disinfectants: Their Use and Evaluation of Effectiveness*, pp. 45-59. Edited by C.H. Collins *et al.* London: Academic Press, Inc.
- STUDENT [W.S. GOSSER] (1908). The Probable Error of a Mean. *Biometrika* **6**, 1-25.

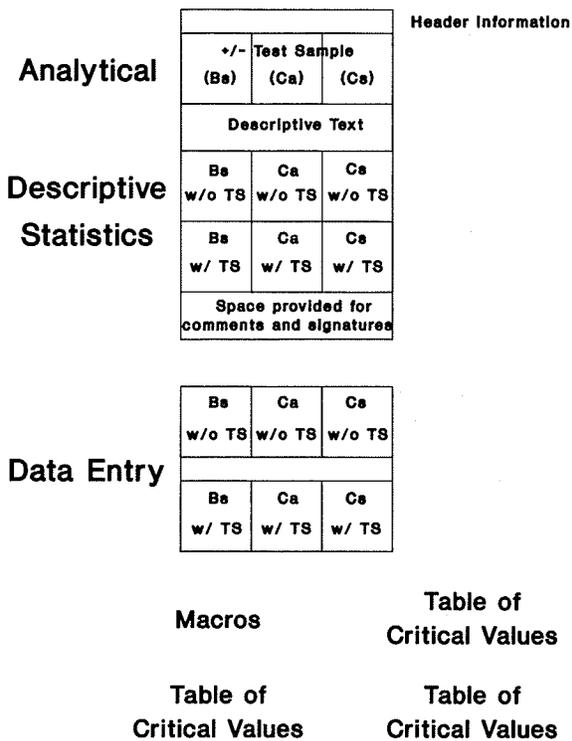


Figure 1 Schematic outline of the spreadsheet. The spreadsheet is designed to present the analysis in the first page of the printout for the three test organisms *Bacillus subtilis* (Bs), *Candida albicans* (Ca), and *Clostridium sporogenes* (Cs). The upper half of the page is the statistical analysis, with the descriptive statistics on the lower half. The second page of printout includes the raw data and the transformed data, with entry space for lot numbers, dates, etc. The lowest portion contains several macros, and the required tables.

- range of cells. This will count text as an entry.
- @IF(x,y,z) - A logical operator (if x is true, then return y, if false, return z)
- @LOG(x) - Determines the logarithm (base 10) of the number, cell reference, or equation.
- @MAX(x) - Returns the maximum value in a range, or a series of discontinuous cells joined by commas.
- @MIN(x) - As with @MAX, but returns the minimum number. Text entries will count as 0, an ERR message as 1.
- @SQRT(x) - Computes the square root of the cell, or equation.
- @VAR(x) - The population variance of a range of cells. The sample variance can be derived by multiplying this value by $n/(n-1)$. The standard deviation of the sample is the square root of the sample variance.
- @VLOOKUP(x,y,z) - Vertical lookup function which states; lookup x in the first column of the range y, then report the value z columns to the right. Both x and z can be numbers, equations, or cell references. Table 1 provides the tables used in this analysis.

Table 1 Necessary tables

Critical t values for 0.01 One-tailed values		Critical t values for 0.05 One-tailed values (used for BETA)	
1	31.821	1	6.314
2	6.965	2	2.92
3	4.541	3	2.353
4	3.747	4	2.132
5	3.365	5	2.015
6	3.143	6	1.943
7	2.998	7	1.895
8	2.896	8	1.86
9	2.821	9	1.833
10	2.764	10	1.812
11	2.718	11	1.796
12	2.681	12	1.782
13	2.65	13	1.771
14	2.624	14	1.761
15	2.602	15	1.753
16	2.583	16	1.746
17	2.567	17	1.74
18	2.552	18	1.734
19	2.539	19	1.729
20	2.528	20	1.725

Critical Values for F Test at ALPHA = 0.01
Degrees of freedom Critical value

1	4052
2	99
3	29.5
4	16
5	11
6	8.47
7	6.99
8	6.03
9	5.35

NOTE: Both samples must be the same size

Cell Formulas

Neutralizer Efficacy (+/- Test Sample) Comparison for *Bacillus subtilis*: see Figure 2.

- A8: *
 - B8: \-
 - C8: \-
 - D8: \-
 - E8: \-
 - F8: \-
 - A9: *
 - B9: '+/- Test Sample (Bs)
 - F9: '|
 - A10: *
 - B10: \-
 - C10: \-
 - F10: '|
 - A11: *
 - B11: 'Pooled Smpl Var =
 - D11: (((C35-1)*C36)+((C46-1)*C47))/(C35+C46-2)
- This equation provides pooled sample variance as the weighted average of the two sample variances (Dowdy & Wearden, 1983).

```

1-A-B-----C-----D-----E-----F
8 * -----
9 * +/- Test Sample (Bs) |
10 * ----- |
11 * Pooled Smpl Var = 0.002 |
12 * Degrees Freedom = 14 |
13 * t = 1.472 |
14 * Critical Value = 2.624 |
15 * The two means are not distinguishable |
16 * |
17 * F Test (@ 0.01) = PASS |
18 * Resolution = 1.023 |
19 * -----

```

Figure 2 Neutralizer toxicity comparison for *Bacillus subtilis*.

This section appears on the summary page providing information on the Student's t Test and the resolving power of the data. It compares data from the CFU grown in the presence and the absence of the test sample. The t statistic is calculated in cell D13. Cell B15 contains an @IF statement that relates the result of the test. Cell D17 contains a nested @IF statement that provides the result of the control calculation for equivalence of variance. The resolution of the assay is defined as the ratio of the two most similar means discernable by the data, and is provided in cell D18. The cell formulas are provided in the text.

```

F11: '|
A12: '*
B12: 'Degrees Freedom =
D12: '+C35+C46-2
F12: '|
A13: '*
C13: 't =
D13: '(C34-C45)/@SQRT((D11/C35)+(D11/C46))
This cell provides the t statistic by dividing the
difference between the means, by the square root
of the sum of: (1) the pooled sample variance divided
by the first sample size and: (2) the pooled sample
variance divided by the second sample size (Stu-
dent, 1908).

F13: '|
A14: '*
B14: 'Critical Value =
D14: '@VLOOKUP(D12,$K$114..$M$133,2)
A table of t values at alpha = 0.01 are listed in cells
K114 through M133, offset by 2 columns.

F14: '|
A15: '*
B15: '@IF(@ABS(D13)<D14,$B$106,$B$107)
This cell reports the results of the t test. If the
absolute value of the calculated t value is less than
the critical t value, the character string "The two
means are not distinguishable" is reported. If
greater, the contents of cell B107 ("The two means
are distinguishable") are reported.

F15: '|
A16: '*
F16: '|

```

```

A17: '*
B17: 'F Test (@ 0.01) =
D17: '@IF((@MAX(C36,C47)/@MIN(C36,C47))<@VLOOKUP
(C35-1,$D$145..$F$153,2),"PASS","FAIL")
This cell reports the results of the F test for equiva-
lence of variance. If the ratio of the larger variance
divided by the smaller variance does not exceed the
critical value (found in the table located at
D145..F153), the string PASS is reported. If it does
exceed the value, the string FAIL is reported (Freund
& Wealpole, 1987).

```

```

F17: '|
A18: '*
B18: ' Resolution =
D18: '10^(@SQRT((2*D11)*((@VLOOKUP(D12,$K$114..$M
$133,2)+@VLOOKUP(D12,$K$140..$M$159,2))^2)/(C35)))
This cell reports the resolution of the assay as
defined by the ratio of the two most similar mean
CFU values (the difference between the most similar
log10 values).

```

```

F18: '|
A19: '*
B19: \-
C19: \-
D19: \-
E19: \-
F19: \-

```

Sample Statistics: *Bacillus subtilis* w/o TS: see Figure 3.

```

A30: '*
B30: \-
C30: \-
D30: \-
E30: \-
F30: \-
A31: '*
B31: 'B. subtilis w/o TS
F31: '|
A32: '*
B32: \-
C32: \-
A33: '*
C33: '^Log
E33: '^CFU
F33: '|
A34: '*
B34: ' Mean:
C34: '@AVG(E76..E85)
This cell provides the mean log10 value for the CFU
recorded from the assay in D76..D85.

E34: '10^C34
The antilog of the transformed data mean was
taken to provide a more accurate approximation of
the geometric mean.

F34: '|
A35: '*
B35: ' Size:
C35: '@COUNT(D76..D85)
This is a count of the data entry column.

```