

INTRODUCTION TO SERIAL ENDPOINT TITRATION

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Skin testing has been performed as an adjunct in the diagnosis of allergic diseases for more than 130 years. Charles Blackley, an English physician, performed the first skin test in 1873.² He applied extracts of grass pollen to denuded forearm skin, producing a wheal in allergic individuals.

In 1911 Leonard Noon attempted to induce the production of pollen antitoxin in allergic patients by injecting these patients with pollen extract.^{13, 19} Noon also attempted to determine the degree of sensitivity of allergic patients to specific allergens. He made various dilutions of antigen extracts and instilled them into the patient's conjunctiva. His endpoint was the concentration of extract resulting in conjunctival inflammation. This work resulted in the development of the Noon unit, which was the quantity of antigenic material extracted from 0.001 mg of timothy grass pollen. Presently, the Noon unit is 1 μ g of antigenic material, and an extract labeled 1:10 w/v contains 100,000 NU/mL. The following relationship is important to keep in mind:

Noon unit = 0.001 mg antigen = 1/2 PNU = 0.1 mL of #4 test solution. The protein nitrogen unit (PNU) is a standardized measure of all protein present in a given extract. The #4 test solution represents an antigen dilution of 1:12,500.

Cooke expanded on Noon's work by establishing an individual's degree of sensitivity to allergens by using intradermal skin challenge tests.³ Cooke assigned patients to four groups based on their skin reactivity to antigenic exposure.

French Hansel is considered the father of otolaryngic allergy.²⁰ He was the first otolaryngologist to perform skin tests in 1930 using multiple

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individual extracts as opposed to single concentrations of antigenic material. His early experiences, published in 1953,⁹ demonstrated that each individual antigen has a unique response in a given patient. The strength of a response to different antigens is not the same for all the antigens encountered by a given individual.²⁰

Phillips previously had demonstrated that the size of the skin wheal following intradermal skin testing correlated well with the dose of the antigen administered.²¹ Hansel thus concluded that multiple intradermal skin tests using individual allergens with varying doses produced greater accuracy of information regarding the degree of the patient's sensitivity.²² This method has become the fundamental concept behind serial endpoint titration (SET). Hansel used intradermal injections of antigens in a 1:10 ratio. He tested individual antigens in varying strength while measuring the response.

Unfortunately, the 1:10 antigenic dilution resulted in abrupt whealing and a high frequency of false-positive responses. In 1937, Herbert Rinkel, an allergist working with Hansel, found that an antigenic dilution ratio of 1:5 administered in progressively increasing increments resulted in a qualitatively and quantitatively superior measure of allergic skin reactivity. The response was constant through three or four dilutions in 72% of patients, avoided false-positive reactions, and allowed for the detection of very low levels of sensitivity. It was acknowledged that a ratio of 1:3 reflected skin reactivity even more accurately, but the mathematics were more difficult than when using 1:5 dilutions.¹ Table 1 shows

Table 1. DOSE COMPARISON BETWEEN SKIN TESTING TECHNIQUES*

Prick (Only) by w/v	Antigen Dose Injected Per Test (μ g)				
	Single Intradermal (with initial prick)		Skin End Point Titration (without prick)		
	by PNU	by w/v	1:10	1:5	1:3
Maximum test dose (for detecting low sensitivity)					
—	—	—	—	100 (#1)	62 (#4)
—	40 (1000)	40 (1:500)	—	—	—
—	—	20 (1:1000)	20 (#1)	20 (#2)	21 (#5)
Intermediate test doses (for detecting moderate sensitivity)					
—	4.0 (100)	4.0 (1:5000)	—	4.0 (#3)	6.8 (#6)
—	—	—	2.0 (#2)	—	2.3 (#7)
—	—	—	—	0.80 (#4)	0.76 (#8)
0.30 (1:10)	0.30 (1:10)	0.30 (1:10)	0.20 (#3)	0.16 (#5)	0.25 (#9)
Initial test doses (for detecting high sensitivity)					
0.06 (1:50)	0.06 (1:50)	0.06 (1:50)	—	—	0.08 (#10)
—	—	—	—	0.03 (#6)	0.03 (#11)
—	—	—	—	—	0.01 (#12)
—	—	—	—	—	0.003 (#13)
—	—	—	—	—	0.001 (#14)
—	—	—	—	—	0.0003 (#15)

*Antigen concentrations or dilutions are in parentheses.

Adapted from Gordon BR: Allergy skin tests for inhalants and foods: Comparison of methods in common use. *Otolaryngol Clin North Am* 31:35-53, 1998; with permission.

the equivalent concentrations of extract used in the currently available skin testing methodologies.

Rinkel emphasized the importance of recognizing the various types of skin responses and knowledge of factors that modify these responses when using serial dilution testing to determine the degree of sensitivity in inhalant allergies. Although these techniques of testing are commonly used today, the final treatment dose determined by these calculations (low dose immunotherapy, also known as the 'Rinkel technique,') is no longer recommended or practiced.

The current terminology, SET or serial dilution titration (SDT), is preferred to avoid confusion with outdated Rinkel treatment techniques.^{13, 23} Other titration techniques also currently are in use (at a 1:3 dilution) by the Food and Drug Administration (FDA) for standardization of allergen extracts (see Table 1).

TECHNIQUE OF SET TESTING

Preparation of the Testing Board

Pollen antigens selected for testing are purchased at 1:20 weight/volume (w/v) concentration or are diluted to this strength. Inhalant allergens, food allergens, and special mixtures are purchased at 1:10 w/v. Using a standard starting concentration results in uniformity of subsequent dilutions, which results in standardization of patient response to diluted concentration of the same antigen.

Antigens to be tested are placed on specially designed boards. A variety of testing boards is available. Boards that accommodate 5-mL vials are the most widely used.¹¹ The ideal board accommodates all testing antigens. To avoid inadvertent injection, concentrates (e.g., 1:20 w/v antigens) should never be placed on the testing board.¹¹

The 5-mL vials of antigen are labeled and arranged on the testing board. Each vial at this stage contains only 4 mL of diluent. To begin, six vials are labeled with the name of the allergen, the dilution numbers 1 through 6, and the date.^{11, 16, 23} This process is repeated for all antigens on the testing board. To make vial #1, 1 mL of concentrate is added to 4 mL of diluent into a 5-mL vial. (Note: for antigens in a 1:10 w/v concentrate, only 0.5 mL of concentrate is used in 4 mL diluent.) The vial's volume is 5 mL at a concentration of 1:100. One milliliter is withdrawn from vial #1 and added to 4 mL of diluent in vial #2 to give a concentration of 1:500 in 5 mL of Timothy grass extract. This process is repeated until vial #6 is made up. The shelf life of the test battery is approximately 2 months.¹⁶

The whole process is repeated until six serial dilutions of each antigen to be tested are made up. Table 2 summarizes this technique.

This time-consuming procedure requires intense concentration and careful measurements. This testing board should always be prepared in the absence of patients and unexpected interruptions.⁶

Table 2. SKIN ENDPOINT TITRATION*

	1:20 Concentration	1:10 Concentration	Resultant Ratio
Dilution #1	4 mL diluent 1 mL allergen concentration	4 mL diluent 0.5 mL allergen concentration	
Total	5 mL	4.5 mL	1:100
From this point on mix both concentrations identically			
Dilution #2	4 mL diluent <u>+ 1 mL from Dilution #1</u>		
Total	5 mL		1:500
Dilution #3	4 mL diluent <u>+ 1 mL from Dilution #2</u>		
Total	5 mL		1:2500
Dilution #4	4 mL diluent <u>+ 1 mL from Dilution #3</u>		
Total	5 mL		1:12,500
Dilution #5	4 mL diluent <u>+ 1 mL from Dilution #4</u>		
Total	5 mL		1:62,500

*1:20 concentrates are used for pollen allergen; 1:10 concentrates are used for inhalant, food, and special mixture antigens.

Establishment of Controls

Positive and negative controls need to be established before SET testing can begin. Histamine serves as the positive control¹² which is prepared by withdrawing and discarding 1 mL of sterile diluent from a 4-mL vial. Two mL of histamine phosphate (0.0275 mg/mL) is then added to the remaining 3 mL. One mL of this solution is added to 4 mL of diluent to create a #3 dilution (1:2500) of the histamine control.¹² An intradermal injection of a #3 dilution of histamine that forms a wheal of 5 mm should result in a wheal of at least 7 mm in diameter within 10 minutes. This response confirms the ability of the skin to react to susceptible antigens with a wheal and flare response.

The negative control is glycerin. Glycerin is used as the preservative in most commercial preparations of allergen concentrates.¹² In an undiluted form, glycerin is provided in a 50% concentration. A concentration this strong is highly reactive with the skin and needs to be diluted. Multiple test doses may need to be given to compare the skin response for the potential concentration of glycerin administered during the injection of the serial dilutions used in testing. The glycerin is diluted to the 1:5 ratio system in the same manner as the 1:20 allergen concentrates (Table 3) to provide the test doses. The #5 dilution is a 0.4% glycerin, and the #1 dilution is a 10% dilution. A control dose of glycerin should be administered that is equivalent to the strongest diluent administered to the patient for the allergy testing. A negative control is a 4-mm wheal of diluent and should not enlarge to more than 5 or 6 mm after 10

Table 3. MIXING THE POSITIVE CONTROL—HISTAMINE

Step	Preparation	Resultant Ratio
1	4-mL diluent vial - 1 mL diluent 3-mL diluent vial	
2	3-mL diluent vial + 2 mL 0.275-mg/mL histamine 5 mL #2 histamine dilution	1:500
3	1 mL of #2 histamine dilution + 4-mL diluent vial 5 mL #3 histamine dilution	1:2500

minutes.¹⁶ A positive skin response to a tested antigen should be compared with the glycerin control. To be a truly positive allergen test, the wheal needs to be at least 2 mm greater in diameter than its equivalent glycerin control.

Testing Procedure

The testing procedure for SET testing is outlined as follows:

1. Control solutions are applied and the skin response is interpreted as described previously (Fig. 1).
2. The upper arms are cleaned with alcohol and dried with clean gauze.
3. Test doses are applied to the outer surface of the arm.
4. A skin-marking pen is used to label the antigens to be tested. The six different dilutions are marked as shown in Fig. 1. Each antigen should be separated from the other antigens by at least 2.0 cm to prevent overlapping of skin responses or an axonal reflex stemming from an adjacent positive reaction.¹¹
5. A 27-gauge, 1/2-inch beveled needle on a 1-mL disposable syringe is used for testing.
6. Approximately 0.05 mL of the first test antigen is removed from the vial into the test syringe.
7. The patient's forearm is then grasped from behind with the left hand (for a right-handed operator), and the skin drawn tight. A #6 dilution of the first antigen is injected intradermally with the bevel of the needle facing upward to give a 4-mm wheal. A true wheal confirms intradermal placement of the needle and ensures consistent results. A 4-mm wheal contains close to 0.01 mL of antigen solution.¹¹
8. The #6 dilution of each antigen is administered in a vertical row as quickly as possible without compromising accuracy.
9. A timer is set for 10 minutes after each set of test dilutions is placed.¹¹

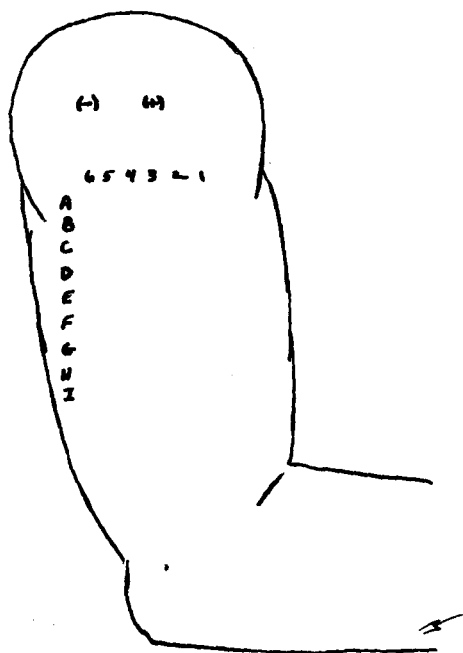


Figure 1. Sample appearance of the layout of injections in an arm undergoing serial endpoint titration. Numbers = antigen dilution; Letters = different antigens; + = positive control; - = negative control.

10. The skin response is measured after 10 minutes. A 4-mm wheal is expected to expand to 5 mm within this time because of hydrostatic pressures. The injection site should lose some firmness and definition. A positive wheal is a site that is 2 mm larger in diameter than the previous site.^{11, 13, 23} The glycerin control is important, as the allergen's response is only considered positive when it is 2 mm greater in diameter than the control.
11. If there is no skin response to the #6 dilutions (i.e., there is no progression of wheal size), the same procedure is repeated for the #4 dilution. Passing over the #5 solution is considered safe, as a positive #5 solution would necessitate the administration of a #4 solution to confirm the positive response. If the #4 solution was positive, a #5 solution may be administered to confirm the appropriate progression of response. Figure 2 demonstrates the pattern of placement of test wheals.
12. Similarly, if the #4 dilution is negative, the #2 dilution may be applied. This step is repeated until the #1 dilution is reached and the endpoint response is established.

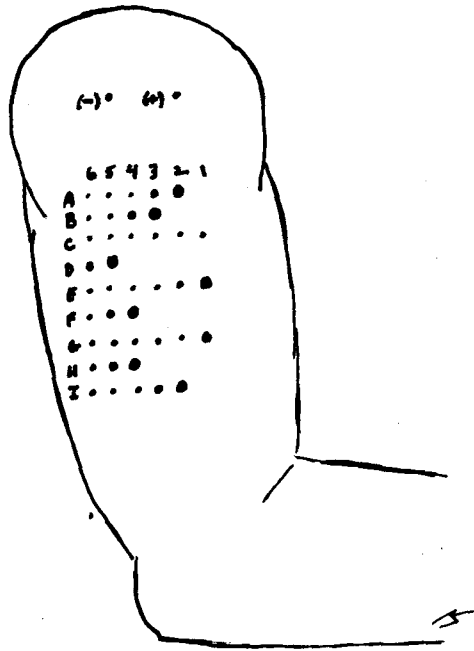


Figure 2. Sample appearance of an arm after the injections for serial endpoint titration are completed. Key: Numbers = antigen dilution; Letters = different antigens; + = positive control; - = negative control.

13. The application of every other dilution shortens testing time and minimizes patient discomfort.
14. If any of these test dilutions result in a positive response, the skipped dilution above or below it must be applied. The goal is to show clear progression in wheal size in intervals of 2 mm over three consecutive dilutions. The sample shown illustrates this principle.

Dilutions	#6	#4	#2
Wheal size (mm)	5	5	9

The next dilution should be #3.

Dilution	#3
Wheal (mm)	7

The endpoint is dilution #3 (i.e., the first positive wheal resulting in a progressive whealing response). Dilution #2 is the confirming wheal.

The next example further describes this technique:

Dilutions	#6	#4
Wheal size (mm)	5	7

The next dilution should be #3.

Dilutions	#3
Wheal size (mm)	9

The endpoint in this example is dilution #4.

Interpretation of Results

A positive skin response to an allergen using SET depends on the establishment of an endpoint, which is the dilution of antigen that results in a wheal at least 2 mm larger than the preceding negative wheal (e.g., 5-mm wheal). The reliability and reproducibility of the whealing response depends largely on injection technique and strict adherence to the technique of SET. Table 4 explains some typical skin responses and endpoints.

The second progressively reacting wheal is the confirming wheal and denotes the completion of testing for that allergen. The first positive wheal does not provide a definitive diagnosis of hypersensitivity and can still represent an unusual skin response. The various patterns of bizarre skin responses are discussed in the next section.

A negative wheal is an initial 4-mm wheal that does not enlarge beyond 5 mm at the end of 10 minutes. Typical SET results are shown in Table 4.

Unusual Skin Responses

The whealing response deviates from the norm in a small number of patients. These variations can be classified into predictable groups, and the endpoint can still be reliably determined once the unusual pattern is recognized.¹¹

The flash response is the most common form of unusual skin response¹¹ that occurs when a series of negative responses is followed by a large positive wheal as shown below:

Dilutions	#6	#5	#4	#3
Wheal size (mm)	5	5	5	15

Table 4. TYPICAL SET RESULTS

Dilution	#6	#5	#4	#3	#2	#1	Endpoint
Wheal size (mm)	4	4	5	7	9		3 (1:2500)
	5	5	7	9			4 (1:12,500)
	5	7	9				5 (1:62,500)

The cause of this phenomenon is not clear, although concomitant food allergies have been implicated. No endpoint can be established with this type of skin response. Testing for that particular antigen is stopped and repeated at a future date. Flash responses rarely recur.¹¹

The second most common pattern of bizarre whealing response is the plateau response as shown:

Dilutions	#6	#5	#4	#3	#2	#1
Wheal size (mm)	5	5	7	7	9	11

The plateau response illustrates the importance of the confirming dilution described previously. The endpoint is the first dilution initiating progressive whealing (i.e., dilution #2). To minimize the possibility of adverse reactions, testing beyond the confirming wheal generally is not performed.

Hourglass responses rarely are seen. This type of skin response occurs when a weak test dilution results in a larger wheal than successive stronger dilutions as shown:

Dilutions	#6	#5	#4	#3	#2	#1
Wheal size (mm)	9	7	5	5	7	9

The true endpoint in this example is #2 dilution. A variant increment whealing response also can occur:

Dilutions	#6	#5	#4	#3
Wheal size (mm)	5	5	8	11

This result means that a positive wheal may be more than 2 mm larger than the preceding wheals. The endpoint in the sample given is dilution #4.

SET AND RAST

The Fadal-Nalebuff modification of the radioallergosorbent test (RAST) scoring system (F/N mRAST) correlates well with SET.¹⁸ Increments of this scoring system are equivalent to the fivefold dilutions used in SET as shown in Table 5.

Because skin tests are slightly more sensitive than RAST in clinical practice, a score one class higher usually is obtained with SET. This result means that a modified RAST score of Class IV will be equivalent to a SET endpoint of #5 dilution. Most other *in vitro* modalities have been designed using F/N mRAST to help maintain the same relationship with SET.⁷ Definite correlation between RAST and the patient's skin response can only be demonstrated using an incremental vial test.^{11, 17} Studies demonstrated good-to-excellent correlation with both skin prick and SET techniques to *in vitro* allergy testing⁴⁻⁷ and to nasal challenges (J. Corey, unpublished data, 1999).

Table 5. A COMPARISON OF ANTIGENIC CONCENTRATIONS BETWEEN STANDARD SET EXTRACTS, STANDARD RAST, AND THE MODIFICATION OF RAST

SET Endpoint	Antigen Concentration	RAST 0	RAST 1
#1	1:100	1:100	1:500
#2	1:500	1:500	1:2500
#3	1:2500	1:2500	1:12,500
#4	1:12,500	1:12,500	1:62,500
#5	1:62,500	1:62,500	1:312,500
#6	1:312,500	1:312,500	1:312,500†

*F/N mRAST; may be true of other RAST and ELISA systems.

†w/v, if concentrate is 1:20.

‡It is rarely necessary to begin treatment at strengths weaker than #6 dilution.

§Aggressive regimen, not recommended for novices, in which vials are prepared using RAST core as endpoint.

||Usual regimen in which endpoint is considered one dilution weaker than RAST score (RAST 1).

Adapted from Mabry RL: Blending skin endpoint titration and *in vitro* methods in clinical practice. Otolaryngol Clin North Am 25:61-70, 1992; with permission.

SET AND IMMUNOTHERAPY

It has been well established that the endpoint wheal by definition contains 0.01 mL of antigenic extract. The confirmatory wheal also contains 0.01 mL of extract but is five times more concentrated. It is equivalent to 0.05 mL of the extract producing the endpoint. Therefore, the patient has received 0.06 mL of the extract during testing and a small amount in the negative wheals.¹⁵ In the absence of an untoward reaction occurring during testing, it is safe to administer 0.05 mL of the endpoint dilution as the starting dose of the immunotherapy.

The concentration of extract in the treatment vial is made up as follows. Because SET is based on successive fivefold dilutions, 50 times of one dilution equals 10 times of the next strongest dilution. All calculations are based on 10 doses of 50 times each for convenience in calculations and simplification of the preparation of treatment SETs.¹² To obtain reduced volume but retain antigen concentration, the following calculations are used. For endpoint dilution #4:

$$1 \times = 0.01 \text{ mL \#4}$$

$$50 \times = 0.05 \text{ mL \#4}$$

$$\times 10 \text{ doses} = 5 \text{ mL \#4}$$

$$\text{or} = 1.0 \text{ mL \#4}$$

$$\text{or} = 0.2 \text{ mL \#2}$$

Multiple allergen vials may be prepared by using these calculations (Table 6).

This calculation results in a treatment set containing all of the antigens required at reactive strengths as measured by SET.¹² Further details of vial preparation are available in most otolaryngologic allergy text books.^{11, 16}

Table 6. CALCULATIONS FOR TREATMENT VIAL PREPARATION

Endpoint	Reduced	Doses	Amount in Vial (mL)
#3 50x	0.02 No. 1	10	0.2
#5 50x	0.02 No. 3	10	+ 0.2
#4 50x	0.02 No. 2	10	+ 0.2
#6 50x	0.02 No. 1	10	+ 0.2
#3 50x	0.02 No. 1	10	+ 0.2
	Sum of all allergens		1.0
	Diluent		+ 1.5
	Total volume in treatment vial		2.5

Safety

Skin testing and treatment using SET techniques has been shown to be safe with a complication rate of 0.005%.¹⁰ The customization of skin testing extract vials for treatment is thought to account for the low reaction rate, allowing a safe, rapid escalation to an appropriate treatment dose.²³

Over the years, misinformation regarding SET resulted in confusion over the validity and reproducibility of this form of skin testing. Some allergists have been skeptical about SET-based immunotherapy, citing low nontherapeutic doses as a reason for concern. Low dose immunotherapy is not a part of the SET technique of testing and has not been recommended or practiced widely for many years. Studies that compare testing techniques show each technique has comparable safety, reproducibility, sensitivity, and specificity characteristics.⁴⁻⁷ Advantages of prick testing over SET include speed of testing and ease of teaching the technique. Advantages of SET include a better reproducibility (standardization) and a (semi) quantitative result that can be used to calculate customized starting doses of specific immunotherapy. SET also may be slightly safer.¹⁰ Disadvantages include a longer patient testing time and a greater amount of time needed to learn the technique. The method of titration (1:3) used by the FDA is probably most accurate but too time consuming for routine use.¹¹

Unfortunately, inadequate instructions or misinterpretation of results also may be responsible for inertia on the part of some allergists in accepting this modality of testing. Many otolaryngologists use SET to diagnose and treat allergies routinely.

SUMMARY

SET allows fine-tuned quantification of the skin response in allergy testing. The 1:5 dilution allows easy calculation for test and treatment vial preparation, good reproducibility, and accuracy in scoring and safety. This fivefold system also correlates well with the F/N mRAST scoring system. Interpretation of results is simple and standardized

with skin responses progressing from clearly negative to progressively positive as increasing strengths of the same antigen are applied. The quantification of the patients' level of sensitivity to various antigens allows immunotherapy to be started at the strongest safe dose. It also allows for safe, rapid escalation to doses needed to elicit the desired immune response. This customization of the immunotherapy schedule ensures the shortest duration of therapy, minimal discomfort, rapid relief of symptoms, and maximal safety. As it is currently practiced, SET gives results comparable with skin prick tests and use followed by intradermal tests relative to safety, reproducibility, sensitivity, and specificity.

SET, a method of skin testing used by many otolaryngologists, is a safe, reliable, and reproducible technique when properly used by well-trained, well-informed, and experienced practitioners.

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