PerkinElmer™ LAS, Inc.

ELAST® ELISA
Amplification System

Catalog Number
NEP116001EA
NEP116E001EA

For Laboratory Use
CAUTION: A research chemical for research purposes

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INTRODUCTION

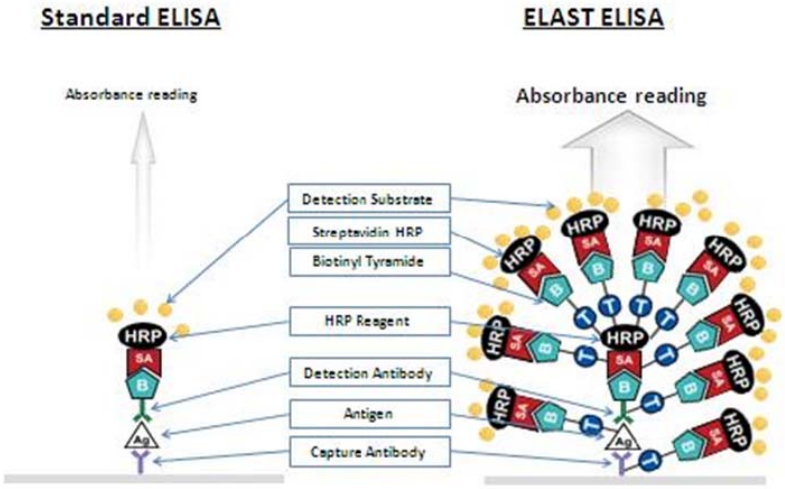
ThePerkinElmer Life Sciences, Inc. ELAST® (ELISA Amplification system) is designed to amplify the signal generated by the enzyme horseradish peroxidase (HRP) when applied to solid phase analytical methods such as the ELISA. The final enhanced signal is primarily due to the catalytic activity of additional HRP deposited on the solid phase

PRINCIPLE OF THE PROCEDURE

PerkinElmer Life Sciences ELAST is based on the catalyzed reporter deposition technology described by Bobrow et al. (1,2). When H_2O_2 and biotinyl-tyramide (a biotin-phenolic compound) are added to an assay system containing immobilized HRP, the enzyme catalyzes the activation of the phenolic group resulting in covalent binding to electron rich moieties on the solid phase. Subsequent reaction with streptavidin-HRP results in the binding of additional HRP to the now biotinylated solid phase. Restated, the ELISA reporter enzyme, HRP, catalyzes the covalent binding of biotinyl-tyramide to the solid phase of the assay vessel. Subsequent reaction with Streptavidin-HRP causes additional HRP to be bound to the solid phase resulting in signal amplification.

In practice, the ELAST procedure is inserted into the user's protocol after the HRP reporter incubation (and washing) and before the addition of the chromogenic substrate.

Figure 1



Other than the optimization suggestions made in the Procedures section below, under many circumstances no changes to the ELISA assay format currently in use need be made as long as appropriate blocking agents are used for the solid phase. The researcher can use any number of methods for HRP reporter addition. For example, an anti-analyte-HRP conjugate or a secondary reporter such as an anti-rabbit or anti-mouse IgG-HRP conjugate can be used. The procedure will also work with biotinylated antibody/streptavidin-HRP systems or hapten/anti-hapten-HRP formats.

REAGENTS IN THE ELAST KIT (NEP116001EA)

BIOTINYL-TYRAMIDE SOLUTION (FP317) -One (1) bottle containing 2 ml of biotinyl- tyramide in ethanol**.**

AMPLIFICATION DILUENT CONCENTRATE (2X) (FP485) -One (1) bottle containing 70 m1 of a borate buffer with hydrogen peroxide.

STREPTAVIDIN-HRP CONCENTRATE (FP105) -One (1) vial containing 0.22 ml of streptavidin-horseradish peroxidase in citrate buffer with added protein and preservative.

Note: The ELAST evaluation size kit (NEP116E001EA) contains the same reagents in smaller amounts to process 1 96-well microtiter plate.

STORAGE RECOMMENDATIONS

All kit components should be kept refrigerated at 2°- 8°C.

SAFETY

OSHA WARNING

ETHYL ALCOHOL
CAUTION
MAY CAUSE RESPIRATORY TRACT, SKIN, AND EYE IRRITATION.
CENTRAL NERVOUS SYSTEM DEPRESSANT.
HIGHLY FLAMMABLE
Vapors are heavier than air and may travel to a source of ignition and

PROP 65 WARNING

This product contains a chemical known to the state of California to cause Reproductive Toxicity.

For Best Results

Read the entire manual before attempting to use the kit.

Blocking: Proper blocking, of the solid phase is critical to the efficiency of amplification. Use only the recommended blocking reagent. The use of bovine serum albumin (BSA) as the protein blocker is preferred. A suggested formulation is listed in the Reagent Formulations section.

Prepare all working dilutions within 30 minutes of use. Prepare only enough for the assay being run. Discard any excess in appropriate containers.

Use only the reagent lots assigned to the kit. Do not interchange vials or bottle caps.

Do not use the kit beyond the expiration date on the kit label.

This kit has been formulated specifically for use in ELISA formats. Use for other applications may not produce the desired amplified signal.

PROCEDURES

GENERAL INFORMATION

Before proceeding to use the ELAST kit, it is important to optimize the unamplified assay to achieve the lowest background possible (preferably 0.01 OD units, or lower). This can be achieved by decreasing detector reagent concentrations or incubation times.

While the most important parameter affecting the extent of amplification with the ELAST kit is the streptavidin-HRP dilution, it is essential to understand the direct relationship of the streptavidin-HRP dilution to the biotinyl-tyramide concentration. For full assay optimization, both steps should be cross matched. To help users, a section of Examples demonstrating how the various components and/ or conditions affect the resultant assay sensitivity is included in this manual.

Modulation of the extent of amplification can also be achieved by changing the concentration of the HRP reporter or other members of the reporter system in the unamplified assay (e.g., unlabeled anti-analyte antibody). This may be desirable in order to save critical assay reagents.

The protocol below is written for use with 96 well microplates. If other vessels are utilized, adjust volumes as necessary .

ELISA AMPLIFICATION STEPS

I REAGENT PREPARATION

The ELISA is performed as usual through the HRP reporter incubation step and subsequent washing. DO NOT ADD SUBSTRATE. A suitable wash buffer and antibody diluent are described in the "Reagent Formulations" section.

A. Prepare the biotinyl-tyramide (B-T) Working Solution:

Dilute an appropriate amount of the Amplification Diluent Concentrate 1: 1 with deionized H20. (As 100 µl per well will be needed, 10 m1 of final dilution will be adequate for an entire 96 well plate).

Add 10 µl of biotinyl-tyramide solution per ml of the diluted Amplification Diluent from step Al. (For one 96 well plate use 100 µl of B-T to 10 ml of diluent.)

Initially, amplify the assay by using this fixed dilution of the biotinyl-tyramide solution. This concentration will be adequate for most applications and is recommended as a starting point. More or less may be used to modulate the extent of amplification.

II AMPLIFICATION

A. Add 100 µl of the B-T Working Solution to each well of the microplate and incubate for 15 minutes at room temperature.

B. Wash the wells at least 4x with PBS-T[1]

C. Dilute the streptavidin-HRP 1:500 (2 µl /ml) in 1% BSA-PBS-T[1] (For one 96 well plate, use 20 µl to 10 ml of diluent)

[1]Described in the "Reagent Formulations" section

This 1:500 dilution is recommended as a starting point. The extent of amplification can be modulated by varying the Streptavidin-HRP concentration (see example 1)

D. Add 100 µl to each well of the microplate and incubate for 15 to 30 minutes at room temperature.

E. Wash the wells at least 4x with PBS-T.

F. Add the customary ELISA substrate and complete the assay "as usual".

Examples

1. Amplification of an HIV -1 p24 ELISA

Determining: optimal Streptavidin-HRP dilution

ThePerkinElmer HIV-l p24 Core Profile ELISA (NEKO60) was run as described in the kit manual. (**Note**: the NEK060 kit has been replaced with NEK050001KT). This is a typical sandwich ELISA where the analyte (HIV-l p24) is captured by an immobilized monoclonal antibody and detected with a biotinylated polyclonal/ streptavidin- HRP reporter system.

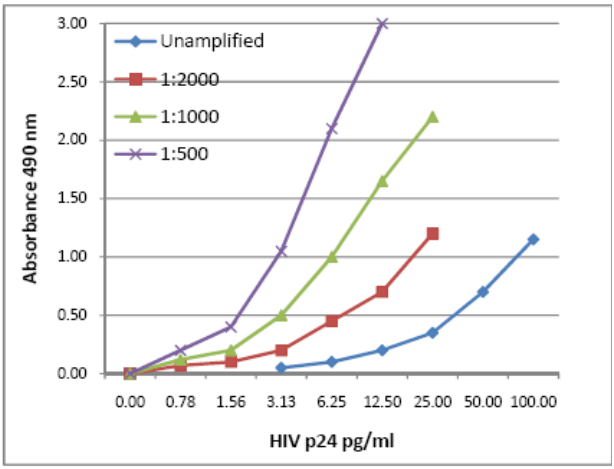
The standard curve range (normally 0 to 100 pg/ml) was changed to 0 to 25 pg/ml for the amplified assay. Briefly, antigen was captured for 2 h at 37°C; biotinylated detector antibody was incubated for 1 h at 37°C; streptavidin-HRP (diluted as indicated in the kit manual) was incubated for 15 min at 37°C. All washes were performed as recommended in the kit. Following the HIV-l p24 ELISA kit streptavidin-HRP incubation and washing, optimization using the ELAST amplification reagents was run as follows.

Step 1. The Amplification Diluent Concentrate was diluted 1:1 with deionized H_20, and biotinyl-tyramide was added to 10 µl /m1. 100 µl was added to all wells for amplification and incubated for 15 min. at room temperature. The wells were washed as recommended in the HIV-l p24 ELISA manual (two 5-cycle washes of at least 300 µl per well per wash using automated microplate washer).

Step 2. The ELAST streptavidin-HRP concentrate was diluted 1/500, 1/1000, and 1/2000 in 1% BSA-PBS-T (see the Reagent Formulations section). One dilution was used for each standard curve. 100 µl was added to the wells and incubated for 15 min. at room temperature. The wells were washed as recommended in the HIV-1 p24 ELISA manual.

OPD substrate was added and incubated for 30 min. at room temperature, then stopped with the kit stop solution. The absorbance at 490 nm was determined- Results are shown in Fig. 2.

Figure 2



Conclusions: The 1/500 dilution yielded the highest absolute signal (over 20 times the unamplified assay) as well as net signal (background subtracted). On the other hand, the *1/1000* dilution yields a greater than 10 fold signal increase with the highest signal noise ratio due primarily to the lower background. Therefore, the best improvement in assay sensitivity is achieved with the 1/1000 dilution.

NOTE: Commercial kits are formulated and optimized to give a fixed signal for each level of analyte concentration in an unamplified assay. Use of a different lot of the same commercial kit or of the ELAST amplification kit may require some reoptimization.

2. Amplification of an Interleukin-2 ELISA

Varying Biotinyl-Tyramide dilution

The PerkinElmer Interleukin-2 (IL-2) ELISA (NEK057) (No longer available) was run as described in the kit manual, with one change; the IL-2 kit Streptavidin-HRP was used at a 1/100 dilution as opposed to a 1/50 as recommended in the kit. This IL-2 assay is a sandwich ELISA where the analyte is captured by an immobilized polyclonal antibody and detected with a biotinylated polyclonal/streptavidin-HRP reporter system. The IL-2 ELISA NEK057 is optimized to give maximum linear range at the expense of assay sensitivity (in contrast to the HIV-1 p24 ELISA which is designed to maximize sensitivity while maintaining minimum assay time)

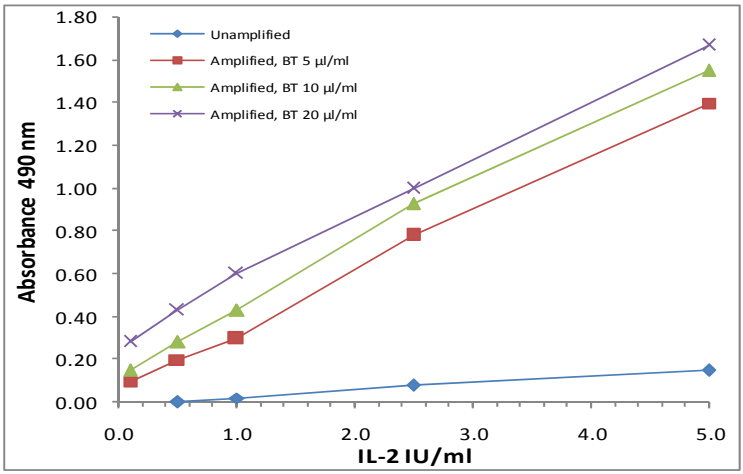
The standard curve range (normally 0 to 100 IU/ml) was changed to 0 to 5 IU/ml for the amplified assay. Briefly, antigen was captured overnight at 4°C; biotinylated detector was incubated for 2 h at 37° C; streptavidin-HRP (diluted 1/100 instead of the recommended 1/50) was incubated for 2 h at 37°C. All washes were performed manually. Following the IL-2 ELISA kit streptavidin-HRP incubation and washing, optimization using the ELAST amplification reagents was run as follows.

Step 1. The Amplification Diluent Concentrate was diluted 1: 1 with deionized H_20, and biotinyl-tyramide was added to 5 µl/m1, 10 µl /m1, and 20 µl /m1. 100 µl of the diluted material was added to all wells for amplification followed by incubation for 15 minutes at room temperature. The wells were washed four times.

Step 2. The ELAST Streptavidin-HRP concentrate was diluted 1/1000 in 1% BSA-PBS-T (see the Reagent Formulations section). 100 µl was added to the wells and incubated for 15 min. at room temperature. The wells were washed four times.

OPD substrate was added and incubated for 30 min. at room temperature, and stopped with kit stop solution. The absorbance at 490 nm was determined. Results are shown in Fig. 3.

Figure 3



Conclusions: From Fig. 3, it is clear that the optimal BT concentration would be 5 µl /ml. Using higher concentrations does not improve assay sensitivity based on net signal and signal/noise ratio. The amplified assay reflects the type of optimization used in this assay (i.e., range vs. sensitivity) by also giving a relatively shallow response (higher blanks and lower signals) which is shifted approximately 10 fold lower in concentration from the unamplified assay.

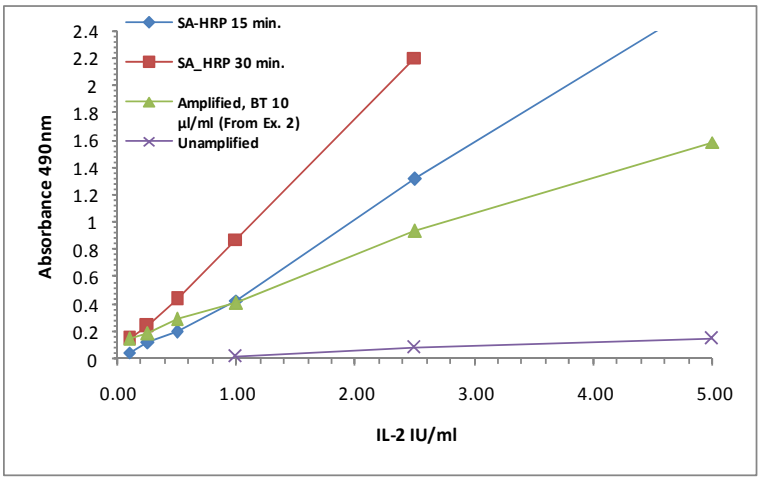
3. Improvements to the Amplified IL-2 ELISA

Modifying ELISA Conditions for Optimal Amplification

The IL-2 ELISA described in Example 2 yielded a very shallow response due to the type of optimization of the original assay. It was found that changing three parameters (concentration, time and diluent composition) of the IL-2 SA-HRP incubation step improved the amplified assay. The IL-2-SA-HRP and diluent were replaced with a more concentrated SA-HRP diluted in 1% BSA-PBS-T. The SA-HRP incubation was for 15 min. at 37°C (as compared to 2 hr. at 37°C in the IL-2 kit). All other IL-2 assay steps remained the same. The assay was amplified using biotinyl-tyramide at 10 µl/ml and (ELAST) SA-HRP at a 1/500 dilution for 15 and 30 min. at room temperature.

The results are shown in Fig. 4. Included, for comparison, is the amplified assay from Example 2 where biotinyl-tyramide was used at 10 µl/m1.

Figure 4



Conclusion: The increased slope of the response curve clearly shows improved sensitivity of the modified assay. If higher absorbances are desired, the ELAST SA-HRP can be incubated for 30 min. instead of 15 min.

4. Amplification of a Salmonella ELISA

A sandwich ELISA for the detection of Salmonella antigen was developed utilizing a goat anti-Salmonella capture antibody and a peroxidase labeled goat anti-Salmonella detector antibody. Affinity purified unlabeled and peroxidase labeled anti-Salmonella antibodies, and Salmonella typhimurium positive control were purchased from Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD.

Optimization of the unamplified assay to maximize signal-to-noise ratio resulted in the following assay parameters:

A. Microplates were coated with l00 µl unlabeled capture antibody at 2.5 mg/ml in 0.1M carbonate buffer overnight at 4°C, blocked with 200 µl 2% BSA-PBS, and stored at 4°C until used.

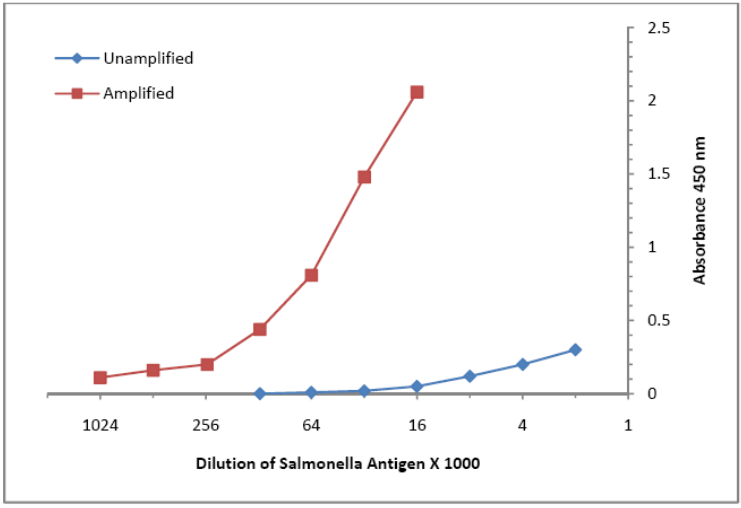
B. Salmonella positive control was diluted in 1% BSA-PBS-T and 100 µl was added to the wells for 2 hr. at room temperature.

C. Peroxidase labeled goat anti-Salmonella antibody was diluted 1/500 in PBS-T containing 40% goat serum and 100 µl was added to the wells for 1hr. at room temperature.

D. A tetramethylbenzidine (TMB) chromogen was incubated for 7 minutes at room temperature, stopped with 3N H_2S0_4/ 1N HCl, and the absorbance at 450nm determined.

For amplification, the ELAST reagent incubations were inserted between steps 3 and 4. Biotinyl-tyramide was diluted to 10 µl/mL and incubated for 15 min. at room temperature. Streptavidin-HRP was diluted 1/500 in 1% BSA-PBS-T and incubated for 30 min. at room temperature. The results are shown in Fig.5.

Figure 5



TROUBLESHOOTING GUIDE

While we feel that a careful reading of Examples 1-4 will give the ELAST user a good grounding in the optimization of assays to achieve high sensitivity , in Table 2 we have summarized the potential problems and listed possible solutions.

Table 2

Observation	Things to try
High Background	Determine the optimal ELAST streptavidin-HRP/biotinyl-tyramide concentrations to give lower backgrounds. *Rework the original assay to drop NSB by identifying specific reagents at fault. Revise diluent, concentration and/ or incubation conditions. *Insufficient washing
Inadequate Amplification	*Was the appropriate blocking reagent used? VERY CRITICAL! *Check the unamplified assay. Did it work right? *Review the relationship between concentrations of BT and SA HRP (Examples 1 and 2) *Were the critical reagents added (BT; SA-HRP) ?
Shallow Response Curve	*Increase concentration of SA-HRP (Examples 1 and 3) *Increase time of SA-HRP incubation (Example 3) *Rework the original assay detection system
Variable Results	*Check the precision of the original assay. As the amplification system: *Requires extra steps *Increases signal in the area of st inherent precision in the original assay (ultra-low levels) ,some increase in CVs are to be expected but this should not be excessive (See Reference 1 in Manual for typical expectations) . *Check the incubation time for the BT and the streptavidin- HRP steps. We have found that a 30 minute incubation of streptavidin-HRP often gives better precision than a 15 minute incubation * May be due to pipeting errors

Phosphate Buffered Saline Reagent Formulations

10X PBS serves as a "base" for many of the buffer formulations used. Two formulations have been used successfully.

Formulation A:
For 1 liter:

NaH_2PO_4 H20	2.03 g (Fisher Cat. No.8369 or equivalent)
Na_2HPO_4	11.49 g (Fisher Cat. No.8374 or equivalent)
NaCl	85 g (Fisher Cat. No.8271 or equivalent)

The pH of the10X solution is 6.7 to 6.9. The pH of the 1X solution should be 7.3 to 7.5 (if not, adjust the 1X) .Storage: Room Temperature

Formulation B:
For 1 liter:

Na_2HPO_4 $7H_20$	21.6 g (Fisher Cat. No.8373 or equivalent)
KH_2 PO_4	2 g (Fisher Cat. No. P285 or equivalent)
NaCl	80 g (Fisher Cat. No.8271 or equivalent)
KCI	2 g (Fisher Cat. No. P217 or equivalent)

The pH of the 10X solution is 6.7 to 6.9. The pH of the 1X solution should be 7.0 to 7.2 (if not, adjust the 1X). Storage: Room Temperature

Alternatively, Dulbecco's Phosphate Buffered Saline without calcium chloride or magnesium chloride (available from commercial sources) may be used.

PBS-Tween 20. 10X (10X PBS-T)

For 1 liter:

10X PBS	995 ml
Tween 20	5 ml

A preservative (1 g/liter) may be added to prolong the life of the reagent. Do not use sodium azide. Storage: Room Temperature

Streptavidin-Enzyme/General Assay Wash Buffer (PBS-T)

For 1 liter:

10X PBS-T	10 ml
H_2O	900 ml

Storage: Room Temperature

ELISA Blocking Reagent (2% BSA-PBS)

For 1 liter:

10X PBS	100 ml
H_2O	800 ml
BSA*	20 g

Adjust the pH to 7.4, add H_20 to 1 liter, and filter through a 0.22 µm filter unit. Storage: 4°C

ELISA Streptavidin-HRP Diluent. 1% BSA-PBS-T

For 1 liter:

10X PBS-T	100 m1
H_2O	800 m1
BSA	10 g

Adjust the pH to 7.4, add H_20 to 1 liter, and filter through a 0.22 µm filter unit. Storage: 4°C

* Bovine Serum Albumin -Sigma A7888 or equivalent.

REFERENCES

1. Bobrow, M.N., Harris, T.D., Shaughnessy, K.J. and Litt, G.J. (1989) Catalyzed reporter deposition, a novel method of signal. amplification. Application to immunoassays. *J. Immunol. Methods* 125,279-285.

2. Bobrow, M.N., Shaughnessy, K.J. and Litt, G.J. (1991). Catalyzed reporter deposition, a novel method of signal amplification. II. Application to membrane immunoassays. *J. Immunol Methods* 137,103-112.

3. Helle, M., Boeije, L., de Groot, E., de Vos, A. and Aarden, L. (1991) Sensitive ELISA for interleukin 6. Detection of IL-6 in biological fluids: synovial fluids and sera. *J. lmmunol Methods* 138,47-56.

LICENSING

RELATED PRODUCTS

HRP Conjugates

Anti-rabbit IgG (goat) HRP	NEF812001EA
Anti-mouse IgG (goat) HRP	NEF822001EA
Anti-human IgG (goat)* HRP	NEF802001EA
Streptavidin HRP	NEL750001EA
Anti-DNP-HRP	FP1128
Antifluorescein-HRP	NEF710001EA

Biotin Conjugates

Anti-rabbit IgG (goat) biotin	NEF813001EA
Anti-mouse IgG (goat) biotin	NEF823001EA
Anti-human IgG (goat) biotin	NEF803001EA

Labeled Streptavidin

Streptavidin Fluorescein	NEL720001EA
Streptavidin Texas Red®	NEL721001EA
Streptavidin Coumarin	NEL722001EA
Streptavidin-HRP	NEL750001EA
Streptavidin-AP	NEL751001EA

TSA Kits for Immunohistochemistry and In Situ Hybridization

TSA Fluorescein System	NEL701A001KT
TSA TMR System	NEL702001001KT
TSA Coumarin System	NEL703001KT
TSA Cyanine 3 System	NEL704A001KT
TSA Biotin System	NEL700A001KT

TSA Plus Kits for Immunohistochemistry and In Situ Hybridization

TSA Plus Fluorescein System	NEL741001KT
TSA Plus TMR System	NEL742001KT
TSA Plus Cyanine 3 System	NEL744001KT
TSA Plus Cyanine 5 System*	NEL745001KT
TSA Plus DNP (AP) System	NEL746B001KT
TSA Plus DNP (HRP) System	NEL747B001KT

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Instruments for ELISA

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