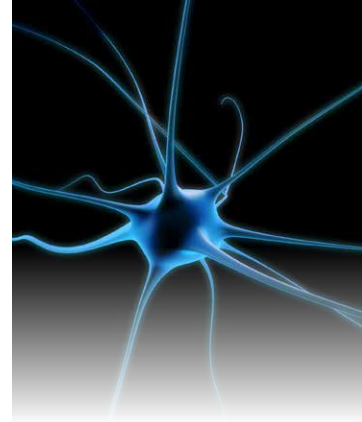
Antec Scientific

Application Note
Neuroscience



ALEXYS Analyzer for Highest Sensitivity in Neurotransmitter Analysis

**Monoamines and Metabolites**
Noradrenaline
Dopamine
Serotonin
5-hydroxyindole acetic-acid (5-HIAA)
3,4-dihydroxyphenylacetic-acid (DOPAC)
homovanillic acid (HVA)

**OPA derivatized amines and amino acids**
GABA and Glutamate
Histamine (LNAAs)
4-aminobutyrate (GABA)
Glutamate (Glu)
LNAAs

**Choline and Acetylcholine**
Choline (Ch)
Acetylcholine (ACh)

**Markers for oxidative stress**
3-nitro-L-Tyrosine
8-OH-DPAT

**Glutathione and other thiols**

# ALEXYS Neurotransmitter Analyzer

- **UHPLC, fast and sensitive**
- **Modular, covering most relevant neurotransmitters**
- **New methods for ACh, GABA, and Glu**
- **Neurosep column for Monoamines**


## Summary

The ALEXYS Neurotransmitter analyzer is a modular system that can be customized for a specific neurotransmitter, or group of transmitters. The system consists of the OR 110 degasser unit, LC 110S pump(s), the AS 110S autosampler, the DECADE II EC detector and Clarity data acquisition software.

Complementary kits have been developed for common neurotransmitters such as epinephrine, norepinephrine, serotonin and metabolites, GABA and glutamate or acetylcholine and choline.

## Introduction

With the introduction of UHPLC the improved peak efficiency of the sub 2 micron particles has become available. According to the van Deemter equation, these small stationary phase particles not only result in a significant gain in efficiency, but the efficiency does not diminish at increased flow rates or linear velocities. This offers new possibilities to further improve neurotransmitter analyses with respect to separation efficiency, retention time and detection sensitivity.

A new ALEXYS Analyzer has been developed for a number of well-known neurotransmitters and metabolites which uses a UHPLC system with sub-2 µm packed columns to optimize plate numbers, retention times and detection sensitivity. Detection limits down to the lower pmole/L range have been achieved for several neurotransmitters. When OPA derivatization is involved the LOD is around 10 nmole/L (GABA and glutamate, amino acids), in case of enzymatic conversion the LOD is around 0.5 nmole/L (acetylcholine).

**Figure 1:** ALEXYS Neurotransmitters Analyzer.

**UHPLC methods**

UHPLC is characterized by extremely efficient separations in a short period of time and plate numbers of more than 100,000 /m can be obtained using sub 2 um particles. As a consequence, every detail in the flow path has to be optimized as it can become a bottle neck in getting the best possible separation performance. Typically, tubing dimensions need to be smaller than 100 um, and loadability (injection volume) is limited. The peak efficiency or plate number N is described as the square of the retention volume (Vr) devided by the peak dispersion σ.

$$N = [\, V_R \,/\, \sigma \,]^2$$

Peak broadening has been described [1, 2] as the sum of all individual dispersion contributions ($\sigma_{tot}^2$) from injection ($\sigma_{inj}^2$), tubing ($\sigma_{tub}^2$), column ($\sigma_{col}^2$) and detection ($\sigma_{det}^2$).

$$\sigma_{tot}^2 = \sigma_{col}^2 + \sigma_{inj}^2 + \sigma_{tub}^2 + \sigma_{det}^2$$

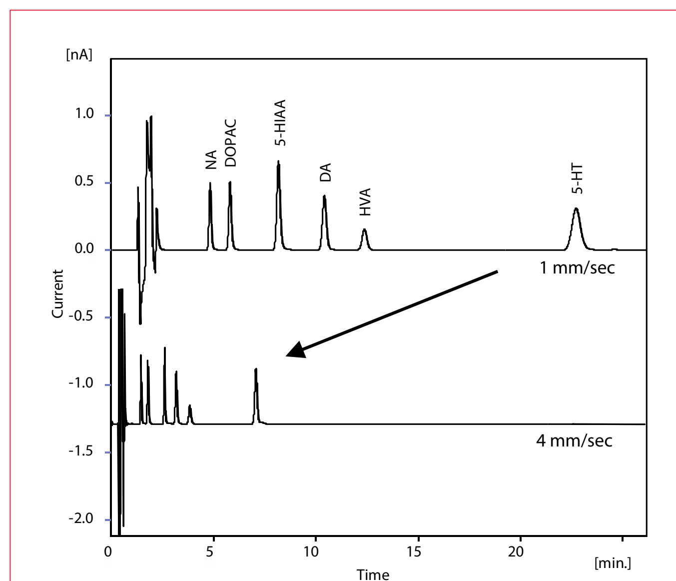



**Figure 2:** Fast HPLC of neurotransmitters with 80,000 -140,000 tp/m.

To fully benefit from the improved column efficiency the others (injection, tubing, detection) must keep up to the same level.

$$\sigma_{tub}^{2} = c\,L\,F\,d^{4}$$

where L is length and d is diameter of tubing, F is flow rate, and the constant c is π / 384 D (D is diffusion coefficient).

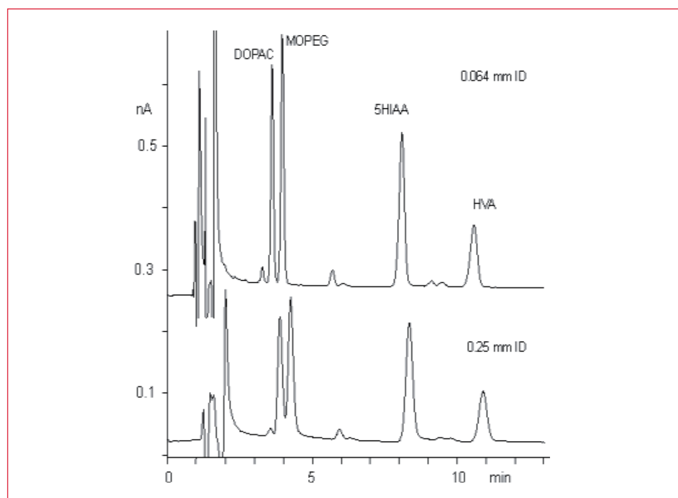



**Figure 3:** Dispersion by tubing. 60 um vs 250 um tubing.

Dispersion by injection is directly proportional to the square of injection volume, and the same holds for detection in relation to detection cell volume.

$$\sigma_{inj}^{2} = a\,V_{inj}^{2}$$
$$\sigma_{det}^{2} = b\,V_{det}^{2}$$
where *a* and *b* are constants.

This has a few practical implications when using UHPLC. Firstly, an increased flow rate in combination with small tubing diameter and a small particle size of stationary phase will contribute considerably to the system pressure. Therefore, the pump, injection valve, all tubing, nuts and ferrules must be rated for high pressure. Where possible the acetonitrile/water mixtures which have a low viscosity are used as mobile phase, instead of methanol/water to minimize the pressure increase. In addition, an elevated temperature (35 – 45 °C) is sometimes applied for the same reason.

Secondly, the fast separation and high plate numbers will have an impact on the signal to noise ratio. The peak height improves with the square root of the plate number, the loadability is limited to a few microliters to maintain the high plate number. Only under stacking conditions larger injection volume can be applied without losing a significant number of plates.

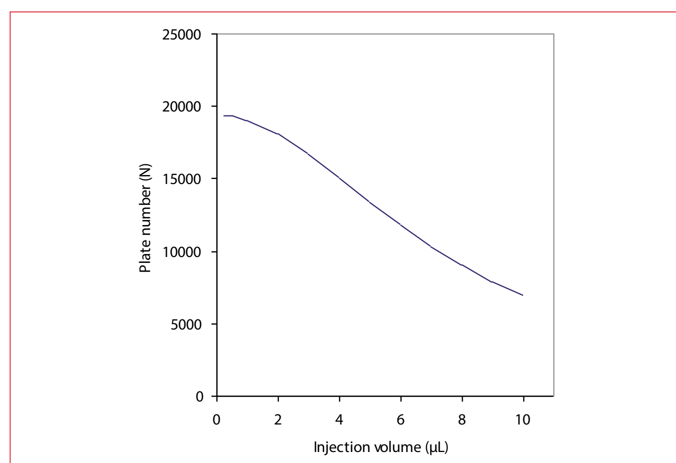
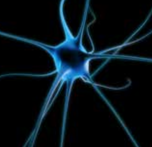



**Figure 4:** Injection volume affects plate count. Plot shows theoretical relationship; for peak eluting at 2 min at a flow rate of 150 uL/min on a column with 20000 plates.



It should be noted that the use of a low pass noise filter is limited. By design, low pass frequency filters will attenuate high frequency signals including narrow peaks. As a rule of thumb the maximum allowed filter setting is related to retention time and inversely related to the square root of N.

$$\tau < c\, t_R/\sqrt{N}$$

where c is 0.32 for max 10% loss in plate number.

For a regular HPLC peak showing 4000 plates at 10 min retention time, the application of a time constant of 3 sec is allowed. In UHPLC a peak with the same plate number but 1.0 min retention time, the maximum time constant is 0.3s. using a stronger filter setting would lead to significant loss of signal. Traditional noise filters will be useless in case of very fast separations. In some cases of very fast peaks a high frequency of data acquisition will be required, which usually increases the noise. This means in some cases a compromise must be found between detection limits, and speed of analysis.

### Sample matrix conditions

Microdialysate samples are 100% aqueous with a high concentration of salts (~150 mM). Collection and storage of samples from microdialysis usually requires precautions to avoid auto oxidation and degradation of substances of interest (e.g. catecholamines are prone to oxidation). Several authors report that their antioxidant mix contain either formic acid, or acetic acid or citric acid in combination with ascorbic acid or another anti-oxidant. This acidic/salty sample matrix has a considerable impact on the system peaks close to the solvent front and elsewhere in the chromatogram. In some cases, the presence of interfering peaks can be manipulated by the octane sulfonic acid (OSA) concentration to the mobile phase. If that approach is not successful other recommendations can be made.

Firstly, to improve detection of peaks close to the solvent front, as a rule of thumb acidified samples should not contain more than 10 mmole/L acid (end concentration).

Secondly, the salt bridge is to be preferred over the ISAAC (In Situ Ag/AgCl) reference electrode when peaks of interest are eluting rapidly. With the salt bridge, the baseline recovers quicker from the solvent front destabilization. Thirdly, the injection volume has an effect, smaller is better.

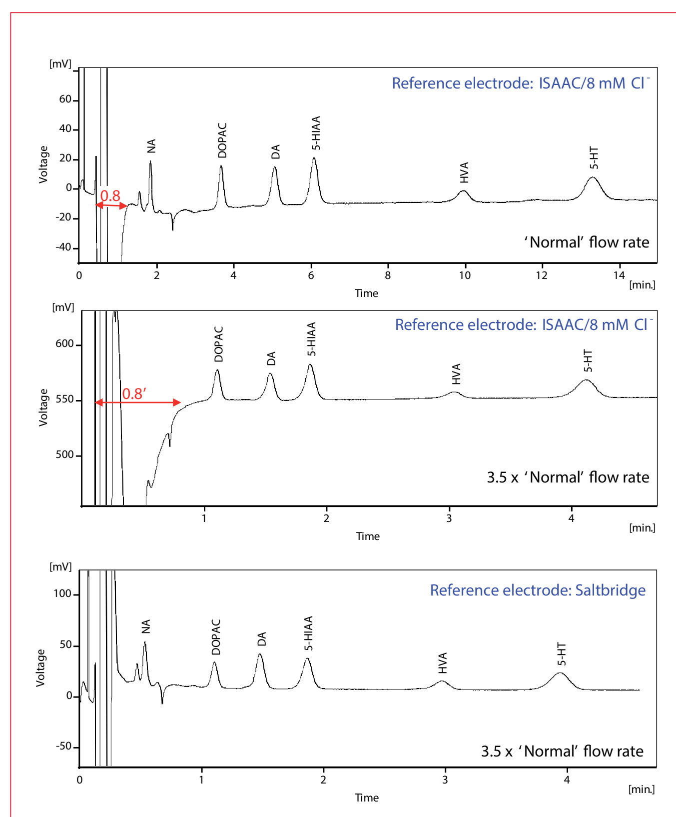



**Figure 5:** Analysis of 10 nmole/L standards in Ringer with 10 mmole/L HAc. In case of ISAAC the NA peak disappears in the front with increased flow rate.

## Optimization of separation conditions

Most neurotransmitters are very polar, and ion pairing chromatography on a (special) C18 column is the separation method of choice in most cases. In addition to the ion pair concentration, the modifier percentage and pH affect separation.

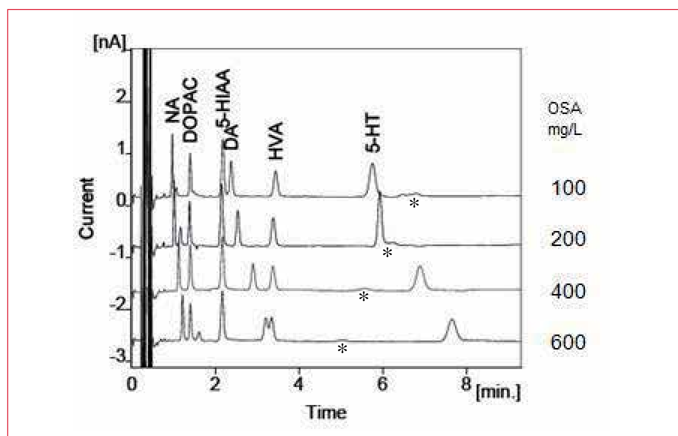



**Figure 6:** Effect of octane sulfonic acid (OSA) concentration in the mobile phase on neurotransmitters and baseline disturbance (*) elution.

Retention of acidic metabolites (e.g. HVA, 5-HIAA, DOPAC) is selectively affected by pH on a C18 column: retention increases with lower pH due to the protonation of the carboxyl group in these components. The retention time of the derivatized amino acids (Glu, Asp, Gly etc) also responds to pH, the response is component dependent. The retention of amines such as dopamine, noradrenaline, serotonin, choline and acetylcholine selectively increases with the ion pair concentration.

Temperature is another parameter that can be modified. Higher temperatures results in faster elution. Separation between peaks can also be affected, as for instance the retention of serotonin is changed more significantly than that of the other components (Fig. 7).

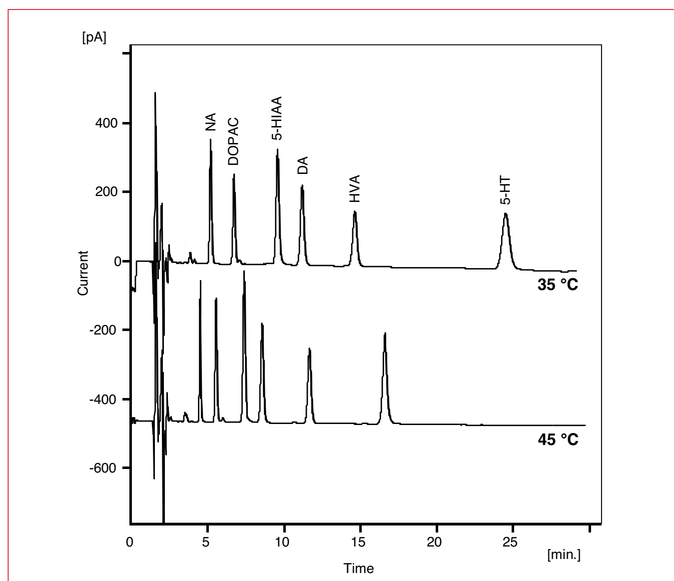
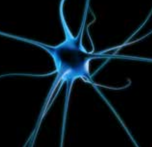



**Figure 7:** Effect of column temperature on separation.

Optimization of separation conditions can be a time consuming part of method development. Antec's application notes already describe the optimized conditions: however, chromatograms of real life samples may show interfering peaks, requiring further optimization. An alternative dual channel approach, that is especially useful for very complex samples has been described elsewhere (Antec application note 213_018).





## Monoamines, NA, DA and 5-HT

For simultaneous analysis of noradrenaline, dopamine and serotonin in microdialysis samples a fast HPLC method has been developed. One of the challenges is to separate NA from the front peak and keep 5-HT within reasonable retention time.

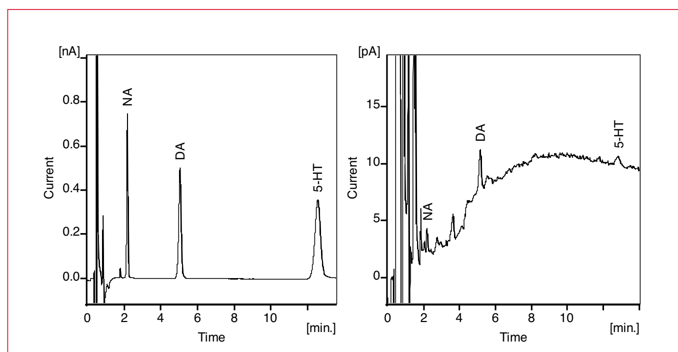



**Figure 8:** On the left analysis of 100 nmole/L standards in acidified Ringer. On the right a real sample: microdialysate of rat brain.

Detection limit of NA, DA and 5-HT is around 100 pmole/L (s/n=3). Linearity shows a correlation coefficient better than 0.999 in the range of 0.1 to 10 nmole/L. The relative standard deviation (RSD) is better than 2% for mid-range concentrations (5 nmole/L).

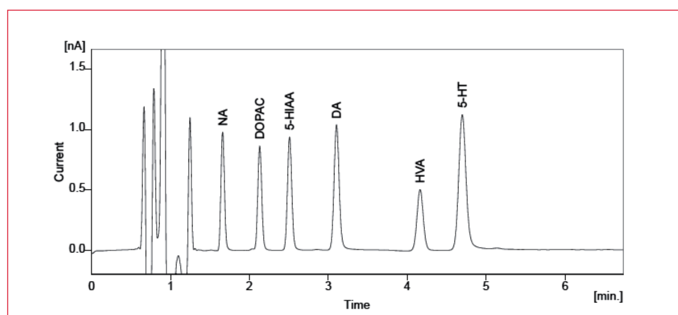



**Figure 9:** Standard mixture of 100 nmole/L neurotransmitters and metabolites.

## GABA and glutamate

The analysis of GABA and glutamate is based on an automated in-needle derivatization routine with OPA reagent, followed by a fast HPLC separation. A short post-analysis step gradient is applied to flush late eluting peaks off the column.

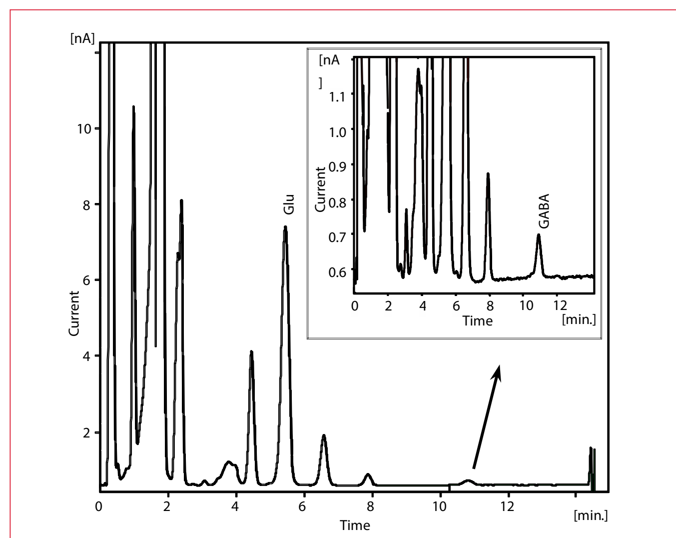



**Figure 10:** Example chromatogram of the analysis of GABA and Glu in pooled rat dialysate from the Nucleus Accumbens.

Detection limit of GABA and Glu is around 10 nmole/L (s/n=3). The linearity of the method was determined in the concentration ranges of 0.5 -2.5 µM and 100 - 500 nM for Glu and GABA respectively. The method showed a good linear detector response with correlation coefficients better than 0.998 for both GABA and Glu.

In principle, all amino acids can be detected with this method. Two other relevant amino acid neurotransmitters are aspartate (Asp) and glycine (Gly), and with some modification to the separation parameters, this method can also be applied to detect those.

## Acetylcholine and choline

For the analysis of acetylcholine, a reversed phase ion-pairing separation method was evaluated. The method has a few interesting features compared to the ion-exchange separation method.

The use of an analytical micro-bore UHPLC C18 column which allows for faster separations and a mobile phase of pH 6.5 is used, which compared to the traditional method at pH 8 improves lifetime of the IMER. Also the working potential is optimized for much better signal-to-noise ratio.

As acetylcholine is not readily detectable, it is first converted to the electrochemically detectable hydrogen peroxide. The enzymatic conversion takes place in a post column AChE/ChOx IMER.

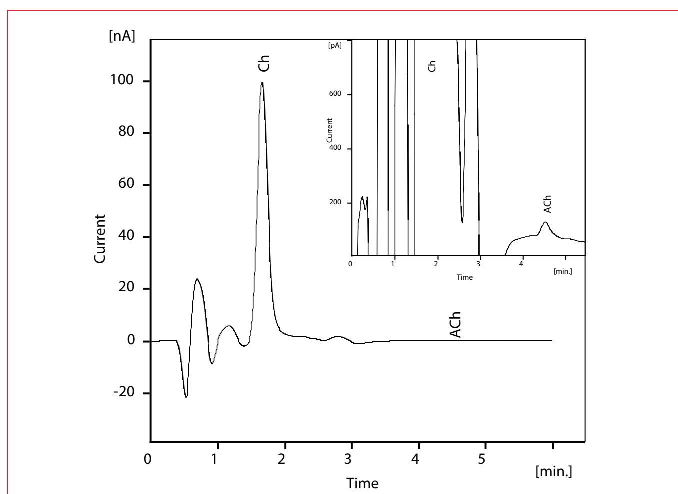



**Figure 11:** Chromatogram of 2 umole/L Choline and 2 nmole/L Acetylcholine in Ringer solution. The inset shows the same chromatogram, but zoomed in on the baseline.

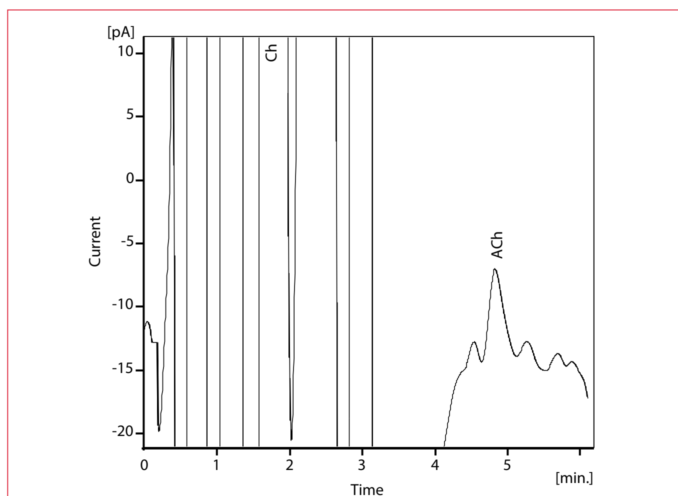
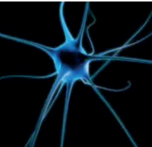



**Figure 12:** Chromatogram of 2 umole/L Choline and 2 nmole/L Acetylcholine in Ringer solution. The inset shows the same chromatogram, but zoomed in on the baseline.

For detection, a glassy carbon working electrode must be coated with peroxidase, and after a drying period of about 4 hours the electrode is ready for use. Detection limit of Acetylcholine is around 0.5 nmole/L (s/n=3). Linearity shows a correlation coefficient better than 0.998 in the tested range of 0.5-20 nmole/L. The relative standard deviation (RSD) is better than 4% (tested with 10 nmole/L).

# Conclusion

Fast and sensitive analysis of a number of neurotransmitters has been demonstrated using the ALEXYS Neurotransmitter Analyzer. It is a modular system with complementary kits for the most common neurotransmitters.





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## Ordering Information

| **Monoamines and metabolites** | |
|---|---|
| 180.0091E | ALEXYS Neurotransmitters BP, 1 ch |
| 191.0035UL | AS 110 autosampler UHPLC cool 6p, LAN |
| 180.0502 | ALEXYS Monoamines kit |

| **GABA and glutamate** | |
|---|---|
| 180.0091E | ALEXYS Neurotransmitters BP, 1 ch |
| 191.0035UL | AS 110 autosampler UHPLC cool 6p, LAN |
| 180.0504 | ALEXYS GABA/Glu kit |
| 180.0602 | LC step-gradient upgrade |

| **Acetylcholine and choline** | |
|---|---|
| 180.0091E | ALEXYS Neurotransmitters BP, 1 ch |
| 191.0035UL | AS 110 autosampler UHPLC cool 6p, LAN |
| 180.0505 | ALEXYS Acetylcholine kit |
| 250.3531 | AChE/ChOx IMER 3mm |

*For research purpose only.* The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

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