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Development of Biacore Assays

Direct Binding Assays

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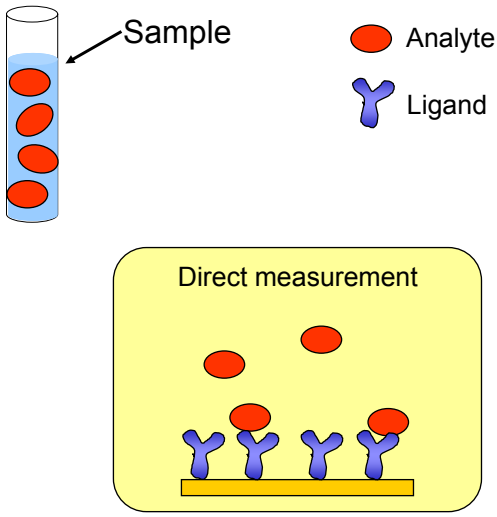

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Direct Binding Assays (continued)

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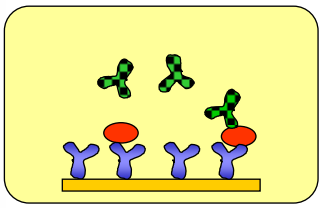
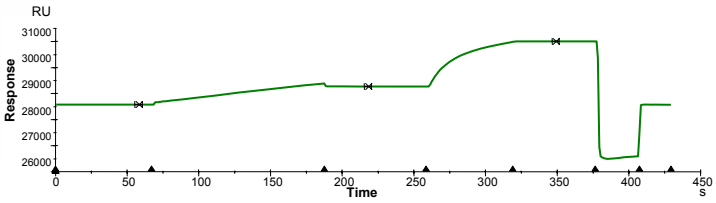
- Direct binding with enhancement (sandwich approach)



Analyte
Ligand
Enhancement molecule



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3

Indirect Assays

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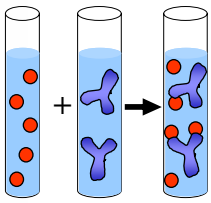
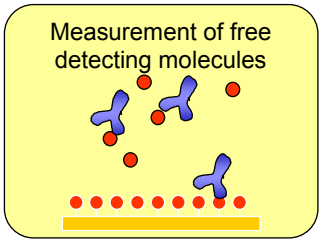
- Inhibition assay (solution competition)



Analyte
Detecting molecule



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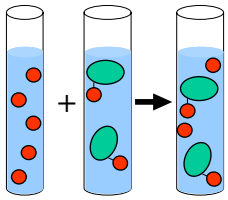
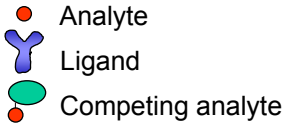
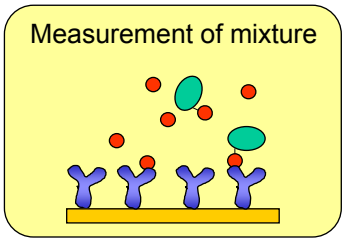
4

Indirect Assays (continued)

- Surface competition assay







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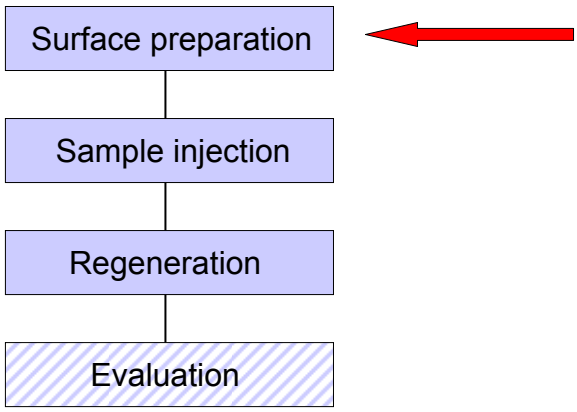
Steps in Biacore Assay Development



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Surface preparation

Immobilization

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- What is immobilization?
  - » Covalent linking of a ligand or capture molecule to the sensor surface
- Points to consider
  - » What to immobilize?
  - » How to immobilize?
  - » What immobilization level is appropriate?
  - » Which Sensor Chip is suitable?

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7

Surface preparation

What to immobilize?

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- Considerations
  - » Molecular weight of interactants
  - » Tagging of interactants
  - » Functional groups
  - » Purity
  - » Valency (number of binding sites)
  - » Binding activity of immobilized interactant must be retained
  - » pI
  - » Available amount
  - » Assay requirements

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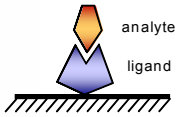
Surface preparation

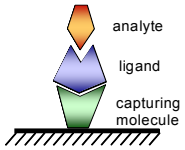
How to immobilize?

- Direct immobilization
  - » Covalent chemistry
  - » Often heterogenous orientation
  - » Higher binding capacity



Amine
Ligand Thiol
Surface Thiol
Maleimide
Aldehyde

- Capturing
  - » Orientation-specific
  - » Selective ligand capture from crude samples
  - » Lower binding capacity



Streptavidin - Biotin
RAM - Mab
Anti-GST - GST
NTA - 6His
Anti-FLAG - FLAG
Anti-His – 6His

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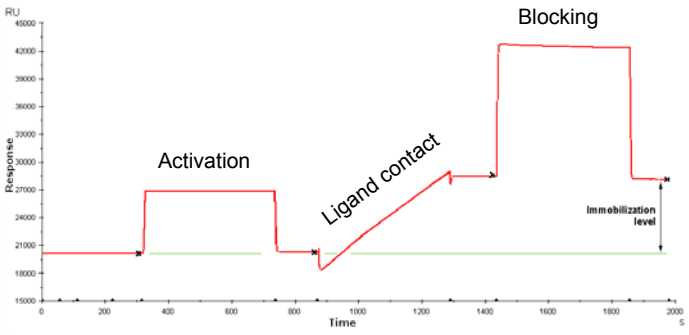
9

Amine Coupling Steps



- Activation = EDC/NHS injection → surface esters
- Ligand contact = reaction with amine groups on ligand
- Blocking = deactivation of free esters with ethanolamine

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10

Choice of immobilization strategy dependent on ligand properties

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- Unstable ligand → Capture
- Impure ligand → Capture
- Covalent coupling results in loss of activity → Try other functional groups (e.g. Thiols)
- Acidic ligands → Capture or alternative chemistry (e.g. Thiol coupling)
- Regeneration is difficult → Capture

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The isoelectric point (pI) of the protein

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- Defined as the pH at which there is no net charge on the protein
- pH < pI: The net charge of the protein will be positive
- pH > pI: The net charge of the protein will be negative

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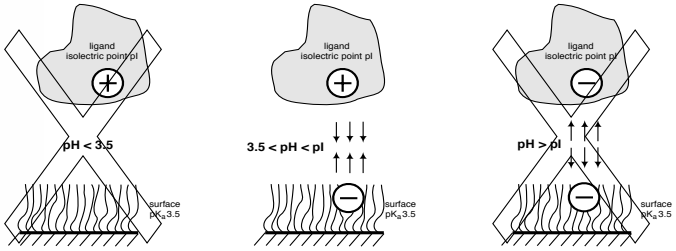
12

Pre-concentration

- Ligand is concentrated at the sensor surface by electrostatic attraction



» Efficient pre-concentration requires that the pH lies between the pk_a of the surface and the isoelectric point (pI) of the ligand.

» Low ionic buffer strength is also important

» Poor pre-concentration can be ***partially*** compensated for by increasing ligand concentration

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Immobilization pH-scouting (1)

- The experimental procedure of finding the appropriate immobilization pH
- At pH >3.5 the dextran matrix carries a net negative charge
- Immobilization buffer pH should be higher than 3.5, but lower than the isoelectric point of the ligand
- For many proteins, 10 mM sodium acetate buffer (pH 4.5) works well

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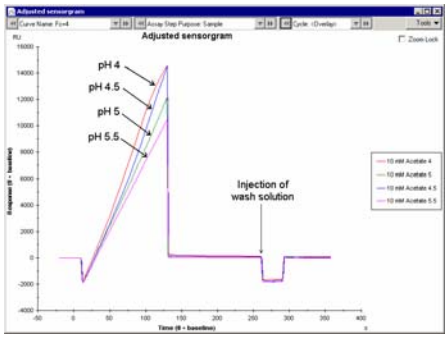
14

Immobilization pH-scouting (2)

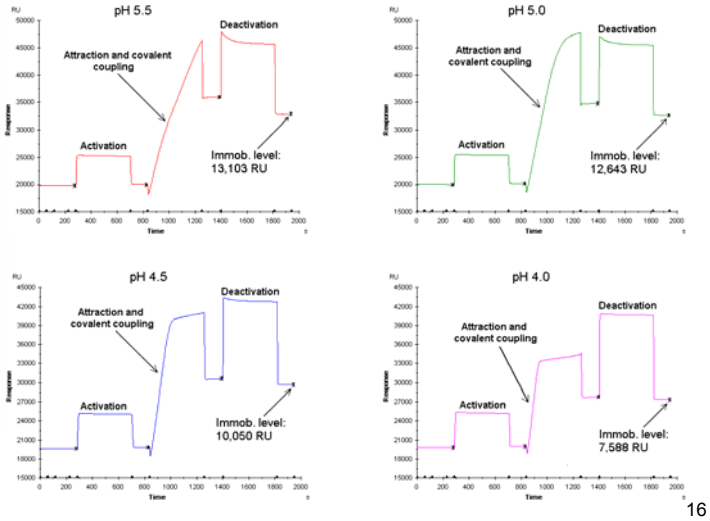
- Inject ligand diluted in buffers with different pH



- Gives useful but incomplete information regarding optimal immobilization conditions…

Immobilization pH-scouting (3)

- For optimization the entire immobilization has to be performed



Immobilization levels

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- The binding capacity of the surface depends on the immobilization level
- Different applications require different immobilization levels
- R_{max} describes the binding capacity of the surface

$$R_{max} = \frac{\text{analyte MW}}{\text{ligand MW}} \times R_L \times S_m$$

R_L = the immobilization level
S_m = the stoichiometric ratio

- The theoretical R_{max} is often higher than the experimental R_{max}

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17

Exercise – Calculation of R_L

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$$R_{max} = \frac{\text{analyte MW}}{\text{ligand MW}} \times R_L \times S_m$$

How much ligand should I immobilize if I want an R_{max} of 100 RU?

analyte MW = 25,000 Da
ligand MW = 150,000 Da
S_m = 1
R_{max} = 100 RU

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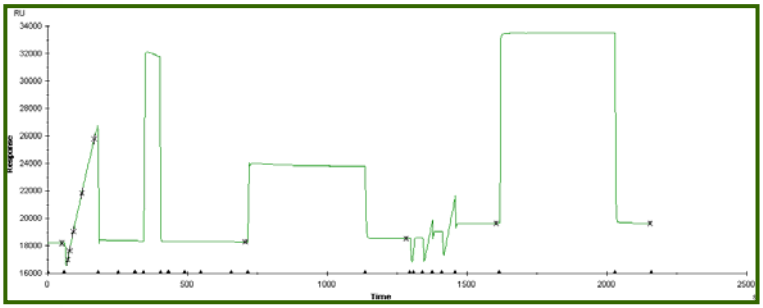
18

A targeting wizard to control immobilization level



- Remove the guesswork!
  - » Pre-concentration to estimate the binding rate
  - » Wash
  - » Activation of the surface
  - » Ligand injection in short pulses until target level is reached (first pulse length determined from pre-concentration rate)
  - » Deactivation

19

Series S Sensor chip surfaces

20

Series S Sensor Chip CM5

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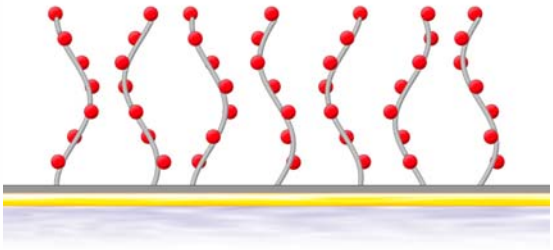

- Carboxymethylated dextran matrix
- The most versatile chip available
- Excellent chemical stability

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21

Series S Sensor Chip CM4

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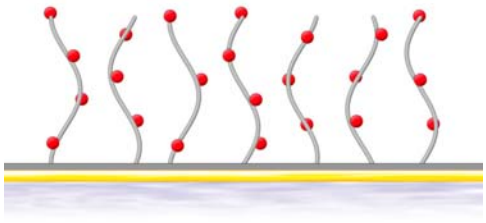

- Carboxymethylated dextran matrix with lower degree of carboxylation than CM5 (less negatively charged)
- Reduces non-specific binding of highly positively-charged molecules that may be found in cell culture, supernatants or cell homogenates
- Convenient for low R_{max} needed in kinetic applications

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Series S Sensor Chip CM3

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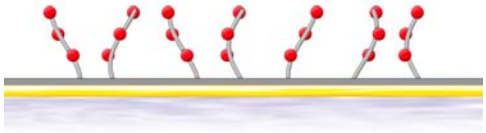

- Carboxymethylated dextran matrix
- Matrix shorter than CM5, but with the same degree of carboxylation
- For work with cells, viruses and studies of multi-component complexes
- Convenient for low immobilization levels

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Series S Sensor Chip C1

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- Flat carboxymethylated surface
- For work with particles such as cells and viruses, and in applications where a dextran matrix is not desired

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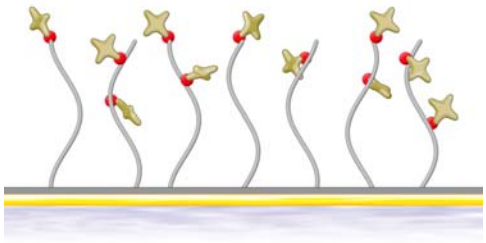
24

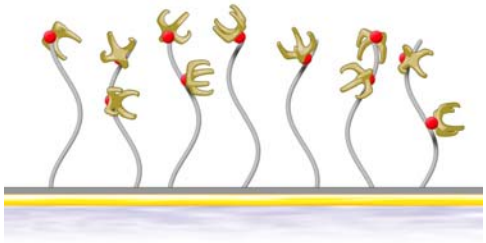
Series S Sensor Chip SA



- Carboxymethylated dextran matrix pre-immobilized with streptavidin
- Captures biotinylated ligands such as carbohydrates, peptides, proteins and DNA
- Ideal for capture of biotinylated DNA fragments

25

Series S Sensor Chip NTA



- Carboxymethylated dextran matrix preimmobilized with NTA
- Capture of His-tagged ligands via metal chelation
- Control steric orientation of ligand component for optimal site exposure
- Generic regeneration

26

Series S Sensor Chip HPA

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- Flat hydrophobic surface
- For lipid monolayers interacting with membrane binding biomolecules
- Alternative to solubilization techniques for studying membrane-associated interactions
- For studies of receptors associated with membrane-like environments interacting with analytes in aqueous buffer

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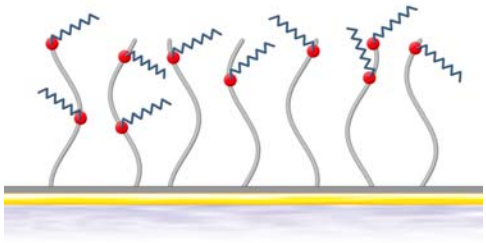
27

Series S Sensor Chip L1

Biacore Training



- Carboxymethylated dextran matrix modified with lipophilic substances
- For rapid and reproducible capture of liposomes with retention of lipid bilayer structure
- No requirement for incorporation of anchoring molecules

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28

Sensor chip surfaces – A summary

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- CM5: The most versatile chip
- CM4: For low R_{max} and for reducing non-specific binding from e.g. crude sample matrix
- CM3: For low immobilization levels and work with cells, viruses and high molecular weight analytes
- C1: For work with cells and particles and when dextran matrix is not desired
- SA: For capture of biotinylated ligands
- NTA: For capture of His-tagged ligands
- HPA: For looking at lipid monolayers interacting with membrane binding biomolecules
- L1: For capture of liposomes with retention of lipid bilayer structure

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29

Steps in Biacore Assay Development

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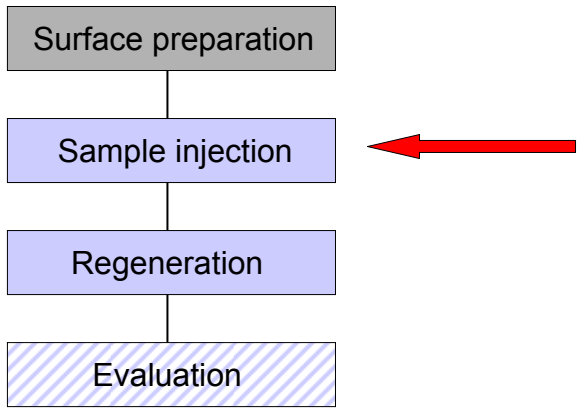

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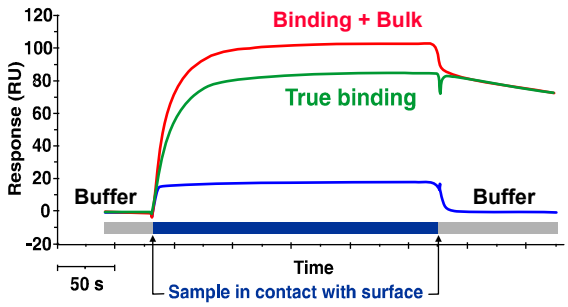
30

Binding and Bulk Effects



- Bulk effects are due to differences in the refractive index of running buffer and sample solution

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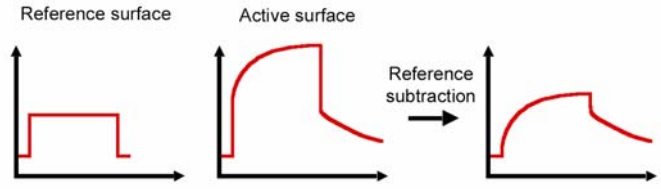
31

Reference surfaces

- If sample matrix and running buffer differ, the bulk contribution can be subtracted by using a reference surface
- Should be placed upstream of the active surface
  - » Flow cells optimized for use in pairs (Fc1 + Fc2, Fc3 + Fc4)
- Reference subtraction is important for assays where the measurement is taken during the sample injection



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32

Design of Reference surfaces

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- Unmodified surface
  - » Is acceptable as a reference surface in many cases
  - » To check for non-specific binding to the dextran matrix
- Activated-deactivated surface
  - » Treating the surface with the immobilization procedure, but omitting the ligand
  - » Decreases the negative charge on the surface and may reduce non-specific binding
- Surface immobilized with dummy ligand
  - » A protein that does not bind the analyte may be immobilized to approx. the same level as the ligand to mimic the active surface as closely as possible

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33

Sample considerations

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- Is the sample homogeneous?
- What is the quality of the analyte?
- Is the analyte active?
- Does the sample aggregate easily?
- Is there non-specific binding?
- Which buffer is most appropriate for my molecules?
- Which injection time should I use?

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34

Buffer requirements

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- Must be 0.2 µm filtered
- Most normal assay buffers are compatible with Biacore
- Include P20 in the running buffer if possible
- Does my molecule of interest require any specific additives?
- If samples require organic solvents to aid solubility
  - » Check solvent compatibility with relevant section in instrument handbook

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35

Test the surface

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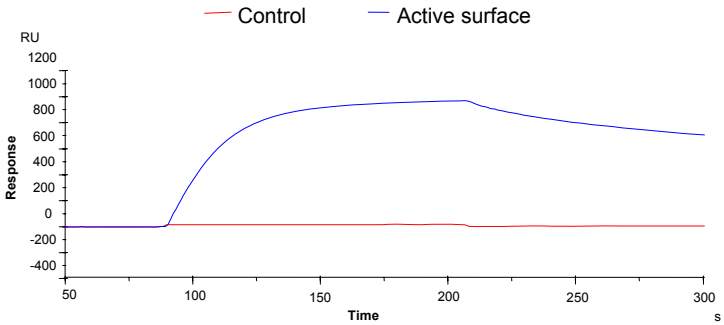

- Initial injection before starting the “real” assay
- Use a generous concentration of analyte
- The sensorgram yields useful information on the interaction
- Useful to assess levels of non-specific binding to the reference surface

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36

Steps in Biacore Assay Development

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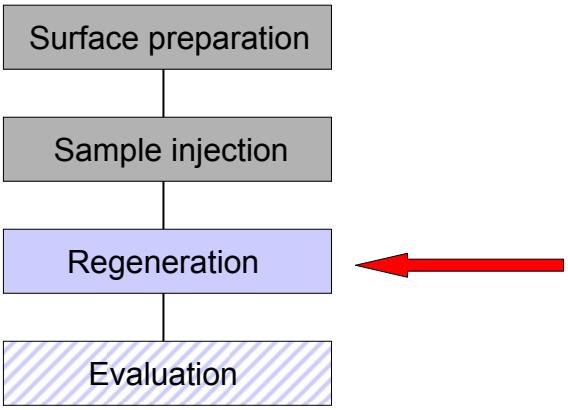

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37

Regeneration

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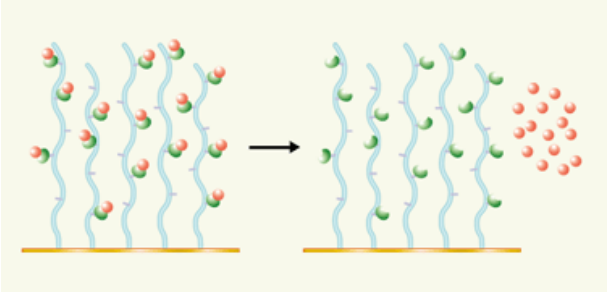
- Removes bound analyte completely from the surface
- The activity of the surface must remain unaffected
- Efficient regeneration is crucial for high-quality data



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38

Testing regeneration conditions

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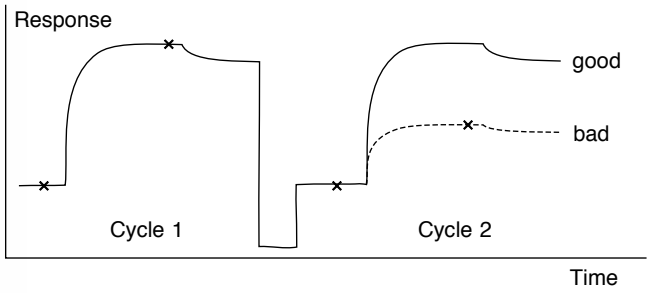

- Efficient regeneration removes all bound analyte
- A second injection of analyte reveals whether the ligand is still fully active
- Repeated cycles of analyte and regeneration injections are required to fully assess the conditions selected

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39

Choosing regeneration conditions

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- Optimal conditions will be specific for the ligand-analyte configuration
- A fairly narrow range of conditions are generally effective with a wide range of interactants
- Suggested alternative starting points for protein ligands:
  1. Low pH (10 mM glycine-HCl, from pH 3 to pH 1.5)
  2. Ethylene glycol (50%, 75% and 100%)
  3. High pH (1-100 mM NaOH)
  4. High ionic strength (up to 5 M NaCl or 4 M $MgCl_2$)
  5. Low concentrations of SDS (up to 0.5 %)

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40

Interpreting trends in analyte binding & baseline responses (1)

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- General guidance
  - » Ideal regeneration: Analyte response is consistent after repeated injections and within 10% of the level in the 1st injection
  - » Too mild conditions: The analyte response decreases and the baseline response increases
  - » Too harsh conditions: The analyte response decreases and the baseline response is constant or decreases

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41

Interpreting trends in analyte binding & baseline responses (2)

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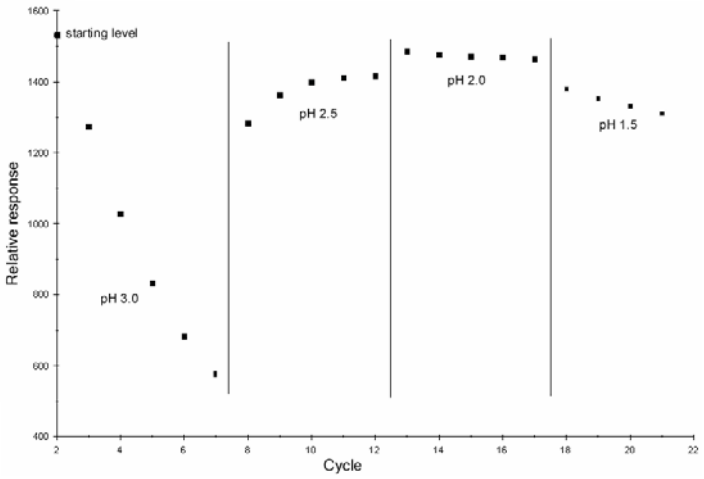
Analyte binding response



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42

Interpreting trends in analyte binding & baseline responses (3)

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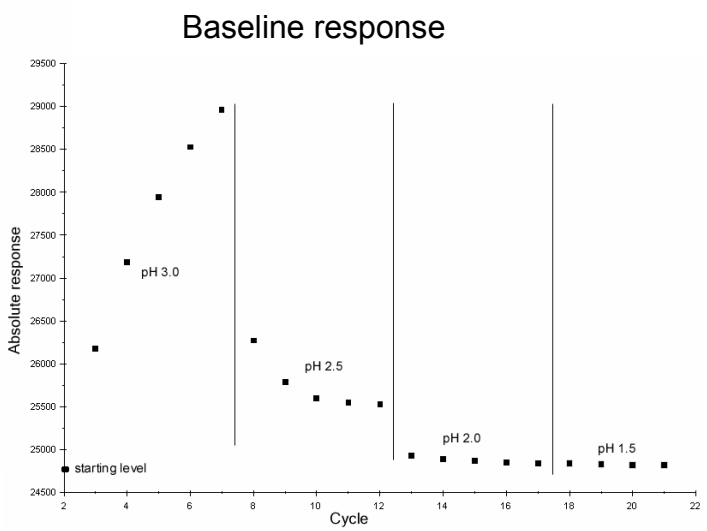

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43

Interpreting trends in analyte binding & baseline responses (4)

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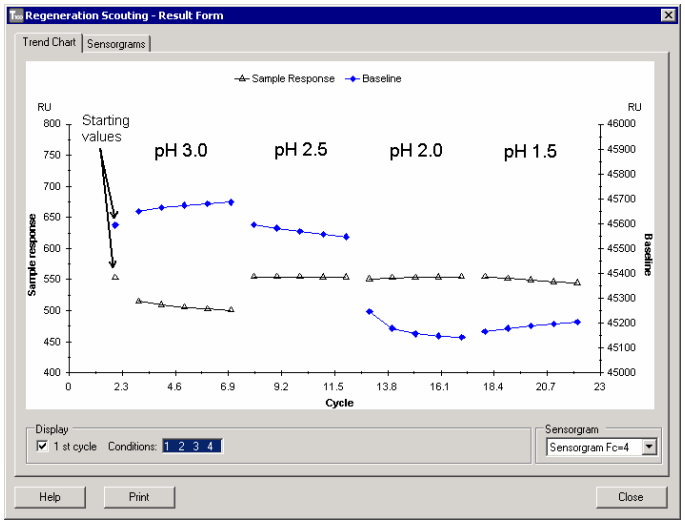

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44

Summary

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- Establish immobilization parameters
  - » What to immobilize
  - » How to immobilize
  - » How much to immobilize
- Evaluate binding capacity of the ligand
- Establish appropriate reference surfaces
- Establish regeneration conditions
- Assess surface stability
  - » Monitor baseline stability
  - » Monitor binding capacity

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