Guide to Running an SPR Experiment

- Follow the start-up procedures described on the "Starting an Experiment" sheet to dock the experimental chip, replace the Milli-Q water with running buffer, prime the instrument, and normalize the chip.
- For a first SPR experiment with a previously uncharacterized interaction, here is a suggested order of operations. This document is meant to serve only as a possible course of action using best practices that have been successful. The individual user is free, and encouraged, to adapt this procedure to match their desired experimental outcome.

1. Immobilize the ligand to a high surface density (3000-5000 RU)

a. A high surface density (3000-5000 RU) ensures that even if subsequent binding of the analyte to the surface is very weak, the binding signal should still be detectable and discernable from the noise and/or bulk shift, i.e. the reference subtracted sensorgram (2-1, 4-3, etc.) will give a signal of at least 20-100 RU.
b. For direct (covalent) immobilizations, the concentration of ligand solution that you should prepare will vary for each protein. Generally, the ligand should be diluted to around 5-20 µg/mL in 10 mM sodium acetate (NaOAc) buffer at the desired pH (pH 5.0 is standard).
c. Activate the surface with a 1:1 mixture of NHS/EDC contact time: 300-600 s at 5 µL/min).
    i. For a 420 s activation @ 5 µL/min, use 50 µL NHS + 50 µL EDC.
    ii. 5 µL/min is the recommended flow rate for immobilizations.
d. Begin the immobilization with a 30 s injection at 5 µL/min. The injection/contact time should be increased for subsequent injections, as necessary, to achieve the desired immobilization level.
i. If very little ligand is immobilized (< 100 RU) during the first 30 s injection, increase the concentration such that you can reach the desired 3000-5000 RU level within 5-10 minutes worth of injections.
e. Deactivate the surface with an ethanolamine injection (contact time: 600-900 s at 5 µL/min), and allow the surface to run on standby mode for at least 30 minutes.

2. Verify specific binding of analyte to the surface

a. Verification of binding can be most easily accomplished by injecting two different concentrations of analyte (e.g., 50 nM and 200 nM diluted in running buffer) for 60-120s at 30 µL/min and regenerating between each injection (see Section 3 for information on determining regeneration conditions).
    i. 30-50 µL/min is the recommended flow rate for analyte binding, dissociation, and regeneration.
b. If reliable, specific binding is observed, there should be a proportional increase in binding signal with increasing concentration.
c. Once ideal regeneration conditions are established (see Section 3), verify the binding signal remains stable for each analyte concentration.

3. Identify the ideal regeneration conditions

a. Always work from the mildest regeneration conditions to the strongest.
b. Avoid injecting regeneration solutions for more than 60 s per injection. Multiple, short injections are preferable to a single, long injection and will tend to damage the surface less, i.e. three 30 s injections are better than one 90 s injection.

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c. An ideal regeneration condition should remove ≥ 90% of the analyte bound to the surface. This can be determined by calculating the difference between the post-regeneration baseline and the pre-analyte injection baseline divided by the total analyte binding signal (the 'binding' report point for the analyte injection). If the difference in the baselines is less than 10% of the total bound, then greater than 90% surface regeneration has been achieved, and the regeneration condition is identified.
d. Once ≥ 90% surface regeneration is achieved, inject the same concentration of analyte for the same amount of time as above to verify that the regeneration conditions did not damage the surface, i.e. if a 60 s injection of 50 nM analyte generated a binding signal of 200 RU, then an identical injection post-regeneration should also generate a binding signal of about 200 RU.
    i. Occasionally, freshly made surfaces may exhibit a degree of non-specific binding or an analyte population that can't be removed following the first one or two injections. If there is difficulty in achieving ≥ 90% regeneration, try injecting the analyte sample again, verify that a similar binding response is generated a previous injection, and inject the regeneration solution again. You may find that the regeneration is now ≥ 90%, and the binding signal stabilizes after the third or fourth injection.
    ii. *Example*:
        1. Baseline: 0 RU
        2. *60s injection of 50 nM analyte*, Binding_1: 250 RU
        3. *Post-regeneration baseline*, Baseline_1: 50 RU (-200 RU, 80% regen)
        4. *60s injection of 50 nM analyte*, Binding_2: 230 RU
        5. *Post-regeneration baseline*, Baseline_2: 5 RU (-225 RU, 98% regen)
        6. *60s injection of 50 nM analyte*, Binding_3: 227 RU
        7. *Post-regeneration baseline*, Baseline_3: 7 RU (-220 RU, 97% regen)

4. *Lower surface density (R_{max} = 100-150 RU) for kinetic titration*

a. At this point, reliable detection of specific binding of the analyte to the surface-immobilized ligand has been established, and an appropriate regeneration condition has been identified.
b. High density surfaces are not suitable for obtaining reliable kinetic information due to the issue of analyte rebinding. For kinetic measurements, a lower density surface must be generated such that the maximum observed binding signal (R_{max}) is around 100 RU. The information gathered from binding to the high density surface can be used to inform the immobilization level required to obtain reliable kinetic data.
c. Use the following equation to calculate the theoretical R_{max} of the high density surface and compare with the experimentally observed R_{max}:
    i. Theoretical R_{max} = ($MW_{analyte}/MW_{ligand}$) x Immobilized Ligand (RU) x Stoichiometry
    ii. The stoichiometry refers to the number of analytes that bind to a single ligand.
    iii. *Example*: Protein analyte (30 kDa) and antibody ligand (150 kDa):
        1. R_{max} = (30,000/150,000) x 3500 RU x 2
        2. Theoretical R_{max} = 1400 RU
        3. Observed R_{max} = 1150 RU (82% of theo. R_{max})
d. During analyte injection on the high density surface, a general sense of how close the binding response to the theoretical R_{max} should have been observed. Binding response of 30% R_{max}? 90%? 110%?

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i. If a binding response greater than the theoretical R_{max} was observed, it is likely a calculation error was made or there is an incorrect assumption regarding the stoichiometry of the interaction or the apparent molecular weight of one of the species.

e. Now the theoretical R_{max} equation can be used to calculate how much ligand to immobilize on a new flow cell to achieve a theoretical R_{max} of 100-150 RU (the ideal for kinetics/affinity measurements)
   i. *Example* (contd.)
      1. Desired Theoretical R_{max} = ($MW_{analyte}/MW_{ligand}$) x [Immobilized Ligand (RU) x % of theoretical R_{max}] x Stoichiometry
      2. Desired Theo. R_{max} = 150 = (30/150) x Immobilized Ligand (RU) x 0.82 x 2
      3. Immobilized Ligand (RU) = 375 RU / 0.82 = 457 RU
      4. Immobilizing 457 RU should give a surface with an observed R_{max} of 150 RU.

5. *Immobilize the ligand on a new flow cell to the desired level*

a. Use the information gathered from your first immobilization in Section 1 to inform your concentrations and injection times to get the desired immobilization level
   i. If injecting 20 µg/mL for 30 s at 5 µL/min resulted in an immobilization of 400 RU, reduce the concentration to < 5 µg/mL in order to not to overshoot the desired immobilization level.

6. *Re-check analyte binding and regeneration*

a. Perform injections of similar analyte concentrations used in Section 2a and verify that the binding response generated is in the expected range (10-150 RU).
b. Make sure that the regeneration conditions are still appropriate by performing a few analyte injections and regenerations as described above in Section 3.

7. *Set-up a kinetic titration using the data collected in step 6*

a. If a 120 s injection of 50 nM analyte gave a response of 100 RU, perhaps set up a titration with the following concentrations: 100, 50, 25, 10, 5, 2.5, 1, 0 nM.
b. It is recommended that titrations be run with increasing concentrations and include at least 5-6 points.
c. It is also a good idea to repeat one of the median concentrations at the end of the titration as an internal control to ensure your regeneration has not damaged the surface after multiple injections.
d. An example cycle run list might look as follows:
   i. Startup – Running Buffer
   ii. Startup – Running Buffer
   iii. 0 nM analyte
   iv. 1 nM analyte
   v. 2.5 nM analyte
   vi. 5 nM analyte
   vii. 10 nM analyte
   viii. 25 nM analyte
   ix. 50 nM analyte
   x. 100 nM analyte
   xi. 5 nM analyte
   xii. 0 nM analyte

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